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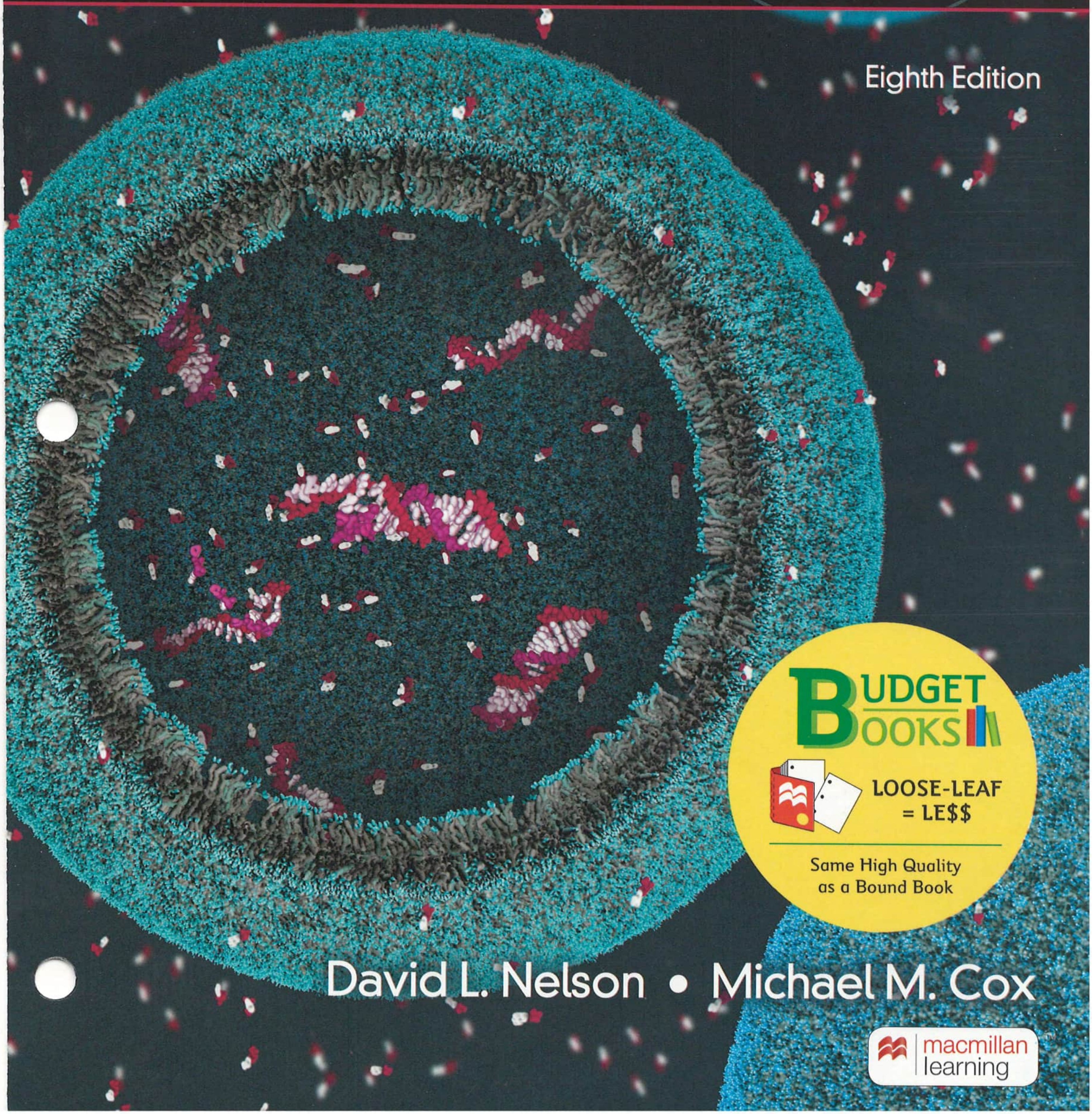
Principles of BIOCHEMISTRY

Eighth Edition



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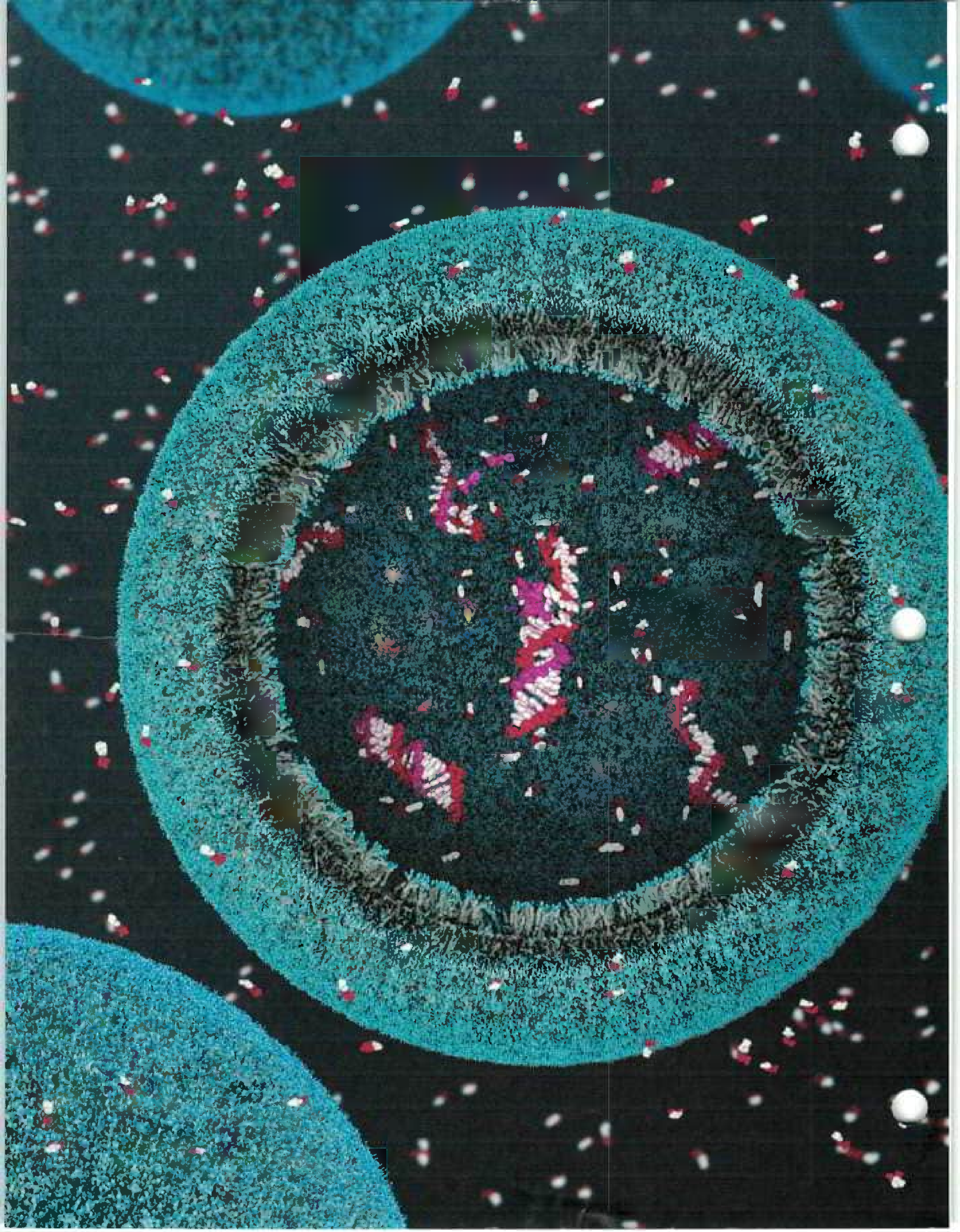

Lehninger
Principles of Biochemistry

EIGHTH EDITION

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Professor Emeritus of Biochemistry
University of Wisconsin–Madison

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In 1946, William Freeman founded W. H. Freeman and Company and published Linus Pauling's *General Chemistry*, which revolutionized the chemistry curriculum and established the prototype for a Freeman text. W. H. Freeman quickly became a publishing house where leading researchers can make significant contributions to mathematics and science. In 1996, W. H. Freeman joined Macmillan and we have since proudly continued the legacy of providing revolutionary, quality educational tools for teaching and learning in STEM.

To Our Teachers

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About the Authors

David Nelson
Michael Cox
Aaron Hoskins

From top left: David L. Nelson, Michael M. Cox, and Aaron A. Hoskins (Photos by David L. Nelson, Michael M. Cox, and Aaron A. Hoskins)

David L. Nelson, born in Fairmont, Minnesota, received his BS in chemistry and biology from St. Olaf College in 1964, and earned his PhD in biochemistry at Stanford Medical School, under Arthur Kornberg. He was a postdoctoral fellow at the Harvard Medical School with Eugene P. Kennedy, who was one of Albert Lehninger's first graduate students. Nelson joined the faculty of the University of Wisconsin–Madison in 1971 and became a full professor of biochemistry in 1982. For eight years he was Director of the Center for Biology Education at the University of Wisconsin–Madison. He became Professor Emeritus in 2013.

Nelson's research focused on the signal transductions that regulate ciliary motion and exocytosis in the protozoan *Paramecium*. For 43 years he taught (with Mike Cox) an intensive survey of biochemistry for advanced biochemistry undergraduates in the life sciences. He has also taught graduate courses on membrane structure and function, as well as on molecular neurobiology. He has received awards for his outstanding teaching, including the Dreyfus Teacher–Scholar Award and the Atwood Distinguished Professorship. In 1991–1992 he was a visiting professor of chemistry and biology at Spelman College. Nelson's second love is history, and in his dotage he teaches the history of biochemistry and collects antique scientific instruments.

Michael M. Cox was born in Wilmington, Delaware. In his first biochemistry course, the first edition of Lehninger's *Biochemistry* was a major influence in refocusing his fascination with biology and inspiring him to pursue a career in biochemistry. After graduate work at Brandeis University with William P. Jencks and postdoctoral work at Stanford with I. Robert Lehman, he moved to the University of Wisconsin–Madison in 1983. He became a full professor of Biochemistry in 1992.

Mike Cox has coordinated an active research team at Wisconsin investigating the function and mechanism of enzymes that act at the interface of DNA replication, repair, and recombination. That work has resulted in over 200 publications to date.

For more than three decades, Cox has taught introductory biochemistry to undergraduates and has lectured in a variety of graduate courses. He organized a course on professional responsibility for first-year graduate students and established a systematic program to draw talented biochemistry undergraduates into the laboratory at an early stage of their college career. He has received multiple awards for both his teaching and his research, including the Eli Lilly Award in Biological Chemistry, election as a AAAS fellow, and the UW Regents Teaching Excellence Award. Cox's hobbies include turning 18 acres of Wisconsin farmland into an arboretum, wine collecting, and assisting in the design of laboratory buildings.

Aaron A. Hoskins was born in Lafayette, Indiana, received his BS in chemistry from Purdue in 2000, and earned his PhD in biological chemistry at Massachusetts Institute of Technology with JoAnne Stubbe. In 2006, he went to Brandeis and University of Massachusetts Medical School as a postdoctoral fellow with Melissa Moore and Jeff Gelles. Hoskins joined the University of Wisconsin–Madison biochemistry faculty in 2011.

Hoskins's PhD research was on de novo purine biosynthesis. At Brandeis and University of Massachusetts, he began to study eukaryotic pre-mRNA splicing. During this time, he developed new single-molecule microscopy tools for studying the spliceosome.

Hoskins's laboratory is focused on understanding how spliceosomes are assembled and regulated and how they recognize introns. Hoskins has won awards for his research, including being named a Beckman Young Investigator and Shaw Scientist. He has taught introductory biochemistry for undergraduates since 2012. Hoskins also enjoys playing with his cat (Louise) and dog (Agatha), yoga/exercise, and tries to read a new book each week.

A Note on the Nature of Science

In this twenty-first century, a typical science education often leaves the philosophical underpinnings of science unstated, or relies on oversimplified definitions. As you contemplate a career in science, it may be useful to consider once again the terms **science**, **scientist**, and **scientific method**.

Science is both a way of thinking about the natural world and the sum of the information and theory that result from such thinking. The power and success of science flow directly from its reliance on ideas that can be tested: information on natural phenomena that can be observed, measured, and reproduced and theories that have predictive value. The progress of science rests on a foundational assumption that is often unstated but crucial to the enterprise: that the laws governing forces and phenomena existing in the universe are not subject to change. The Nobel laureate Jacques Monod referred to this underlying assumption as the "postulate of objectivity." The natural world can therefore be understood by applying a process of inquiry—the scientific method. Science could not succeed in a universe that played tricks on us. Other than the postulate of objectivity, science makes no inviolate assumptions about the natural world. A useful scientific idea is one that (1) has been or can be reproducibly substantiated, (2) can be used to accurately predict new phenomena, and (3) focuses on the natural world or universe.

Scientific ideas take many forms. The terms that scientists use to describe these forms have meanings quite different from those applied by nonscientists. A *hypothesis* is an idea or assumption that provides a reasonable and testable explanation for one or more observations, but it may lack extensive experimental substantiation. A *scientific theory* is much more than a hunch. It is an idea that has been substantiated to some extent and provides an explanation for a body of experimental observations. A theory can be tested and built upon and is thus a basis for further advance and innovation. When a scientific theory has been repeatedly tested and validated on many fronts, it can be accepted as a fact.

In one important sense, what constitutes science or a scientific idea is defined by whether or not it is published in the scientific literature after peer review by other working scientists. As of late 2014, about 34,500 peer-reviewed scientific journals worldwide were publishing some 2.5 million articles each year, a continuing rich harvest of information that is the birthright of every human being.

Scientists are individuals who rigorously apply the scientific method to understand the natural world. Merely having an advanced degree in a scientific discipline does not make one a scientist, nor does the lack of such a degree prevent one from making important scientific contributions. A scientist must be willing to challenge any idea when new findings demand it. The ideas that a scientist accepts must be based on measurable, reproducible observations, and the scientist must report these observations with complete honesty.

The **scientific method** is a collection of paths, all of which may lead to scientific discovery. In the *hypothesis and experiment* path, a scientist poses a hypothesis, then subjects it to experimental test. Many of the processes that biochemists work with every day were discovered in this manner. The DNA structure elucidated by James Watson and Francis Crick led to the hypothesis that base pairing is the basis for information transfer in polynucleotide synthesis. This hypothesis helped inspire the discovery of DNA and RNA polymerases.

Watson and Crick produced their DNA structure through a process of *model building and calculation*. No actual experiments were involved, although the model building and calculations used data collected by other scientists. Many adventurous scientists have applied the process of *exploration and observation* as a path to discovery. Historical voyages of discovery (Charles Darwin's 1831 voyage on H.M.S. *Beagle* among them) helped to map the planet, catalog its living occupants, and change the way we view the world. Modern scientists follow a similar path when they explore the ocean depths or launch probes to other planets. An analog of hypothesis and experiment is *hypothesis and deduction*. Crick reasoned that there must be an adaptor molecule that facilitated translation of the information in messenger RNA into protein. This adaptor hypothesis led to the discovery of transfer RNA by Mahlon Hoagland and Paul Zamecnik.

Not all paths to discovery involve planning. *Serendipity* often plays a role. The discovery of penicillin by Alexander Fleming in 1928 and of RNA catalysts by Thomas Cech in the early 1980s were both chance discoveries, albeit by scientists well prepared to exploit them. *Inspiration* can also lead to important advances. The polymerase chain reaction (PCR), now a central part of biotechnology, was developed by Kary Mullis after a flash of inspiration during a road trip in northern California in 1983.

These many paths to scientific discovery can seem quite different, but they have some important things in common. They are focused on the natural world. They rely on *reproducible observation* and/or *experiment*. All of the ideas, insights, and experimental facts that arise from these endeavors can be tested and reproduced by scientists anywhere in the world. All can be used by other scientists to build new hypotheses and make new discoveries. All lead to information that is properly included in the realm of science. Understanding our universe requires hard work. At the same time, no human endeavor is more exciting and potentially rewarding than trying, with occasional success, to understand some part of the natural world.

The authoritative reference with a framework for understanding.

Lehninger Principles of Biochemistry earned acclaim for its presentation and organization of complex concepts and connections, anchored in the *principles* of biochemistry. This legacy continues in the eighth edition with a new framework that highlights the *principles* and supports student learning.

Overview of key features

The definitive ***Lehninger Principles of Biochemistry***, Eighth Edition, continues to help students navigate the complex discipline of biochemistry with a clear and coherent presentation. Renowned authors David Nelson, Michael Cox, and new coauthor Aaron Hoskins have focused this eighth edition around the fundamental principles to help students understand and navigate the most important aspects of biochemistry. Text features and digital resources in the new **Achieve** platform emphasize this focus on the principles, while coverage of recent discoveries and the most up-to-date research provide fascinating context for learning the dynamic discipline of biochemistry.

ORGANIZED AROUND PRINCIPLES FOR BETTER UNDERSTANDING

- This edition provides a **new learning path** for students, through emphasis on the fundamental principles of biochemistry.

Streamlined for easier navigation

- A **new, vibrant design improves navigation** through the content.
- Based on extensive user feedback, the authors have carefully trimmed topics and subtopics to emphasize crucial content, resulting in shorter chapters and an **overall reduction in book length**.
- **Clear principles** are identified at the outset of each chapter and called out with icons in the narrative of the chapter. The end-of-section summaries parallel the section content.
- Hundreds of new or revised figures make **current research** accessible to the biochemistry student.
- Captions have been streamlined throughout, maintaining the philosophy that the captions should support the understanding of the figure, independent of the text.
- Where possible, **figures have been simplified**, and many figures have step-by-step annotations, reducing caption length.
- **A revised photo program** emphasizes context-rich images.
- The **end-of-chapter problem sets have been revised** to ensure an equivalent experience whether students are using the text or doing homework online through Achieve.

Achieve supports educators and students throughout the full range of instruction, including assets suitable for pre-class preparation, in-class active learning, and post-class study and assessment. The pairing of a powerful new platform with outstanding biochemistry content provides an unrivaled learning experience.

FEATURES OF ACHIEVE INCLUDE:

- A **design guided by learning science research** through extensive collaboration and testing by both students and faculty, including two levels of Institutional Review Board approval.
- A **learning path** of powerful content, including pre-class, in-class, and post-class activities and assessments.
- A **detailed gradebook with insights for just-in-time teaching** and reporting on student achievement by learning objective.
- Easy **integration and gradebook sync with iClicker** classroom engagement solutions.
- Simple **integration with your campus LMS** and **availability through Inclusive Access** programs.

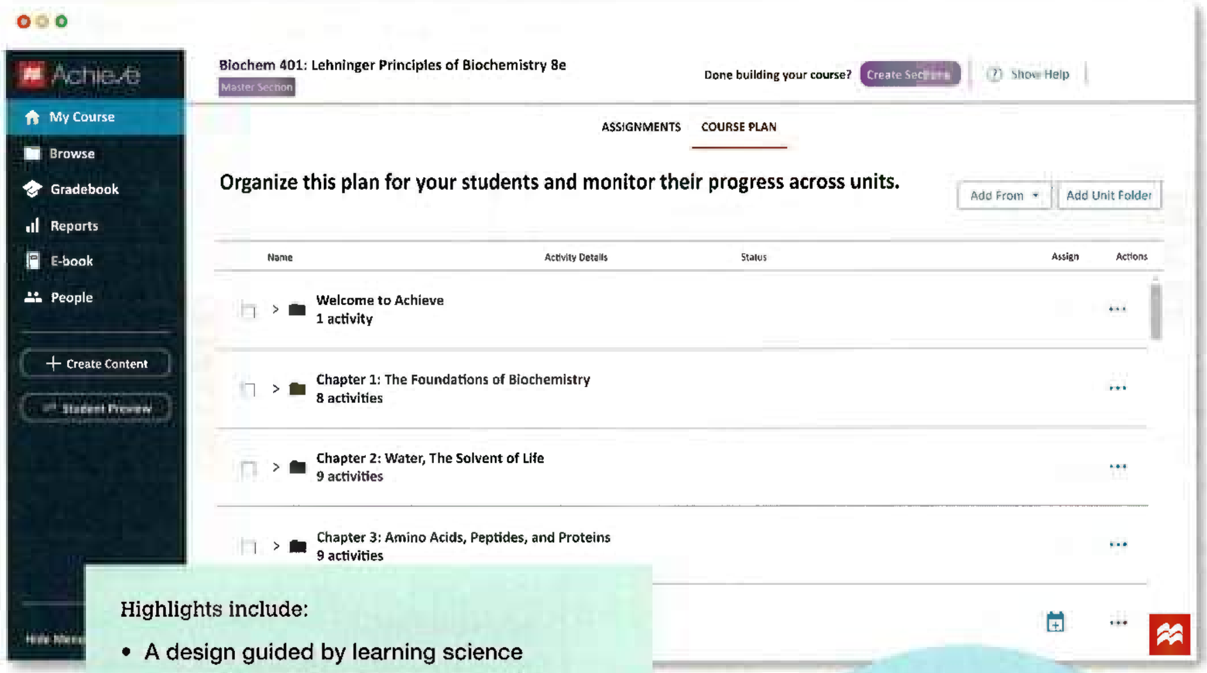
NEW IN ACHIEVE FOR *LEHNINGER PRINCIPLES OF BIOCHEMISTRY,* EIGHTH EDITION:

- **Virtually all end-of-chapter questions are available as online assessments** in Achieve with hints, targeted feedback, and detailed solutions.
- **Skills You Need activities** support students with review and practice of prerequisite skills and concepts from chemistry, biology, and math for each biochemistry chapter.
- **Instructor Activity Guides** provide everything you need to plan and implement activities, including interactive media, clicker questions, and pre- and post-class assessments.
- **Interactive Molecular Figures** allow students to view and interact with textbook illustrations of protein structures online in interactive three-dimensional models for a better understanding of their three-dimensional structures.
- Updated and expanded **instructor resources** and tools.

Achieve

Achieve is the culmination of years of development work put toward creating the most powerful online learning tool for biochemistry students. It houses all of our renowned assessments, multimedia assets, e-books, and instructor resources in a powerful new platform.

Achieve supports educators and students throughout the full range of instruction, including assets suitable for pre-class preparation, in-class active learning, and post-class study and assessment. The pairing of a powerful new platform with outstanding biochemistry content provides an unrivaled learning experience.



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Add Unit Folder

Biochem 401: Lehninger Principles of Biochemistry 8e

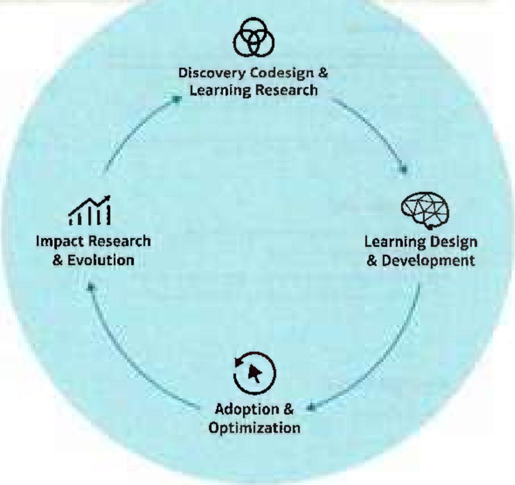
Done building your course?

Organize this plan for your students and monitor their progress across units.

Name	Activity Details	Status	Assign	Actions
Welcome to Achieve 1 activity				···
Chapter 1: The Foundations of Biochemistry 8 activities				···
Chapter 2: Water, The Solvent of Life 9 activities				···
Chapter 3: Amino Acids, Peptides, and Proteins 9 activities				···

Highlights include:

- **A design guided by learning science research.** Codesigned through extensive collaboration and testing by both students and faculty, including two levels of Institutional Review Board approval.
- **A learning path of powerful content** including pre-class, in-class, and post-class activities and assessments. A detailed gradebook with insights for just-in-time teaching and reporting on student achievement by learning objective.
- **Easy integration and gradebook sync** with iClicker classroom engagement solutions.
- **Simple integration** with your campus LMS and availability through **Inclusive Access** programs.



Discovery Codesign & Learning Research
Learning Design & Development
Adoption & Optimization
Impact Research & Evolution

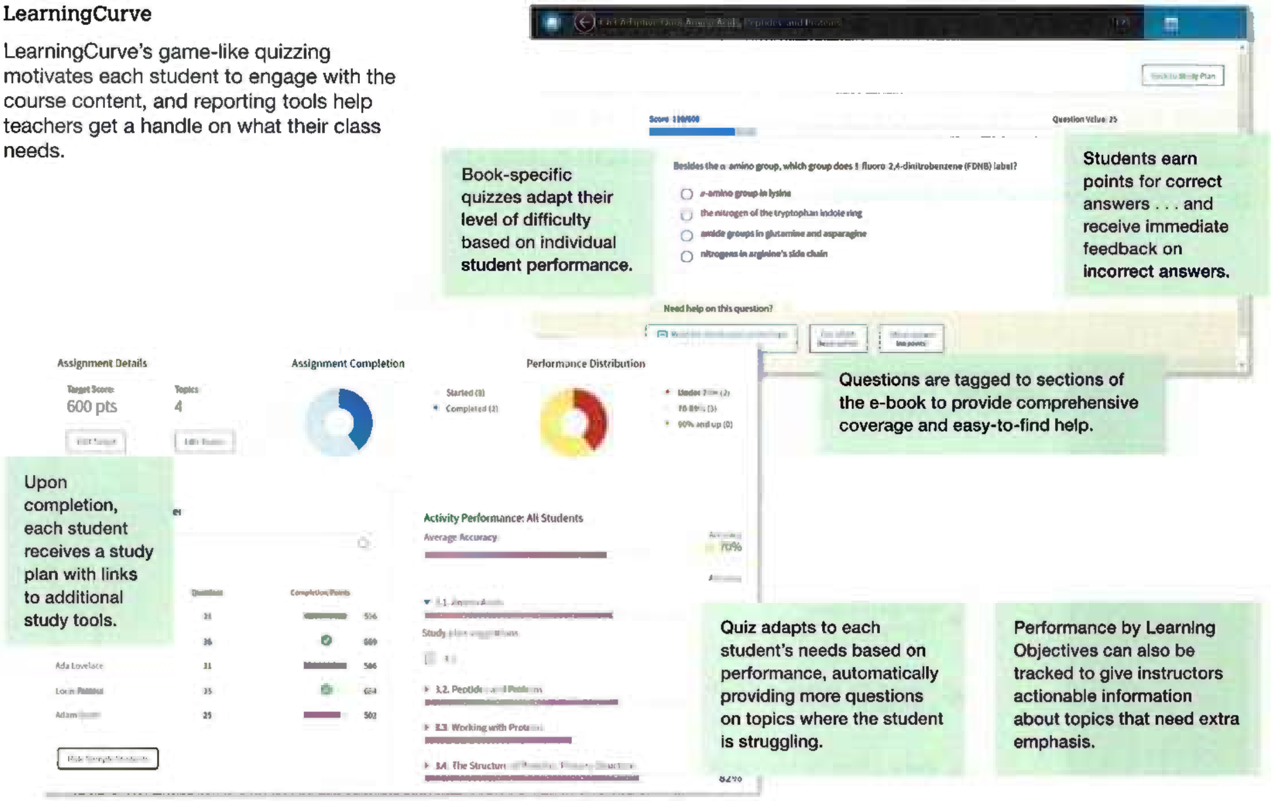
For more information or to sign up for a demonstration of Achieve, contact your local Macmillan representative or visit **macmillanlearning.com/achieve**

Full Learning Path and Flexible Resources

Achieve supports flexible instruction and engages student learning. This intuitive platform includes content for pre-class preparation, in-class active learning, and post-class engagement and assessment, providing an unparalleled environment and resources for teaching and learning biochemistry.

LearningCurve

LearningCurve's game-like quizzing motivates each student to engage with the course content, and reporting tools help teachers get a handle on what their class needs.



Besides the α-amino group, which group does 1-fluoro-2,4-dinitrobenzene (FDNB) label?

- ○ ε-amino group in lysine
- ○ the nitrogen of the tryptophan indole ring
- ○ amide groups in glutamine and asparagine
- ○ nitrogens in arginine's side chain

Need help on this question?

Book-specific quizzes adapt their level of difficulty based on individual student performance.

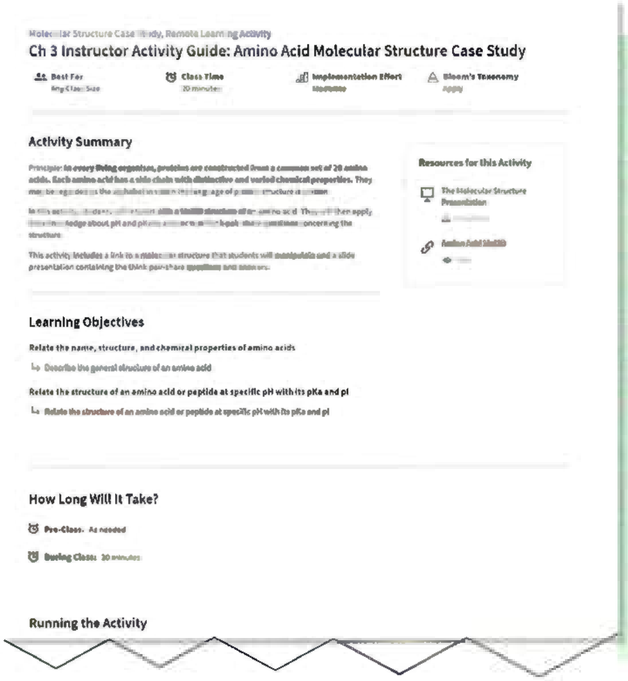
Students earn points for correct answers . . . and receive immediate feedback on incorrect answers.

Questions are tagged to sections of the e-book to provide comprehensive coverage and easy-to-find help.

Upon completion, each student receives a study plan with links to additional study tools.

Quiz adapts to each student's needs based on performance, automatically providing more questions on topics where the student is struggling.

Performance by Learning Objectives can also be tracked to give instructors actionable information about topics that need extra emphasis.



Molecular Structure Case Study, Remote Learning Activity

Ch 3 Instructor Activity Guide: Amino Acid Molecular Structure Case Study

Best For: Any Class Size
Class Time: 20 minutes
Implementation Effort: Moderate
Bloom's Taxonomy: Apply

Activity Summary

Principle: In every living organism, proteins are constructed from a common set of 20 amino acids. Each amino acid has a side chain with distinctive and varied chemical properties. They [illegible]

[illegible]

This activity includes a link to a molecular structure that students will manipulate and a slide presentation containing the think-pair-share questions and answers.

Resources for this Activity

The Molecular Structure Presentation

Learning Objectives

Relate the name, structure, and chemical properties of amino acids

↳ Describe the general structure of an amino acid

Relate the structure of an amino acid or peptide at specific pH with its pKa and pI

↳ Relate the structure of an amino acid or peptide at specific pH with its pKa and pI

How Long Will It Take?

Pre-Class: As needed

During Class: 20 minutes

Running the Activity

In-Class Activities

Active learning exercises provide students with hands-on exploration and help correct common misconceptions.

The activities have been vetted in the classroom and serve as a great off-the-shelf option for incorporating new methods of active learning in your classroom.

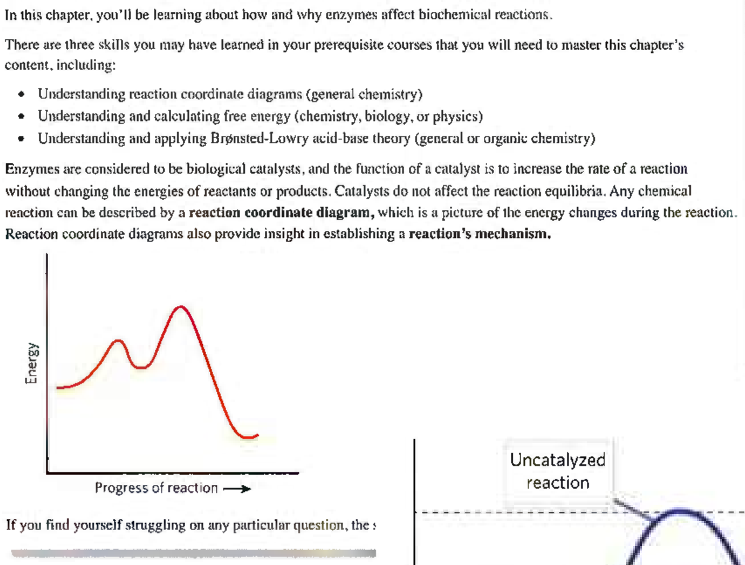
The activities include everything you need, including an instructor's guide, presentation decks, and student handouts.

iClicker

Achieve includes gradebook integration with iClicker, allowing for a seamless pairing of the best classroom response system with the best in-class content.

Achieve MORE

Achieve supports teaching and learning with exceptional content and resources.



In this chapter, you'll be learning about how and why enzymes affect biochemical reactions.

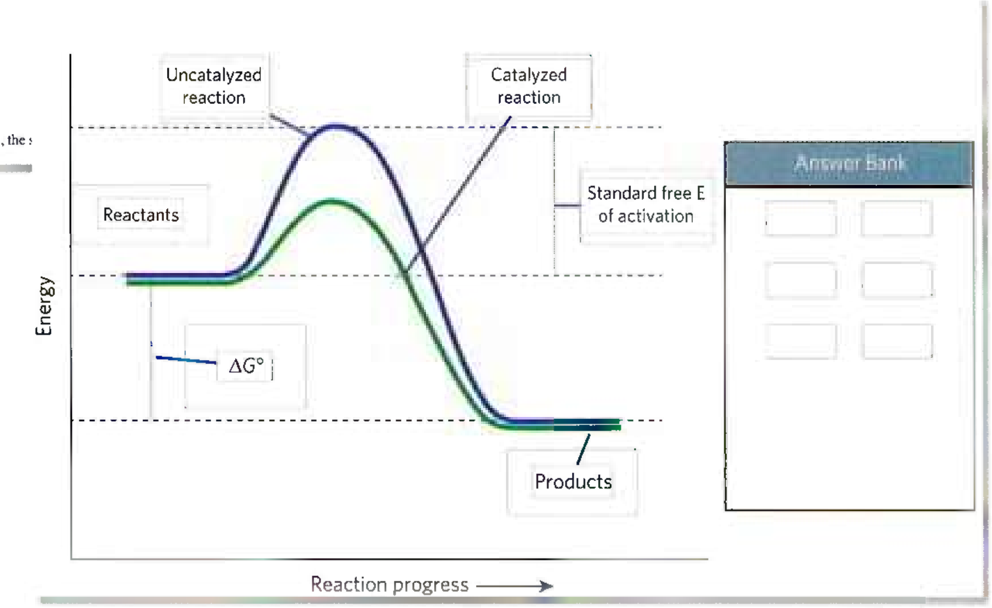
There are three skills you may have learned in your prerequisite courses that you will need to master this chapter's content, including:

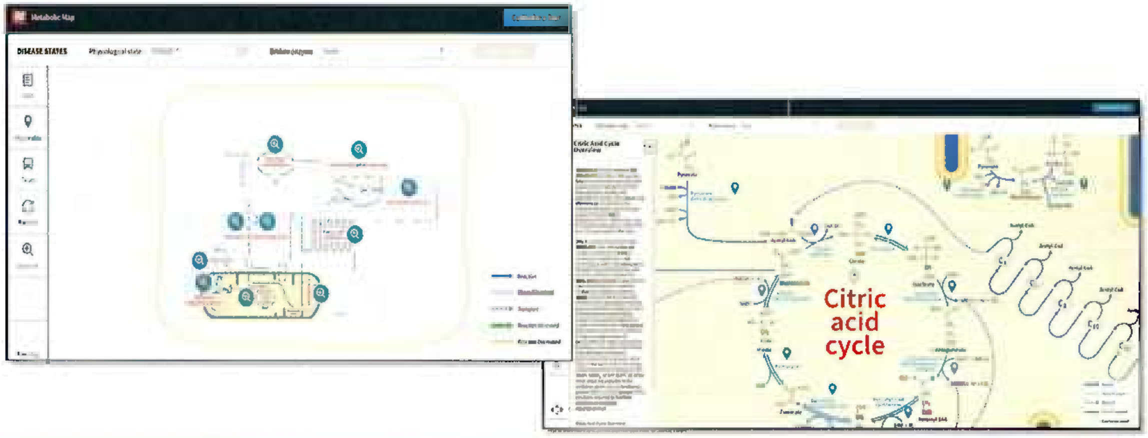
- Understanding reaction coordinate diagrams (general chemistry)
- Understanding and calculating free energy (chemistry, biology, or physics)
- Understanding and applying Brønsted-Lowry acid-base theory (general or organic chemistry)

Enzymes are considered to be biological catalysts, and the function of a catalyst is to increase the rate of a reaction without changing the energies of reactants or products. Catalysts do not affect the reaction equilibria. Any chemical reaction can be described by a **reaction coordinate diagram,** which is a picture of the energy changes during the reaction. Reaction coordinate diagrams also provide insight in establishing a **reaction's mechanism.**

If you find yourself struggling on any particular question, the

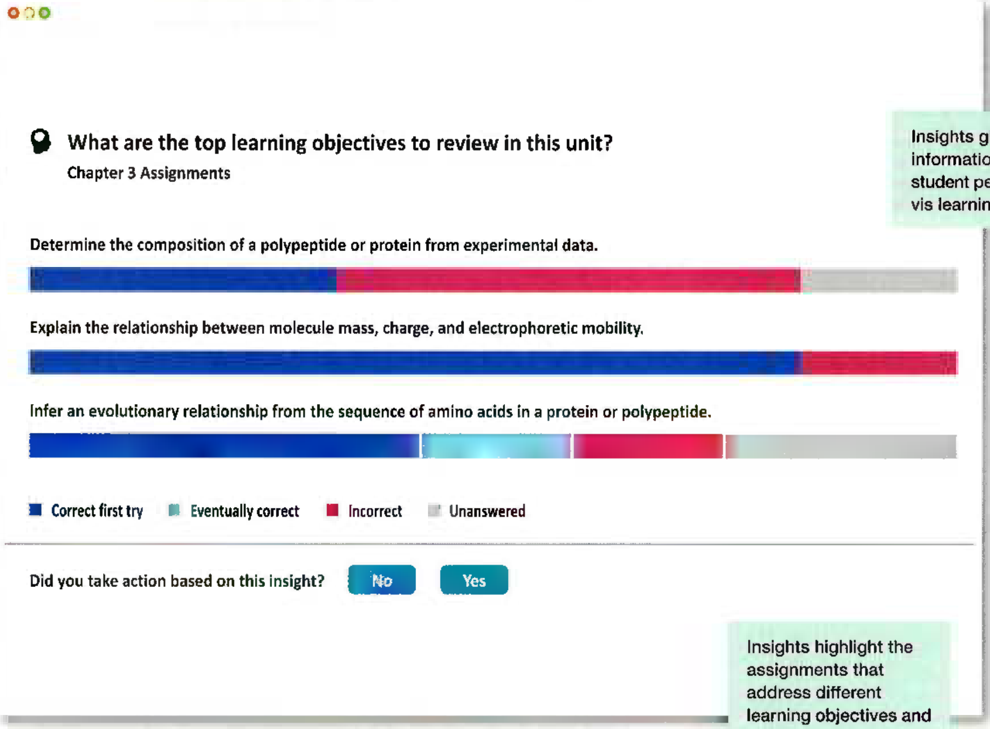
Skills You Need chapter-level activities provide a just-in-time refresher of chemistry, organic chemistry, biology, and math concepts and skills that students should know for each chapter of *Lehninger Principles of Biochemistry*. Designed to be completed in 30 minutes or less, each activity explains how skills and concepts from prerequisite or other courses will be relevant to the chapter, revisits the information, and gives students a chance to evaluate their mastery. In addition, the activities provide short tutorials and identify vocabulary, skills, and concepts requiring more practice.





Interactive Metabolic Map allows students to navigate and zoom between overviews and detailed views of the most commonly taught metabolic pathways. Embedded Tours take students through the pathways step by step, helping them to both visualize concepts in isolation and understand how concepts are connected. By examining the pathways in this way, students are better equipped to understand the meaning of those connections. Assessment questions help students solidify their understanding and provide more structure to their learning. *Metabolic Pathways include: glycolysis, gluconeogenesis, the citric acid cycle, oxidative phosphorylation, the pentose phosphate pathway, fatty acid synthesis, the urea cycle, and β oxidation.

Powerful analytics, viewable in an elegant dashboard, offer instructors a window into student progress. Achieve gives you the insight to address students' weaknesses and misconceptions before they struggle on a test.



What are the top learning objectives to review in this unit?
Chapter 3 Assignments

Determine the composition of a polypeptide or protein from experimental data.

Explain the relationship between molecule mass, charge, and electrophoretic mobility.

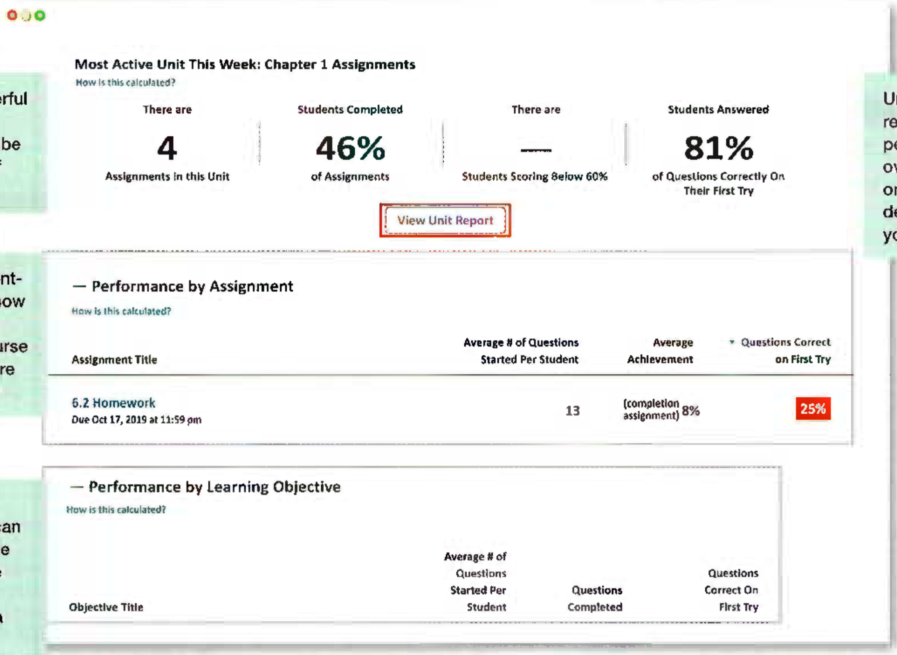
Infer an evolutionary relationship from the sequence of amino acids in a protein or polypeptide.

Did you take action based on this insight?

Insights give instructors information regarding student performance vis a vis learning objectives.

Insights highlight the assignments that address different learning objectives and show visualizations of student performance.

Reports provide powerful views of student performance and can be viewed in a number of different ways.



Most Active Unit This Week: Chapter 1 Assignments

There are	Students Completed	There are	Students Answered
4	46%	—	81%
Assignments in this Unit	of Assignments	Students Scoring Below 60%	of Questions Correctly On Their First Try

— Performance by Assignment

Assignment Title	Average # of Questions Started Per Student	Average Achievement	▾ Questions Correct on First Try
6.2 Homework Due Oct 17, 2019 at 11:59 pm	13	(completion assignment) 8%	25%

— Performance by Learning Objective

Objective Title	Average # of Questions Started Per Student	Questions Completed	Questions Correct On First Try

Unit and subunit reports give quick performance overviews based on the way you've decided to group your assignments.

At-a-glance assignment-level details let you know how far your students have gotten in the course and how quickly they're grasping the material.

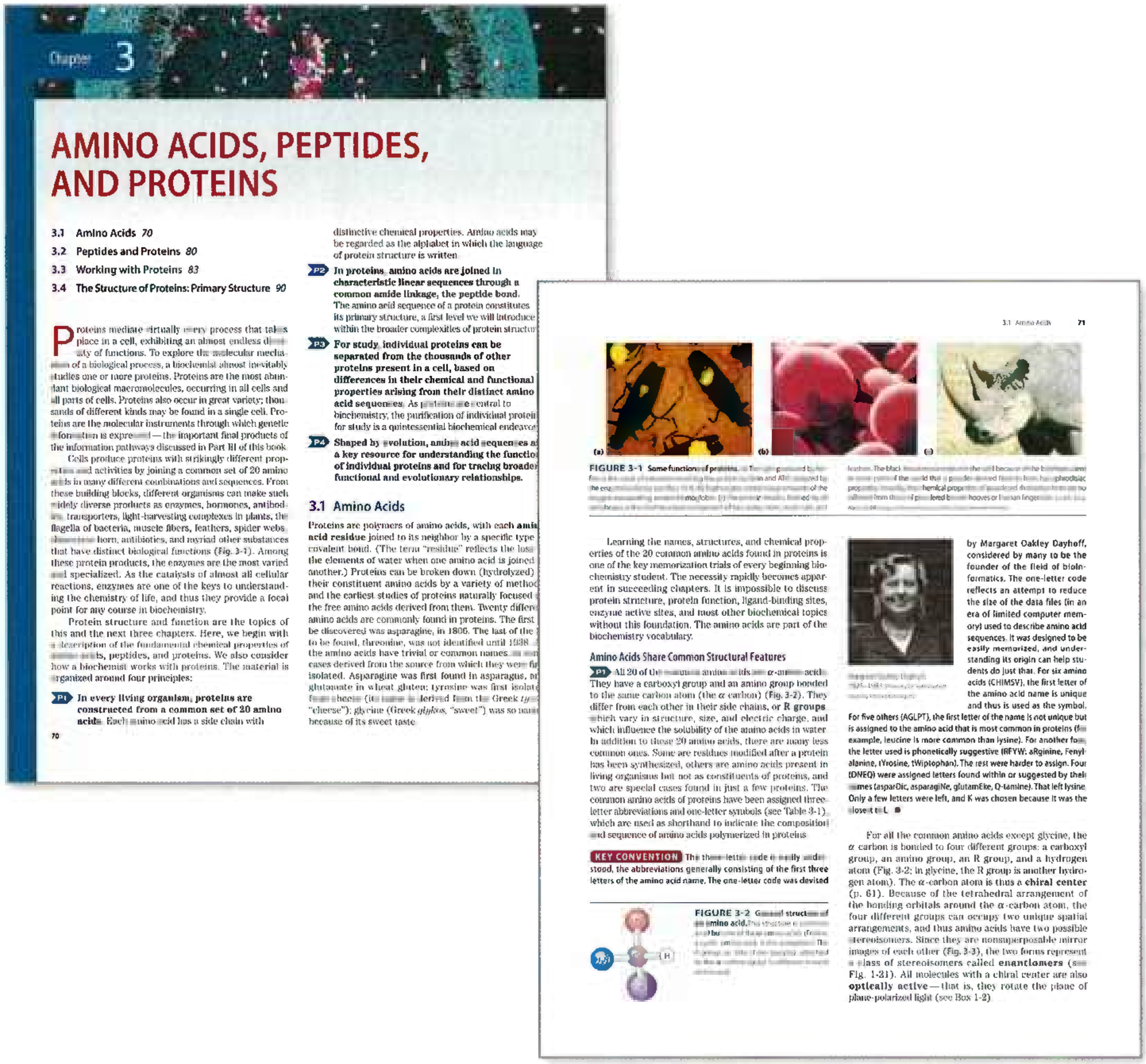
Performance by Learning Objectives can also be tracked to give instructors actionable information about topics that need extra emphasis.

The authoritative reference, with a framework for understanding

The story of principles—the fundamental concepts of biochemistry—provides an organizing framework to build an understanding of concepts and then develop critical connections between them.

Principles help students ground the details of biochemistry to its fundamental concepts. The first page of every chapter lists the principles with a numbered icon **P1**. Throughout the chapter, icons appear where needed to identify content that relates to those principles. **Active Learning Activities** in Achieve provide short opportunities for in-class engagement.

> The summary of principles and the highlighting of these principles throughout the text. This is really helpful! The boxes with additional information, particularly clinical relevance of the material. Most of my students are pre-med and it helps them engage in the material better if there is clinical relevance. The problems at the end of the chapter. These are really beneficial for the students who want to make sure they understand the material."
>
> — Robin Haynes, PhD, *Harvard University*



Chapter 3

AMINO ACIDS, PEPTIDES, AND PROTEINS

3.1 Amino Acids 70
3.2 Peptides and Proteins 80
3.3 Working with Proteins 83
3.4 The Structure of Proteins: Primary Structure 90

Proteins mediate virtually every process that takes place in a cell, exhibiting an almost endless diversity of functions. To explore the molecular mechanism of a biological process, a biochemist almost inevitably studies one or more proteins. Proteins are the most abundant biological macromolecules, occurring in all cells and all parts of cells. Proteins also occur in great variety; thousands of different kinds may be found in a single cell. Proteins are the molecular instruments through which genetic information is expressed—the important final products of the information pathways discussed in Part III of this book.

Cells produce proteins with strikingly different properties and activities by joining a common set of 20 amino acids in many different combinations and sequences. From these building blocks, different organisms can make such widely diverse products as enzymes, hormones, antibodies, transporters, light-harvesting complexes in plants, the flagella of bacteria, muscle fibers, feathers, spider webs, [illegible] horn, antibiotics, and myriad other substances that have distinct biological functions (Fig. 3-1). Among these protein products, the enzymes are the most varied and specialized. As the catalysts of almost all cellular reactions, enzymes are one of the keys to understanding the chemistry of life, and thus they provide a focal point for any course in biochemistry.

Protein structure and function are the topics of this and the next three chapters. Here, we begin with a description of the fundamental chemical properties of amino acids, peptides, and proteins. We also consider how a biochemist works with proteins. The material is organized around four principles:

P1 **In every living organism, proteins are constructed from a common set of 20 amino acids.** Each amino acid has a side chain with distinctive chemical properties. Amino acids may be regarded as the alphabet in which the language of protein structure is written.

P2 **In proteins, amino acids are joined in characteristic linear sequences through a common amide linkage, the peptide bond.** The amino acid sequence of a protein constitutes its primary structure, a first level we will introduce within the broader complexities of protein structur

P3 **For study, individual proteins can be separated from the thousands of other proteins present in a cell, based on differences in their chemical and functional properties arising from their distinct amino acid sequences.** As proteins are central to biochemistry, the purification of individual protein for study is a quintessential biochemical endeavo

P4 **Shaped by evolution, amino acid sequences a a key resource for understanding the functio of individual proteins and for tracing broader functional and evolutionary relationships.**

3.1 Amino Acids

Proteins are polymers of amino acids, with each **amin acid residue** joined to its neighbor by a specific type covalent bond. (The term "residue" reflects the los the elements of water when one amino acid is joined another.) Proteins can be broken down (hydrolyzed) their constituent amino acids by a variety of method and the earliest studies of proteins naturally focused the free amino acids derived from them. Twenty differe amino acids are commonly found in proteins. The first be discovered was asparagine, in 1806. The last of the to be found, threonine, was not identified until 1938. the amino acids have trivial or common names, [illegible] cases derived from the source from which they were fi isolated. Asparagine was first found in asparagus, a glutamate in wheat gluten; tyrosine was first isolat from cheese (its name is derived from the Greek *ty* "cheese"); glycine (Greek *glykos*, "sweet") was so nam because of its sweet taste.

70

3.1 Amino Acids 71

(a) (b) (c)

FIGURE 3-1 Some functions of proteins. [illegible]

Learning the names, structures, and chemical properties of the 20 common amino acids found in proteins is one of the key memorization trials of every beginning biochemistry student. The necessity rapidly becomes apparent in succeeding chapters. It is impossible to discuss protein structure, protein function, ligand-binding sites, enzyme active sites, and most other biochemical topics without this foundation. The amino acids are part of the biochemistry vocabulary.

Amino Acids Share Common Structural Features

P1 All 20 of the common amino acids are α-amino acids. They have a carboxyl group and an amino group bonded to the same carbon atom (the α carbon) (Fig. 3-2). They differ from each other in their side chains, or **R groups**, which vary in structure, size, and electric charge, and which influence the solubility of the amino acids in water. In addition to these 20 amino acids, there are many less common ones. Some are residues modified after a protein has been synthesized, others are amino acids present in living organisms but not as constituents of proteins, and two are special cases found in just a few proteins. The common amino acids of proteins have been assigned three-letter abbreviations and one-letter symbols (see Table 3-1), which are used as shorthand to indicate the composition and sequence of amino acids polymerized in proteins.

KEY CONVENTION The three-letter code is easily understood, the abbreviations generally consisting of the first three letters of the amino acid name. The one-letter code was devised by Margaret Oakley Dayhoff, considered by many to be the founder of the field of bioinformatics. The one-letter code reflects an attempt to reduce the size of the data files (in an era of limited computer memory) used to describe amino acid sequences. It was designed to be easily memorized, and understanding its origin can help students do just that. For six amino acids (CHIMSV), the first letter of the amino acid name is unique and thus is used as the symbol. For five others (AGLPT), the first letter of the name is not unique but is assigned to the amino acid that is most common in proteins (for example, leucine is more common than lysine). For another four, the letter used is phonetically suggestive (RFYW: aRginine, Fenylalanine, tYrosine, tWiptophan). The rest were harder to assign. Four (DNEQ) were assigned letters found within or suggested by their names (asparDic, asparagiNe, glutamEke, Q-tamine). That left lysine. Only a few letters were left, and K was chosen because it was the closest to L. ■

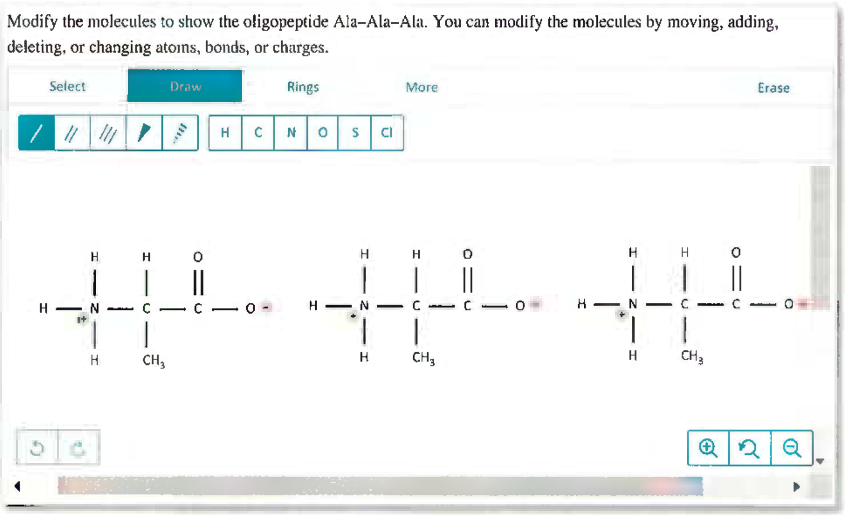
Margaret Oakley Dayhoff [illegible]

For all the common amino acids except glycine, the α carbon is bonded to four different groups: a carboxyl group, an amino group, an R group, and a hydrogen atom (Fig. 3-2; in glycine, the R group is another hydrogen atom). The α-carbon atom is thus a **chiral center** (p. 61). Because of the tetrahedral arrangement of the bonding orbitals around the α-carbon atom, the four different groups can occupy two unique spatial arrangements, and thus amino acids have two possible stereoisomers. Since they are nonsuperposable mirror images of each other (Fig. 3-3), the two forms represent a class of stereoisomers called **enantiomers** (see Fig. 1-21). All molecules with a chiral center are also **optically active**—that is, they rotate the plane of plane-polarized light (see Box 1-2).

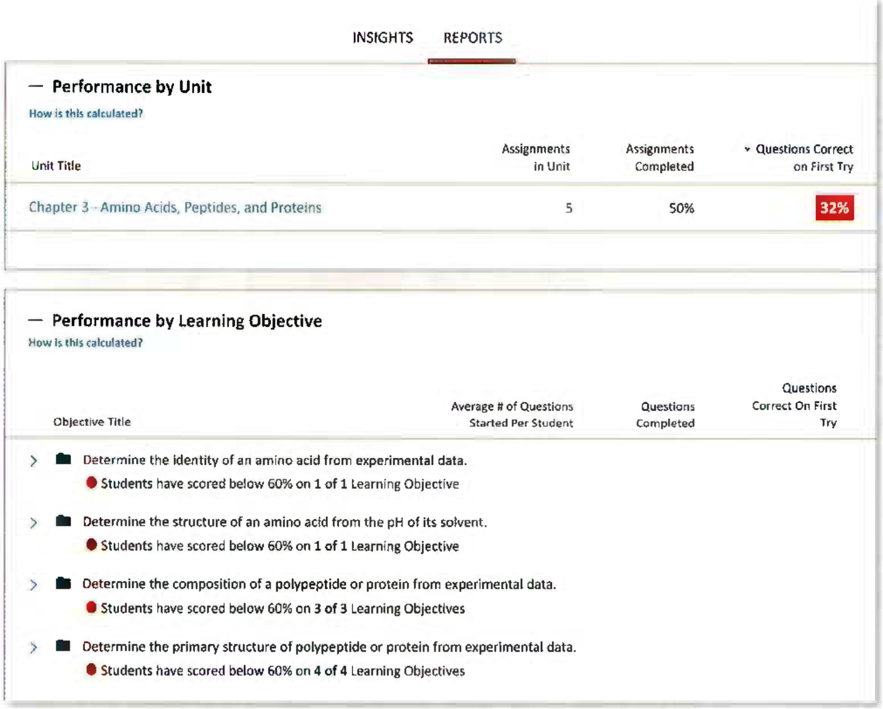
FIGURE 3-2 General structure of an amino acid. [illegible]

Achieve supports retention and assessment for *Lehninger Principles of Biochemistry*.

The principles framework extends to resources that support retention and assessment both in the text and in the accompanying Achieve platform. Students are encouraged to check understanding and retention through LearningCurve Adaptive Quizzing. All assessment questions, including all end-of-chapter problems, are included in the Achieve problem library, making it easy to assign the content you want and provide students with support through hints, targeted feedback, and detailed solutions. Assessments are tagged to principles-driven learning objectives, which can be easily viewed in the reporting and analytics of Achieve.



Modify the molecules to show the oligopeptide Ala–Ala–Ala. You can modify the molecules by moving, adding, deleting, or changing atoms, bonds, or charges.



— Performance by Unit

How is this calculated?

Unit Title	Assignments in Unit	Assignments Completed	Questions Correct on First Try
Chapter 3 - Amino Acids, Peptides, and Proteins	5	50%	32%

— Performance by Learning Objective

How is this calculated?

Objective Title	Average # of Questions Started Per Student	Questions Completed	Questions Correct On First Try
Determine the identity of an amino acid from experimental data. ● Students have scored below 60% on 1 of 1 Learning Objective			
Determine the structure of an amino acid from the pH of its solvent. ● Students have scored below 60% on 1 of 1 Learning Objective			
Determine the composition of a polypeptide or protein from experimental data. ● Students have scored below 60% on 3 of 3 Learning Objectives			
Determine the primary structure of polypeptide or protein from experimental data. ● Students have scored below 60% on 4 of 4 Learning Objectives			

> “I have found that on-line homework systems (like Sapling) that provide immediate feedback to students as they are working on the problems have really improved my students’ understanding and grades.”
>
> — David Bartley, PhD, *Adrian College*

Tools and Resources to Support Teaching

Course Preparation

- **Transition Guide** for navigating the changes between editions
- **Migration Tool** to move your assignments from your previous Sapling Course into your new Achieve course
- **Specialized Indices** for topics covered throughout the text, including Nutrition and Evolution
- **Section Management Courses** for making copies of your course when teaching multiple sections or to serve as a coordinator for other instructors' sections

Class Preparation

- **Skills You Need** assignments refresh students on content from courses frequently taken as prerequisites
- **Standalone slide decks** for content, images, and clicker questions that can be used as is or edited
- **Interactive e-book,** including assignable sections and chapters
- **LearningCurve Adaptive Quizzing** assignments to ensure reading comprehension

Instruction

- **Editable all-in-one Lecture Slides** that include content, images, clicker questions, multimedia tools, and activities
- **Cloud-based iClicker** in-class response system
- **Instructor Activity Guides,** developed with instructors and tied to the principles framework, include both instructor material and assessable student material
- **Interactive Metabolic Map** and **Animated Mechanism Videos, problem-solving videos**, and **case studies** are integrated into Lecture Slides and available as stand-alone resources

Practice and Assessment

- **Two editable, curated homework assignments,** including an assignment that matches the order and questions in the text and an assignment tied to the principles framework that uses questions from the text and other sources
- **Question Bank** with thousands of additional questions to create an assignment from scratch or add to a curated assignment
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- **Case Study** assignments
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Reporting and Analytics

- **Insights** on top learning objectives and assignments to review are surfaced just-in-time (7-day period)
- **Detailed reporting** by class, individual students, and learning objectives
- **Gradebook that syncs with iClicker** for an easy, all-in-one gradebook

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Chapter 1

THE FOUNDATIONS OF BIOCHEMISTRY

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About 14 billion years ago, the universe arose as a cataclysmic explosion of hot, energy-rich subatomic particles. Within seconds, the simplest elements (hydrogen and helium) were formed. As the universe expanded and cooled, material condensed under the influence of gravity to form stars. Some stars became enormous and then exploded as supernovae, releasing the energy needed to fuse simpler atomic nuclei into the more complex elements. Atoms and molecules formed swirling masses of dust particles, and their accumulation led eventually to the formation of rocks, planetoids, and planets. Thus were produced, over billions of years, Earth itself and the chemical elements found on Earth today. About 4 billion years ago, life arose on Earth — simple microorganisms with the ability to extract energy from chemical compounds and, later, from sunlight, which they used to make a vast array of more complex **biomolecules** from the simple elements and compounds on the Earth's surface. We and all other living organisms are made of stardust.

Biochemistry asks how the remarkable properties of living organisms arise from thousands of different biomolecules. When these molecules are isolated and examined individually, they conform to all the physical and chemical laws that describe the behavior of inanimate matter — as do all the processes occurring in living organisms. The study of biochemistry shows how the collections of inanimate molecules that constitute living organisms interact to maintain and perpetuate life governed solely by the same physical and chemical laws that govern the nonliving universe.

In each chapter of this book, we organize our discussion around central principles or issues in biochemistry. In this chapter, we consider the features that define a living organism, and we develop these principles:

P1 **Cells are the fundamental unit of life.** Although they vary in complexity and can be highly specialized for their environment or function within a multicellular organism, they share remarkable similarities.

P2 **Cells use a relatively small set of carbon-based metabolites to create polymeric machines, supramolecular structures, and information repositories.** The chemical structure of these components defines their cellular function. The collection of molecules carries out a program, the end result of which is reproduction of the program and self-perpetuation of that collection of molecules — in short, life.

P3 **Living organisms exist in a dynamic steady state, never at equilibrium with their surroundings.** Following the laws of thermodynamics, living organisms extract energy from their surroundings and employ it to maintain homeostasis and do useful work. Essentially all of the energy obtained by a cell comes from the flow of electrons, driven by sunlight or by metabolic redox reactions.

P4 **Cells have the capacity for precise self-replication and self-assembly using chemical information stored in the genome.** A single bacterial cell placed in a sterile nutrient medium can give rise to a billion identical "daughter" cells in 24 hours. Each cell is a faithful copy of the original, its construction directed entirely by information contained in the genetic material of the original cell. On a larger scale, the progeny of vertebrate animals share a striking resemblance to their parents, also the result of their inheritance of parental genes.

P5 **Living organisms change over time by gradual evolution.** The result of eons of evolution is an enormous diversity of life forms, fundamentally related through their shared ancestry, which can be seen at the molecular level in the similarity of gene sequences and protein structures.

Despite these common properties and the fundamental unity of life they reveal, it is difficult to make generalizations about living organisms. Earth has an enormous diversity of organisms living in a wide range of habitats, from hot springs to Arctic tundra, from animal intestines to college dormitories. These habitats are matched by a correspondingly wide range of specific biochemical adaptations, achieved within a common chemical framework. For the sake of clarity, in this book we sometimes risk certain generalizations, which, though not perfect, remain useful; we also frequently point out the exceptions to these generalizations, which can prove illuminating.

Biochemistry describes in molecular terms the structures, mechanisms, and chemical processes shared by all organisms and provides organizing principles that underlie life in all its diverse forms. Although biochemistry provides important insights and practical applications in medicine, agriculture, nutrition, and industry, its ultimate concern is with the wonder of life itself.

In this introductory chapter we give an overview of the cellular, chemical, physical, and genetic backgrounds of biochemistry and the overarching principle of evolution—how life emerged and evolved into the diversity of organisms we see today. As you read through the book, you may find it helpful to refer back to this chapter at intervals to refresh your memory of this background material.

1.1 Cellular Foundations

The unity and diversity of organisms become apparent even at the cellular level. The smallest organisms consist of single cells and are microscopic. Larger, multicellular organisms contain many different types of cells, which vary in size, shape, and specialized function. P1 Despite these obvious differences, all cells of the simplest and most complex organisms share certain fundamental properties, which can be seen at the biochemical level.

Cells Are the Structural and Functional Units of All Living Organisms

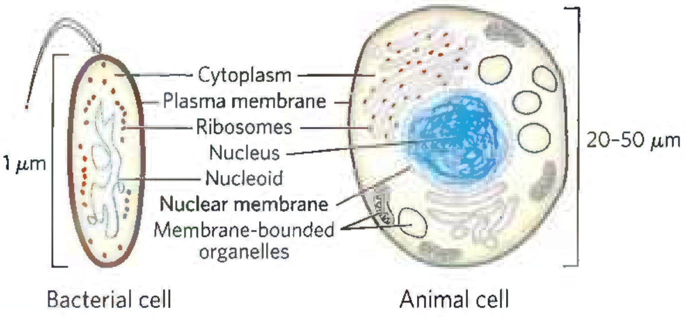


FIGURE 1-1 The universal features of living cells. All cells have a nucleus or nucleoid containing their DNA, a plasma membrane, and cytoplasm. Eukaryotic cells contain a variety of membrane-bounded organelles (including mitochondria and chloroplasts) and large particles (ribosomes, for example).

Cells of all kinds share certain structural features (Fig. 1-1). The **plasma membrane** defines the periphery of the cell, separating its contents from the surroundings. It is composed of lipid and protein molecules that form a thin, tough, pliable, hydrophobic barrier around the cell. The membrane is a barrier to the free passage of inorganic ions and most other charged or polar molecules. Transport proteins in the plasma membrane allow the passage of certain ions and molecules, receptor proteins transmit signals into the cell, and membrane enzymes participate in some reaction pathways. Because the individual lipids and proteins of the plasma membrane are not covalently linked, the entire structure is remarkably flexible, allowing changes in the shape and size of the cell. As a cell grows, newly made lipid and protein molecules are inserted into its plasma membrane; cell division produces two cells, each with its own membrane. This growth and cell division (fission) occurs without loss of membrane integrity.

The internal volume enclosed by the plasma membrane, the **cytoplasm** (Fig. 1-1), is composed of an aqueous solution, the cytosol, and a variety of suspended particles with specific functions. These particulate components (membranous organelles such as mitochondria and chloroplasts; supramolecular structures such as **ribosomes** and **proteasomes**, the sites of protein synthesis and degradation) sediment when cytoplasm is centrifuged at 150,000 *g* (*g* is the gravitational force of Earth). What remains as the supernatant fluid is defined as the **cytosol**, a highly concentrated solution containing enzymes and the RNA (ribonucleic acid) molecules that encode them; the components (amino acids and nucleotides) from which these macromolecules are assembled; hundreds of small organic molecules called **metabolites**, intermediates in biosynthetic and degradative pathways; **coenzymes**, compounds essential to many enzyme-catalyzed reactions; and inorganic ions (K^+, Na^+, Mg^{2+}, and Ca^{2+}, for example).

All cells have, for at least some part of their life, either a **nucleoid** or a **nucleus**, in which the **genome**—the complete set of genes, composed of DNA (deoxyribonucleic acid)—is replicated and stored, with its associated proteins. The nucleoid, in bacteria and archaea, is not separated from the cytoplasm by a membrane; the nucleus, in **eukaryotes**, is enclosed within a double membrane, the nuclear envelope. Cells with nuclear envelopes make up the large domain Eukarya (Greek *eu*, "true," and *karyon*, "nucleus"). Microorganisms without nuclear membranes, formerly grouped together as **prokaryotes** (Greek *pro*, "before"), are now recognized as comprising two very distinct groups: the domains Bacteria and Archaea, described below.

Cellular Dimensions Are Limited by Diffusion

Most cells are microscopic, invisible to the unaided eye. Animal and plant cells are typically 5 to 100 μm in diameter, and many unicellular microorganisms are only 1 to 2 μm long (see the end of the book for information on units and their abbreviations). What limits the dimensions of a cell? The lower limit is probably set by the minimum number of each type of biomolecule required by the cell. The smallest cells, certain bacteria known as mycoplasmas, are 300 nm in diameter and have a volume of about 10^{-14} mL. A single bacterial ribosome is about 20 nm in its longest dimension, so a few ribosomes take up a substantial fraction of the volume in a mycoplasmal cell.

The upper limit of cell size is probably set by the rate of transport of nutrients into the cell and waste products out. As the size of a cell increases, its surface-to-volume ratio decreases. For a spherical cell, the surface area is a function of the square of the radius (r^2), whereas its volume is a function of r^3. A bacterial cell the size of *Eschericia coli* is so small, and the ratio of its surface area to its volume is so large, that every part of its cytoplasm is easily reached by nutrients moving across the membrane and into the cell. With increasing cell size, surface-to-volume ratio decreases, until metabolism consumes nutrients faster than transmembrane carriers can supply them. Many types of animal cells have a highly folded or convoluted surface that increases their surface-to-volume ratio and allows higher rates of uptake of materials from their surroundings (**Fig. 1-2**).

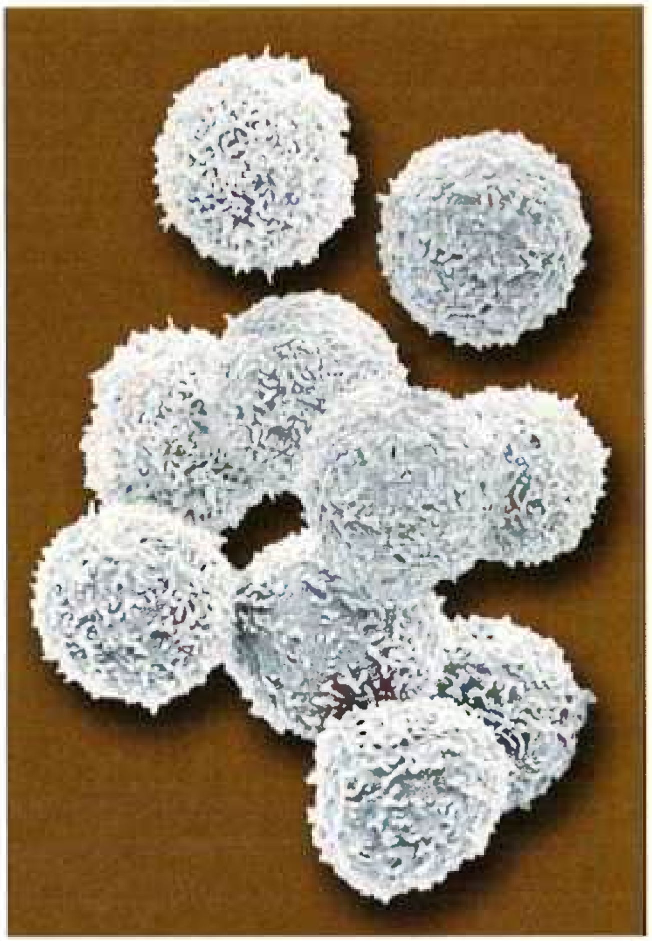


FIGURE 1-2 Most animal cells have intricately folded surfaces. The human lymphocytes in this artificially colored scanning electron micrograph are about 10–12 μm in diameter. Their convoluted surfaces give them a much larger surface area than a sphere of the same diameter. [Steve Gschmeissner/Science Source.]

Organisms Belong to Three Distinct Domains of Life

The development of techniques for determining DNA sequences quickly and inexpensively has greatly improved our ability to deduce evolutionary relationships among organisms. Similarities between gene sequences in various organisms provide deep insight into the course of evolution. In one interpretation of sequence similarities, all living organisms fall into one of three large groups (domains) that define three branches of the evolutionary tree of life originating from a common progenitor (**Fig. 1-3**). Two large groups of single-celled microorganisms can be distinguished on genetic and biochemical grounds: **Bacteria** and **Archaea**. Bacteria inhabit soils, surface waters, and the tissues of other living or decaying organisms. Many of the Archaea, recognized as a distinct domain by the microbiologist Carl Woese in the 1980s, inhabit extreme environments—salt lakes, hot springs, highly acidic bogs, and the ocean depths. The available evidence suggests that the Archaea and Bacteria diverged early in evolution. All eukaryotic organisms, which make up the third domain, **Eukarya**, evolved from the same branch that gave rise to the Archaea; eukaryotes are therefore more closely related to archaea than to bacteria.

Within the domains of Archaea and Bacteria are subgroups distinguished by their habitats. In **aerobic** habitats with a plentiful supply of oxygen, some resident organisms derive energy from the transfer of electrons from fuel molecules to oxygen within the cell. Other environments are **anaerobic**, devoid of oxygen, and microorganisms adapted to these environments obtain energy by transferring electrons to nitrate (forming N_2), sulfate (forming H_2S), or CO_2 (forming CH_4). Many organisms that have evolved in anaerobic environments are *obligate* anaerobes: they die when exposed to oxygen. Others are *facultative* anaerobes, able to live with or without oxygen.

Organisms Differ Widely in Their Sources of Energy and Biosynthetic Precursors

P3 We can classify organisms according to how they obtain the energy and carbon they need for synthesizing cellular material (as summarized in **Fig. 1-4**). There

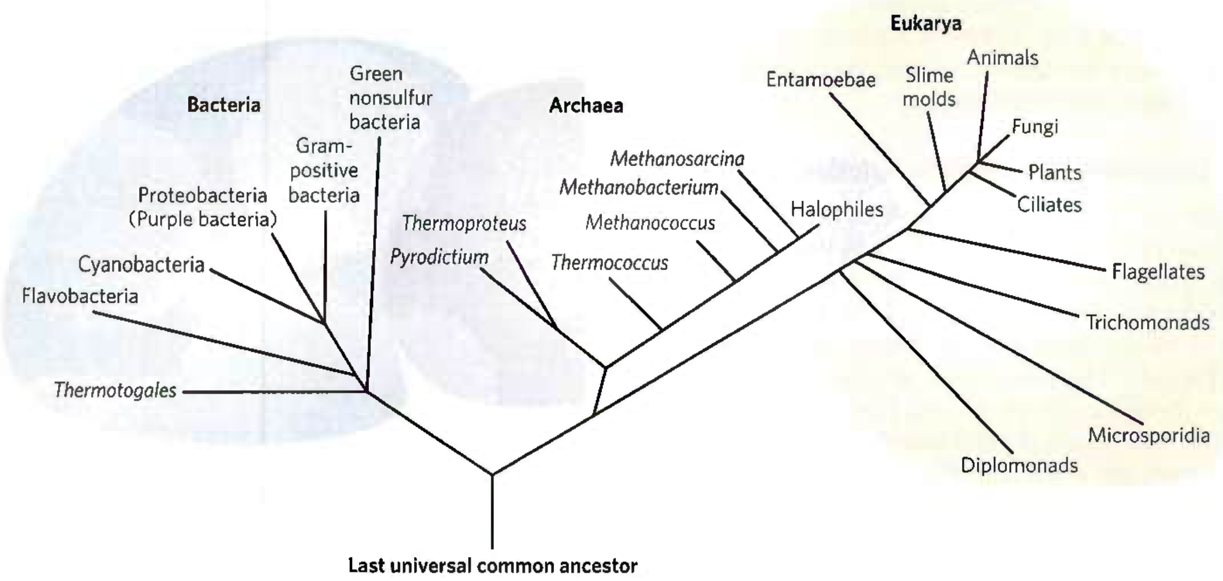

Eukarya
Animals
Entamoebae
Slime molds
Fungi
Plants
Ciliates
Flagellates
Trichomonads
Microsporidia
Diplomonads
Bacteria
Green nonsulfur bacteria
Gram-positive bacteria
Proteobacteria (Purple bacteria)
Cyanobacteria
Flavobacteria
Thermotogales
Archaea
Methanosarcina
Methanobacterium
Methanococcus
Halophiles
Thermoproteus
Pyrodictium
Thermococcus
Last universal common ancestor

FIGURE 1-3 **Phylogeny of the three domains of life.** Phylogenetic relationships are often illustrated by a "family tree" of this type. The basis for this tree is the similarity in nucleotide sequences of the ribosomal RNAs of each group. [Information from C. R. Woese, *Microbiol. Rev.* 51:221, 1987, Fig. 4.]

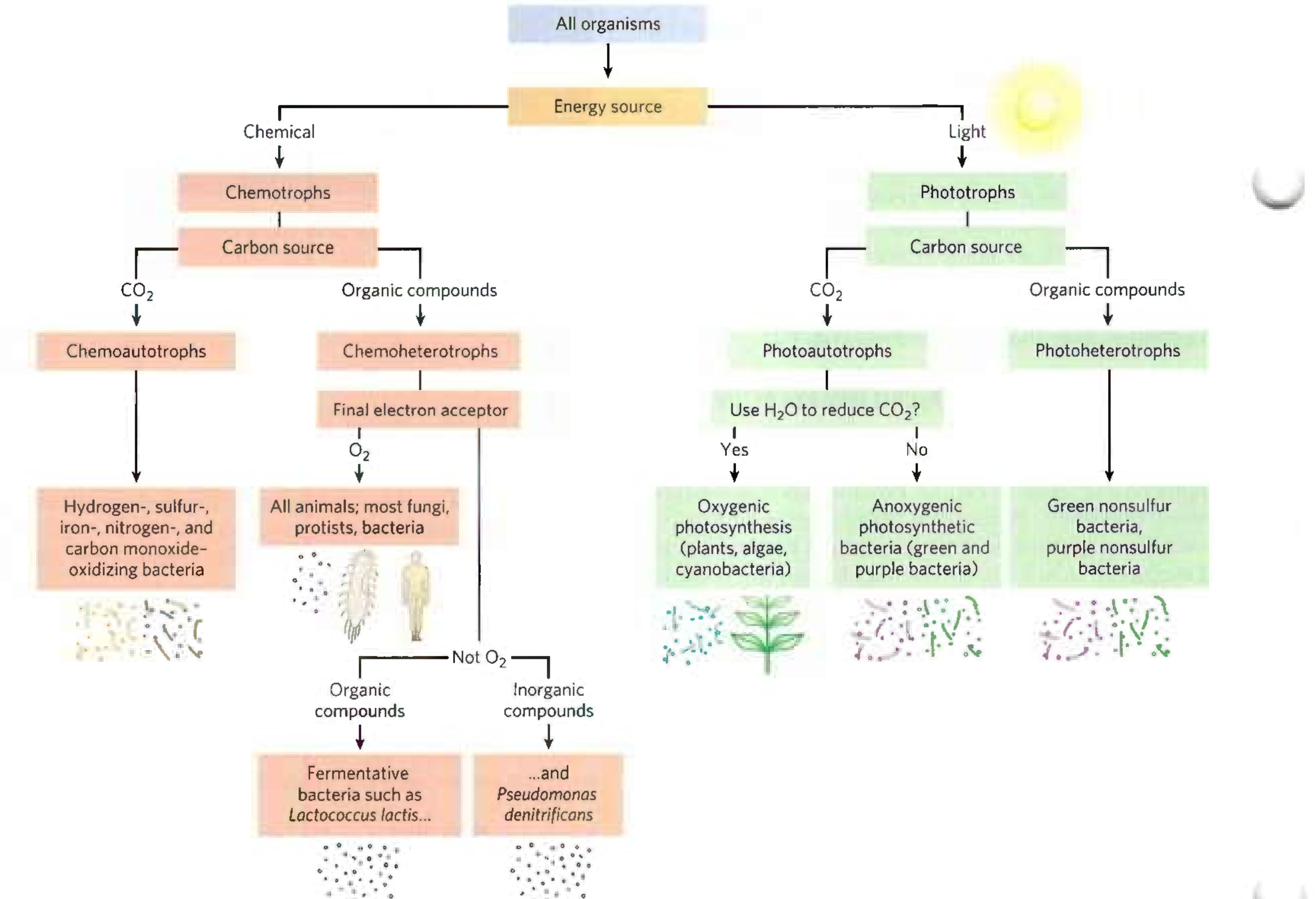

All organisms
Energy source
Chemical
Light
Chemotrophs
Phototrophs
Carbon source
Carbon source
CO_2
Organic compounds
CO_2
Organic compounds
Chemoautotrophs
Chemoheterotrophs
Photoautotrophs
Photoheterotrophs
Final electron acceptor
Use H_2O to reduce CO_2?
O_2
Yes
No
Hydrogen-, sulfur-, iron-, nitrogen-, and carbon monoxide-oxidizing bacteria
All animals; most fungi, protists, bacteria
Oxygenic photosynthesis (plants, algae, cyanobacteria)
Anoxygenic photosynthetic bacteria (green and purple bacteria)
Green nonsulfur bacteria, purple nonsulfur bacteria
Not O_2
Organic compounds
Inorganic compounds
Fermentative bacteria such as *Lactococcus lactis*...
...and *Pseudomonas denitrificans*

FIGURE 1-4 All organisms can be classified according to their source of energy (sunlight or oxidizable chemical compounds) and their source of carbon for the synthesis of cellular material.

are two broad categories based on energy sources: **phototrophs** (Greek *trophē*, "nourishment") trap and use sunlight, and **chemotrophs** derive their energy from oxidation of a chemical fuel. Some chemotrophs oxidize inorganic fuels — HS^- to S^0 (elemental sulfur), S^0 to SO_4^-, NO_2^- to NO_3^-, or Fe^{2+} to Fe^{3+}, for example. Phototrophs and chemotrophs may be further divided into those that can synthesize all of their biomolecules directly from CO_2 (**autotrophs**) and those that require some preformed organic nutrients made by other organisms (**heterotrophs**). We can describe an organism's mode of nutrition by combining these terms. For example, cyanobacteria are photoautotrophs; humans are chemoheterotrophs. Even finer distinctions can be made, and many organisms can obtain energy and carbon from more than one source under different environmental or developmental conditions.

Bacterial and Archaeal Cells Share Common Features but Differ in Important Ways

The best-studied bacterium, *Escherichia coli*, is a usually harmless inhabitant of the human intestinal tract. The *E. coli* cell (**Fig. 1-5a**) is an ovoid about 2 μm long and a little less than 1 μm in diameter, but other bacteria may be spherical or rod-shaped, and some are substantially larger. *E. coli* has a protective outer membrane and an inner plasma membrane that encloses the cytoplasm and the nucleoid. Between the inner and outer membranes is a thin but strong layer of a high molecular weight polymer (peptidoglycan) that gives the cell its shape and rigidity. The plasma membrane and the layers outside it constitute the **cell envelope**. The plasma membranes of bacteria consist of a thin bilayer of lipid molecules penetrated by proteins. Archaeal plasma membranes have

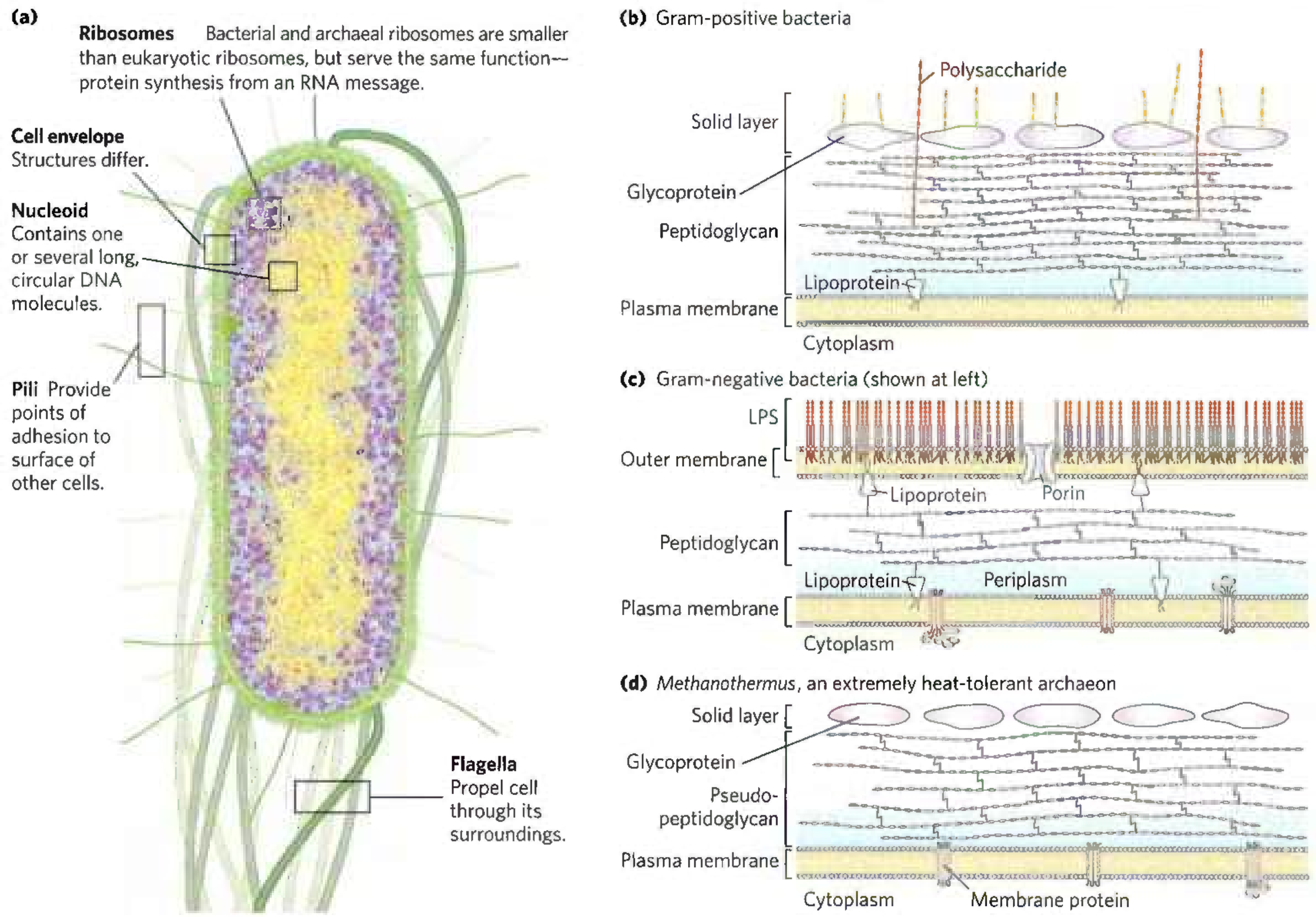

(a)
Ribosomes Bacterial and archaeal ribosomes are smaller than eukaryotic ribosomes, but serve the same function—protein synthesis from an RNA message.
Cell envelope Structures differ.
Nucleoid Contains one or several long, circular DNA molecules.
Pili Provide points of adhesion to surface of other cells.
Flagella Propel cell through its surroundings.
(b) Gram-positive bacteria
Polysaccharide
Solid layer
Glycoprotein
Peptidoglycan
Lipoprotein
Plasma membrane
Cytoplasm
(c) Gram-negative bacteria (shown at left)
LPS
Outer membrane
Lipoprotein
Porin
Peptidoglycan
Lipoprotein
Periplasm
Plasma membrane
Cytoplasm
(d) Methanothermus, an extremely heat-tolerant archaeon
Solid layer
Glycoprotein
Pseudo-peptidoglycan
Plasma membrane
Cytoplasm
Membrane protein

FIGURE 1-5 Some common structural features of bacterial and archaeal cells. (a) This correct-scale drawing of *E. coli* serves to illustrate some common features. (b) The cell envelope of gram-positive bacteria is a single membrane with a thick, rigid layer of peptidoglycan on its outside surface. A variety of polysaccharides and other complex polymers are interwoven with the peptidoglycan, and surrounding the whole is a porous "solid layer" composed of glycoproteins. (c) *E. coli* is gram-negative and has a double membrane. Its outer membrane has a lipopolysaccharide (LPS) on the outer surface and phospholipids on the inner surface. This outer membrane is studded with protein channels (porins) that allow small molecules, but not proteins, to diffuse through. The inner (plasma) membrane, made of phospholipids and proteins, is impermeable to both large and small molecules. Between the inner and outer membranes, in the periplasm, is a thin layer of peptidoglycan, which gives the cell shape and rigidity, but does not retain Gram's stain. (d) Archaeal membranes vary in structure and composition, but all have a single membrane surrounded by an outer layer that includes either a peptidoglycan-like structure or a porous protein shell (solid layer), or both. [(a) David S. Goodsell. (b, c, d) Information from S.-V. Albers and B. H. Meyer, *Nature Rev. Microbiol.* 9:414, 2011, Fig. 2.]

a similar architecture, but the lipids can be strikingly different from those of bacteria (see Fig. 10-6).

Bacteria and archaea have group-specific specializations of their cell envelopes (Fig. 1-5b–d). Some bacteria, called gram-positive because they are colored by Gram's stain (introduced by Hans Christian Gram in 1884), have a thick layer of peptidoglycan outside their plasma membrane but lack an outer membrane. Gram-negative bacteria have an outer membrane composed of a lipid bilayer into which are inserted complex lipopolysaccharides and proteins called porins that provide transmembrane channels for the diffusion of low molecular weight compounds and ions across this outer membrane. The structures outside the plasma membrane of archaea differ from organism to organism, but they, too, have a layer of peptidoglycan or protein that confers rigidity on their cell envelopes.

P2 The cytoplasm of *E. coli* contains about 15,000 ribosomes, various numbers (from 10 to thousands) of copies of each of 1,000 or so different enzymes, perhaps 1,000 organic compounds of molecular weight less than 1,000 (metabolites and cofactors), and a variety of inorganic ions. The nucleoid contains a single, circular molecule of DNA, and the cytoplasm (like that of most bacteria) contains one or more smaller, circular segments of DNA called **plasmids**. In nature, some plasmids confer resistance to toxins and antibiotics in the environment. In the laboratory, these DNA segments are especially amenable to experimental manipulation and are powerful tools for genetic engineering (see Chapter 9).

Other species of bacteria, as well as archaea, contain a similar collection of biomolecules, but each species has physical and metabolic specializations related to its environmental niche and nutritional sources. Cyanobacteria, for example, have internal membranes specialized to trap energy from light (see Fig. 20-23). Many archaea live in extreme environments and have biochemical adaptations to survive in extremes of temperature, pressure, or salt concentration. Differences in ribosomal structure gave the first hints that Bacteria and Archaea constituted separate domains. Most bacteria (including *E. coli*) exist as individual cells, but often associate in biofilms or mats, in which large numbers of cells adhere to each other and to some solid substrate beneath or at an aqueous surface. Cells of some bacterial species (the myxobacteria, for example) show simple social behavior, forming many-celled aggregates in response to signals between neighboring cells.

Eukaryotic Cells Have a Variety of Membranous Organelles, Which Can Be Isolated for Study

Typical eukaryotic cells (**Fig. 1-6**) are much larger than bacteria — commonly 5 to 100 μm in diameter, with cell volumes a thousand to a million times larger than those of bacteria. The distinguishing characteristics of eukaryotes are the nucleus and a variety of membrane-enclosed organelles with specific functions. These organelles include **mitochondria**, the site of most of the energy-extracting reactions of the cell; the **endoplasmic reticulum** and **Golgi complexes**, which play central roles in the synthesis and processing of lipids and membrane proteins; **peroxisomes**, in which very-long-chain fatty acids are oxidized and reactive oxygen species are detoxified; and **lysosomes**, filled with digestive enzymes to degrade unneeded cellular debris. In addition to these, plant cells contain **vacuoles** (which store large quantities of organic acids) and **chloroplasts** (in which sunlight drives the synthesis of ATP (adenosine triphosphate) in the process of photosynthesis). Also present in the cytoplasm of many cells are granules or droplets containing stored nutrients such as starch and fat.

In a major advance in biochemistry, Albert Claude, Christian de Duve, and George Palade developed methods for separating organelles from the cytosol and from each other — an essential step in investigating their structures and functions. In a typical cell fractionation (**Fig. 1-7**), cells or tissues in solution are gently disrupted by physical shear. This treatment ruptures the plasma membrane but leaves most of the organelles intact. The homogenate is then centrifuged; organelles such as nuclei, mitochondria, and lysosomes differ in size and therefore sediment at different rates.

These methods were used to establish, for example, that lysosomes contain degradative enzymes, mitochondria contain oxidative enzymes, and chloroplasts contain photosynthetic pigments. The isolation of an organelle enriched in a certain enzyme is often the first step in the purification of that enzyme.

The Cytoplasm Is Organized by the Cytoskeleton and Is Highly Dynamic

Fluorescence microscopy reveals several types of protein filaments crisscrossing the eukaryotic cell, forming an interlocking three-dimensional meshwork, the **cytoskeleton**. Eukaryotes have three general types of cytoplasmic filaments — actin filaments, microtubules, and intermediate filaments (**Fig. 1-8**) — differing in width (from about 6 nm to 22 nm), composition, and specific function. All types provide structure and organization to the cytoplasm and shape to the cell. Actin filaments and microtubules also help to produce the motion of organelles or of the whole cell.

Each type of cytoskeletal component consists of simple protein subunits that associate noncovalently to form filaments of uniform thickness. These filaments are not permanent structures; they undergo constant disassembly into their protein subunits and reassembly into filaments. Their locations in cells are not rigidly fixed but may change dramatically with mitosis, cytokinesis, amoeboid motion, or other changes in cell shape. The assembly, disassembly, and location of all types of filaments are regulated by other proteins, which serve to link or bundle the filaments or to move cytoplasmic organelles along

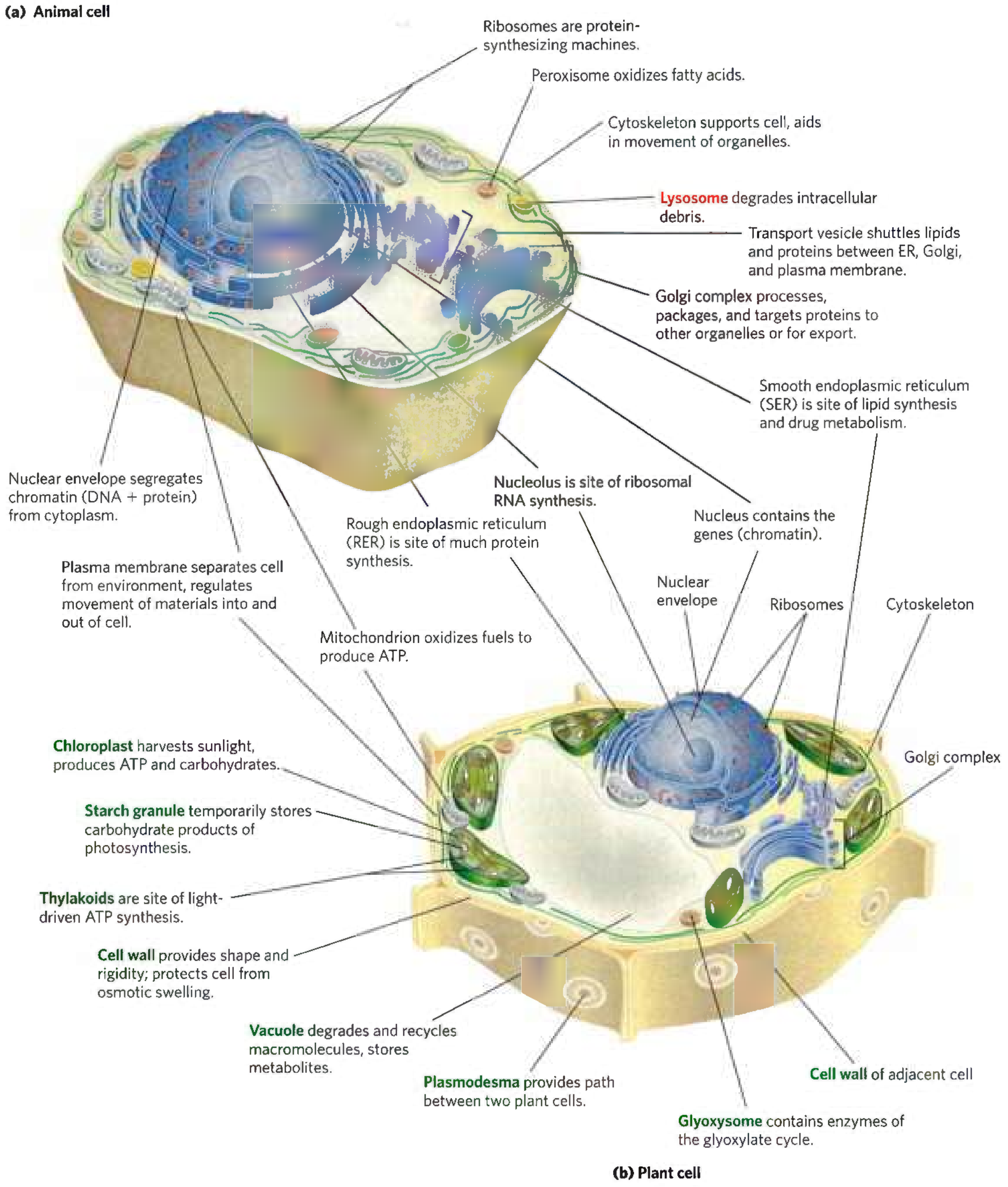

(a) Animal cell
Ribosomes are protein-synthesizing machines.
Peroxisome oxidizes fatty acids.
Cytoskeleton supports cell, aids in movement of organelles.
Lysosome degrades intracellular debris.
Transport vesicle shuttles lipids and proteins between ER, Golgi, and plasma membrane.
Golgi complex processes, packages, and targets proteins to other organelles or for export.
Smooth endoplasmic reticulum (SER) is site of lipid synthesis and drug metabolism.
Nuclear envelope segregates chromatin (DNA + protein) from cytoplasm.
Nucleolus is site of ribosomal RNA synthesis.
Rough endoplasmic reticulum (RER) is site of much protein synthesis.
Nucleus contains the genes (chromatin).
Plasma membrane separates cell from environment, regulates movement of materials into and out of cell.
Nuclear envelope
Ribosomes
Cytoskeleton
Mitochondrion oxidizes fuels to produce ATP.
Chloroplast harvests sunlight, produces ATP and carbohydrates.
Golgi complex
Starch granule temporarily stores carbohydrate products of photosynthesis.
Thylakoids are site of light-driven ATP synthesis.
Cell wall provides shape and rigidity; protects cell from osmotic swelling.
Vacuole degrades and recycles macromolecules, stores metabolites.
Plasmodesma provides path between two plant cells.
Cell wall of adjacent cell
Glyoxysome contains enzymes of the glyoxylate cycle.
(b) Plant cell

FIGURE 1-6 Eukaryotic cell structure. Schematic illustrations of two major types of eukaryotic cell: (a) a representative animal cell and (b) a representative plant cell. Plant cells are usually 10 to 100 μm in diameter — larger than animal cells, which typically range from 5 to 30 μm. Structures labeled in red are unique to animal cells; those labeled in green are unique to plant cells. Eukaryotic microorganisms (such as protists and fungi) have structures similar to those in plant and animal cells, but many also contain specialized organelles not illustrated here.

the filaments. (Bacteria contain actinlike proteins that serve similar roles in those cells.)

The filaments disassemble and then reassemble elsewhere. Membranous vesicles bud from one organelle and fuse with another. Organelles move through the cytoplasm along protein filaments, their motion powered by energy-dependent motor proteins. The **endomembrane system** (see Fig. 11-4) segregates specific metabolic

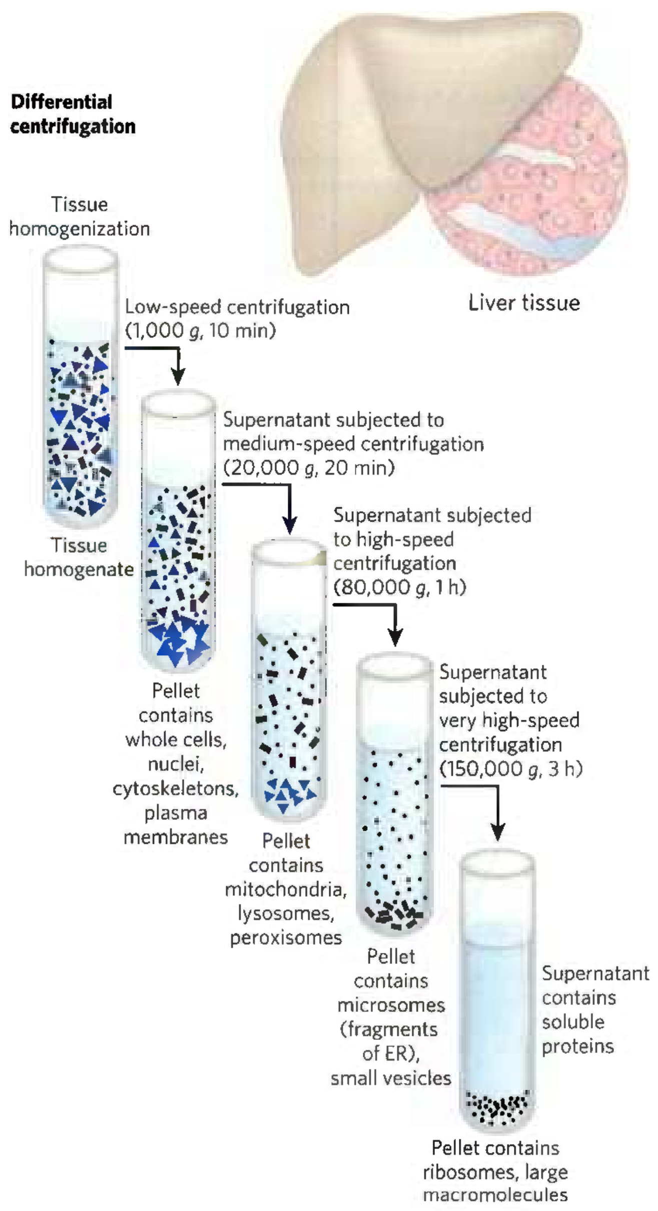

Differential centrifugation
Tissue homogenization
Liver tissue
Low-speed centrifugation (1,000 *g*, 10 min)
Tissue homogenate
Supernatant subjected to medium-speed centrifugation (20,000 *g*, 20 min)
Pellet contains whole cells, nuclei, cytoskeletons, plasma membranes
Supernatant subjected to high-speed centrifugation (80,000 *g*, 1 h)
Pellet contains mitochondria, lysosomes, peroxisomes
Supernatant subjected to very high-speed centrifugation (150,000 *g*, 3 h)
Pellet contains microsomes (fragments of ER), small vesicles
Supernatant contains soluble proteins
Pellet contains ribosomes, large macromolecules

FIGURE 1-7 Subcellular fractionation of tissue. A tissue such as liver is first mechanically homogenized to break cells and disperse their contents in an aqueous buffer. The sucrose medium has an osmotic pressure similar to that in organelles, thus balancing diffusion of water into and out of the organelles, which would swell and burst in a solution of lower osmolarity (see Fig. 2-12). The large and small particles in the suspension can be separated by centrifugation at different speeds. Larger particles sediment more rapidly than small particles, and soluble material does not sediment. By careful choice of the conditions of centrifugation, subcellular fractions can be separated for biochemical characterization. [Information from B. Alberts et al., *Molecular Biology of the Cell*, 2nd edn, p. 165, Garland Publishing, 1989.]

processes and provides surfaces on which certain enzyme-catalyzed reactions occur. **Exocytosis** and **endocytosis**, mechanisms of transport (out of and into cells, respectively) that involve membrane fusion and fission, provide paths between the cytoplasm and the surrounding medium, allowing the secretion of substances produced in the cell and uptake of extracellular materials.

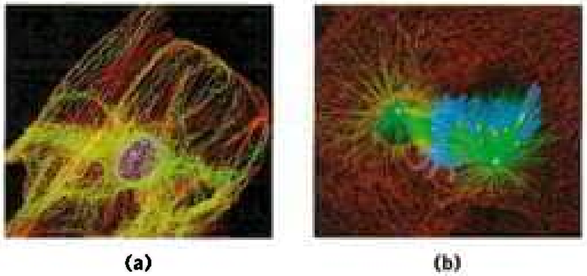


FIGURE 1-8 The three types of cytoskeletal filaments: actin filaments, microtubules, and intermediate filaments. Cellular structures can be labeled with an antibody (that recognizes a characteristic protein) covalently attached to a fluorescent compound. The stained structures are visible when the cell is viewed with a fluorescence microscope. (a) In this cultured fibroblast cell, bundles of actin filaments are stained red; microtubules, radiating from the cell center, are stained green; and chromosomes (in the nucleus) are stained blue. (b) A newt lung cell undergoing mitosis. Microtubules (green), attached to structures called kinetochores (yellow) on the condensed chromosomes (blue), pull the chromosomes to opposite poles, or centrosomes (magenta), of the cell. Intermediate filaments, made of keratin (red), maintain the structure of the cell. [(a) James J. Faust and David G. Capco. (b) Dr. Alexey Khodjakov, Wadsworth Center, New York State Department of Health.]

This structural organization of the cytoplasm is far from random. The motion and positioning of organelles and cytoskeletal elements are under tight regulation, and at certain stages in its life, a eukaryotic cell undergoes dramatic, finely orchestrated reorganizations, such as the events of mitosis. The interactions between the cytoskeleton and organelles are noncovalent, reversible, and subject to regulation in response to various intracellular and extracellular signals.

Cells Build Supramolecular Structures

Macromolecules and their monomeric subunits differ greatly in size. An alanine molecule is less than 0.5 nm long. **P2** A molecule of hemoglobin, the oxygen-carrying protein of erythrocytes (red blood cells), consists of nearly 600 amino acid subunits in four long chains, folded into globular shapes and associated in a structure 5.5 nm in diameter. In turn, proteins are much smaller than ribosomes (about 20 nm in diameter), which are much smaller than organelles such as mitochondria, typically 1 μm in diameter. It is a long jump from simple biomolecules to cellular structures that can be seen with the light microscope. **Figure 1-9** illustrates the structural hierarchy in cellular organization.

The monomeric subunits of proteins, nucleic acids, and polysaccharides are joined by covalent bonds. In supramolecular complexes, however, macromolecules are held together largely by noncovalent interactions—much weaker, individually, than covalent bonds. Among these noncovalent interactions are hydrogen bonds; ionic interactions (between charged groups); and aggregations of nonpolar groups in aqueous solution, brought about by van der Waals interactions (also called London

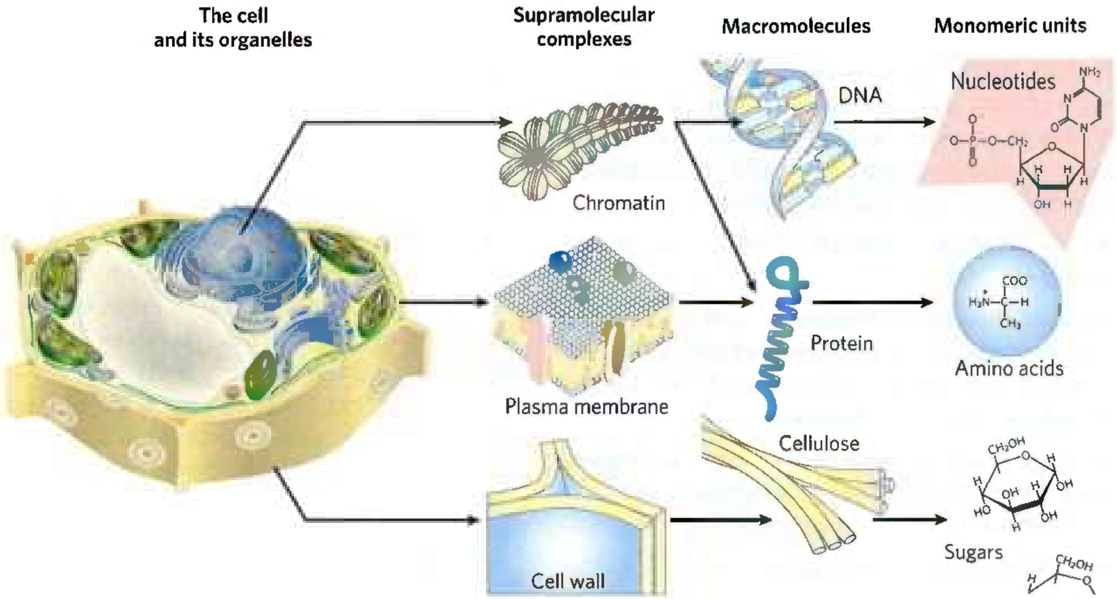


FIGURE 1-9 Structural hierarchy in the molecular organization of cells. The organelles and other relatively large components of cells are composed of supramolecular complexes, which in turn are composed of smaller macromolecules and even smaller molecular subunits. For example, the nucleus of this plant cell contains chromatin, a supramolecular complex that consists of DNA and basic proteins (histones). DNA is made up of simple monomeric subunits (nucleotides), as are proteins (amino acids). [Information from W. M. Becker and D. W. Deamer, *The World of the Cell*, 2nd edn, Fig. 2-15, Benjamin/Cummings Publishing Company, 1991.]

forces) and by the hydrophobic effect—all of which have energies much smaller than those of covalent bonds. (These noncovalent interactions are described in Chapter 2.) The large numbers of weak interactions between macromolecules in supramolecular complexes stabilize these assemblies, producing their unique structures.

In Vitro Studies May Overlook Important Interactions among Molecules

One approach to understanding a biological process is to study purified molecules in vitro (from the Latin, meaning "in glass"—in the test tube), without interference from other molecules present in the intact cell—that is, in vivo (from the Latin, meaning "in the living"). Although this approach has been remarkably revealing, we must keep in mind that the inside of a cell is quite different from the inside of a test tube. The "interfering" components eliminated by purification may be critical to the biological function or regulation of the molecule that is being purified. For example, in vitro studies of pure enzymes are commonly done at very low enzyme concentrations in thoroughly stirred aqueous solutions. In the cell, an enzyme is dissolved or suspended in the gel-like cytosol with thousands of other proteins, some of which bind to that enzyme and influence its activity. Some enzymes are components of multienzyme complexes in which reactants are channeled from one enzyme to another, never entering the bulk solvent. When all of the known macromolecules in a cell are represented in their known dimensions and concentrations (**Fig. 1-10**), it is clear that the cytosol is very crowded and that diffusion of macromolecules within the cytosol must be slowed by collisions with other large structures. In short, a given molecule may behave quite differently in the cell than it behaves in vitro. A central challenge of biochemistry is to understand the influences of cellular organization and macromolecular associations on the function of individual enzymes and other biomolecules—to understand function in vivo as well as in vitro.

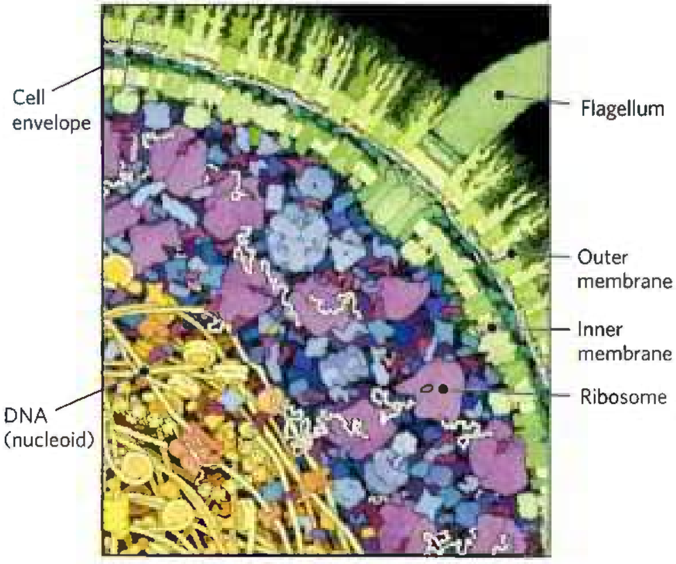


FIGURE 1-10 The crowded cell. This drawing is an accurate representation of the relative sizes and numbers of macromolecules in one small region of an *E. coli* cell. [© David S. Goodsell 1999.]

SUMMARY 1.1 *Cellular Foundations*

- All cells share certain fundamental properties: they are bounded by a plasma membrane; have a cytosol containing metabolites, coenzymes, inorganic ions, and enzymes; and have a set of genes contained within a nucleoid (bacteria and archaea) or a nucleus (eukaryotes).
- The size of cells is limited by the need to deliver oxygen to all parts of the cell.
- By comparing their DNA sequences, researchers can place organisms in three domains: Bacteria, Archaea, and Eukarya. Archaea and Eukarya are more closely related to each other than either is to Bacteria.
- All organisms require a source of energy to perform cellular work. Phototrophs obtain energy from sunlight; chemotrophs obtain energy from chemical fuels.
- Bacterial and archaeal cells contain cytosol, a nucleoid, and plasmids, all within a cell envelope.
- Eukaryotes contain a nucleus and a variety of membrane-enclosed organelles with specialized functions, which can be studied in the isolated organelles.
- Cytoskeletal proteins assemble into long filaments that give cells shape and rigidity and serve as rails along which cellular organelles move throughout the cell. The membrane-bounded compartments constitute an interconnected and dynamic endomembrane system.
- Supramolecular complexes held together by noncovalent interactions are part of a hierarchy of structures, some visible with the light microscope.
- Studying isolated cellular components in vitro simplifies the experimental system, but such study may overlook important interactions that occur in the living cell.

1.2 Chemical Foundations

Biochemistry aims to explain biological form and function in chemical terms. During the first half of the twentieth century, parallel biochemical investigations of glucose breakdown in yeast and in animal muscle cells revealed remarkable chemical similarities between these two apparently very different cell types; for example, the breakdown of glucose in yeast and in muscle cells involved the same 10 chemical intermediates and the same 10 enzymes. Subsequent studies of many other biochemical processes in many different organisms have confirmed the generality of this observation, neatly summarized in 1954 by the biochemist Jacques Monod: "What is true of *E. coli* is true of the elephant." The current understanding that all organisms share a common evolutionary origin is based in part on this observed universality of chemical intermediates and transformations, often termed "biochemical unity."

Fewer than 30 of the more than 90 naturally occurring chemical elements are known to be essential to organisms. Most of the elements in living matter have a relatively low atomic number; only three have an atomic number above that of selenium, 34 (**Fig. 1-11**). The four most abundant elements in living organisms, in terms of percentage of total number of atoms, are hydrogen, oxygen, nitrogen, and carbon, which together make up more than 99% of the mass of most cells. They are the lightest elements capable of efficiently forming one, two, three, and four bonds, respectively; in general, the lightest elements form the strongest bonds. The trace elements represent a miniscule fraction of the weight of the human body, but all are essential to life, usually because they are essential to the function of specific proteins, including many enzymes. The oxygen-transporting capacity of the hemoglobin molecule, for example, is absolutely dependent on four iron ions that make up only 0.3% of the molecule's mass.

Biomolecules Are Compounds of Carbon with a Variety of Functional Groups

The chemistry of living organisms is organized around carbon, which accounts for more than half of the dry weight of cells. Carbon can form single bonds with hydrogen atoms and can form both single bonds and double bonds with oxygen and nitrogen atoms (**Fig. 1-12**). Of greatest significance in biology is the ability of carbon atoms to form very stable single bonds with up to four other carbon

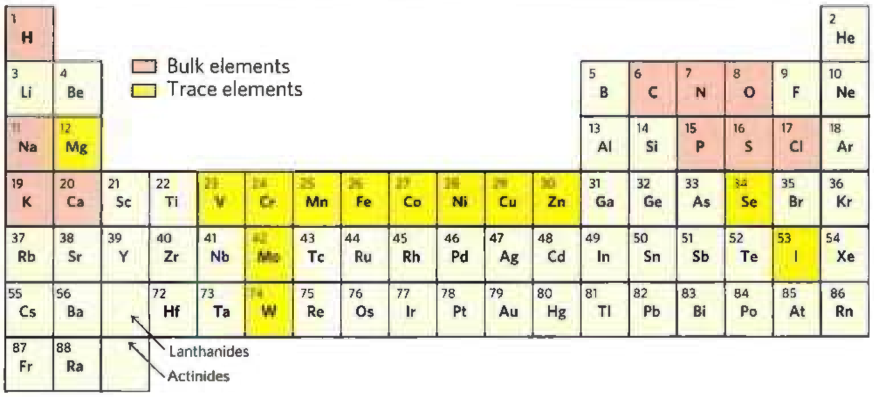


FIGURE 1-11 Elements essential to animal life and health. Bulk elements (shaded light red) are structural components of cells and tissues and are required in the diet in gram quantities daily. For trace elements (shaded yellow), the requirements are much smaller: for humans, a few milligrams per day of Fe, Cu, and Zn, and even less of the others. The elemental requirements for plants and microorganisms are similar to those shown here; the ways in which they acquire these elements vary.

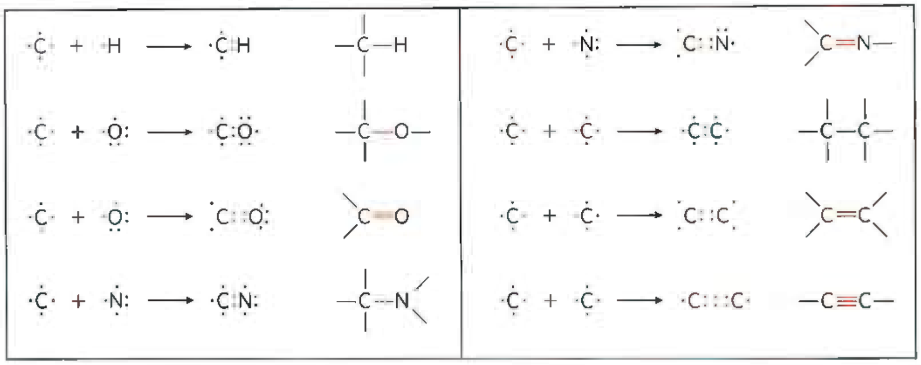


FIGURE 1-12 Versatility of carbon bonding. Carbon can form covalent single, double, and triple bonds (all bonds in red), particularly with other carbon atoms. Triple bonds are rare in biomolecules.

atoms. Two carbon atoms also can share two (or three) electron pairs, thus forming double (or triple) bonds.

The four single bonds that can be formed by a carbon atom project from the nucleus to the four apices of a tetrahedron (**Fig. 1-13**), with an angle of about 109.5° between any two bonds and an average bond length of 0.154 nm. There is free rotation around each single bond, unless very large or highly charged groups are attached to both carbon atoms, in which case rotation may be restricted. A double bond is shorter (about 0.134 nm) and rigid, and it allows only limited rotation about its axis.

Covalently linked carbon atoms in biomolecules can form linear chains, branched chains, and cyclic structures. It seems likely that the bonding versatility of carbon, with itself and with other elements, was a major factor in the selection of carbon compounds for the molecular machinery of cells during the origin and evolution of living organisms. No other chemical element can form molecules of such widely different sizes, shapes, and composition.

Most biomolecules can be regarded as derivatives of hydrocarbons, with hydrogen atoms replaced by a variety of functional groups that confer specific chemical properties on the molecule, forming various families of organic compounds. Typical of these are alcohols, which have one or more hydroxyl groups; amines, with amino groups; aldehydes and ketones, with carbonyl groups; and carboxylic acids, with carboxyl groups (**Fig. 1-14**). Many biomolecules are polyfunctional, containing two or more types of functional groups (**Fig. 1-15**), each with its own chemical characteristics and reactions. The chemical "personality" of a compound is determined by the chemistry of its functional groups and their disposition in three-dimensional space.

Cells Contain a Universal Set of Small Molecules

Dissolved in the aqueous phase (cytosol) of all cells is a collection of perhaps several thousand different small organic molecules (M_r ~100 to ~500), with intracellular concentrations ranging from nanomolar to >10 mM (see Fig. 13-31). (See **Box 1-1** for an explanation of the various ways of referring to molecular weight.) These are the central metabolites in the major pathways occurring in nearly every cell—the metabolites and pathways that have been conserved throughout the course of evolution. This collection of molecules includes the common amino acids, nucleotides, sugars and their phosphorylated derivatives, and mono-, di-, and tricarboxylic acids. The molecules may be polar or charged and most are water-soluble. They are trapped in the cell because the plasma membrane is impermeable to them, although specific membrane transporters can catalyze the movement of some molecules into and out of the cell or between compartments in eukaryotic cells. The universal occurrence of the same set of compounds in living cells reflects the evolutionary conservation of metabolic pathways that developed in the earliest cells.

There are other small biomolecules, specific to certain types of cells or organisms. For example, vascular

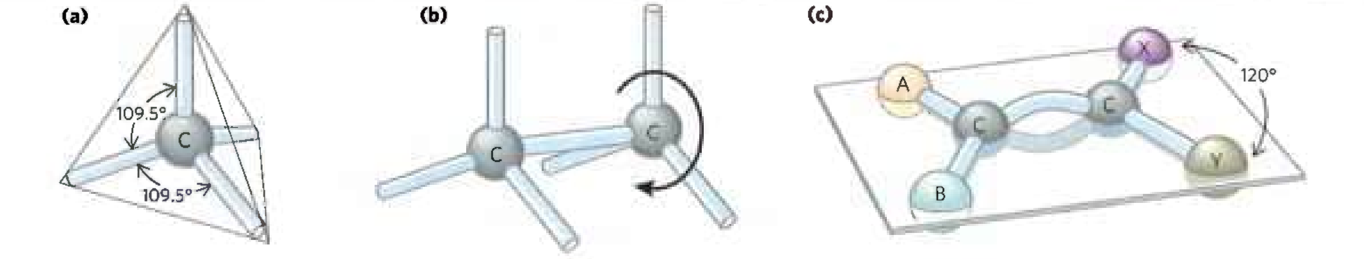


FIGURE 1-13 Geometry of carbon bonding. (a) Carbon atoms have a characteristic tetrahedral arrangement of their four single bonds. (b) Carbon–carbon single bonds have freedom of rotation, as shown for the compound ethane ($CH_3—CH_3$). (c) Double bonds are shorter and do not allow free rotation. The two doubly bonded carbons and the atoms designated A, B, X, and Y all lie in the same rigid plane.

plants contain, in addition to the universal set, small molecules called **secondary metabolites**, which play roles specific to plant life. These metabolites include compounds that give plants their characteristic scents and colors, and compounds such as morphine, quinine, nicotine, and caffeine that are valued for their physiological effects on humans but have other purposes in plants.

The entire collection of small molecules in a given cell under a specific set of conditions has been called the **metabolome**, in parallel with the term "genome." **Metabolomics** is the systematic characterization of the metabolome under very specific conditions (such as following administration of a drug, or a biological signal such as insulin).

Macromolecules Are the Major Constituents of Cells

Many biological molecules are **macromolecules**, polymers with molecular weights above ~5,000 that are assembled from relatively simple precursors (**Fig. 1-16**). Shorter polymers are called **oligomers** (Greek *oligos*, "few"). Proteins, nucleic acids, and polysaccharides are macromolecules composed of monomers with molecular weights of 500 or less. Synthesis of macromolecules is a major energy-consuming activity of cells. Macromolecules themselves may be further assembled into supramolecular complexes, forming functional units such as ribosomes. **Table 1-1** shows the major classes of biomolecules in an *E. coli* cell.

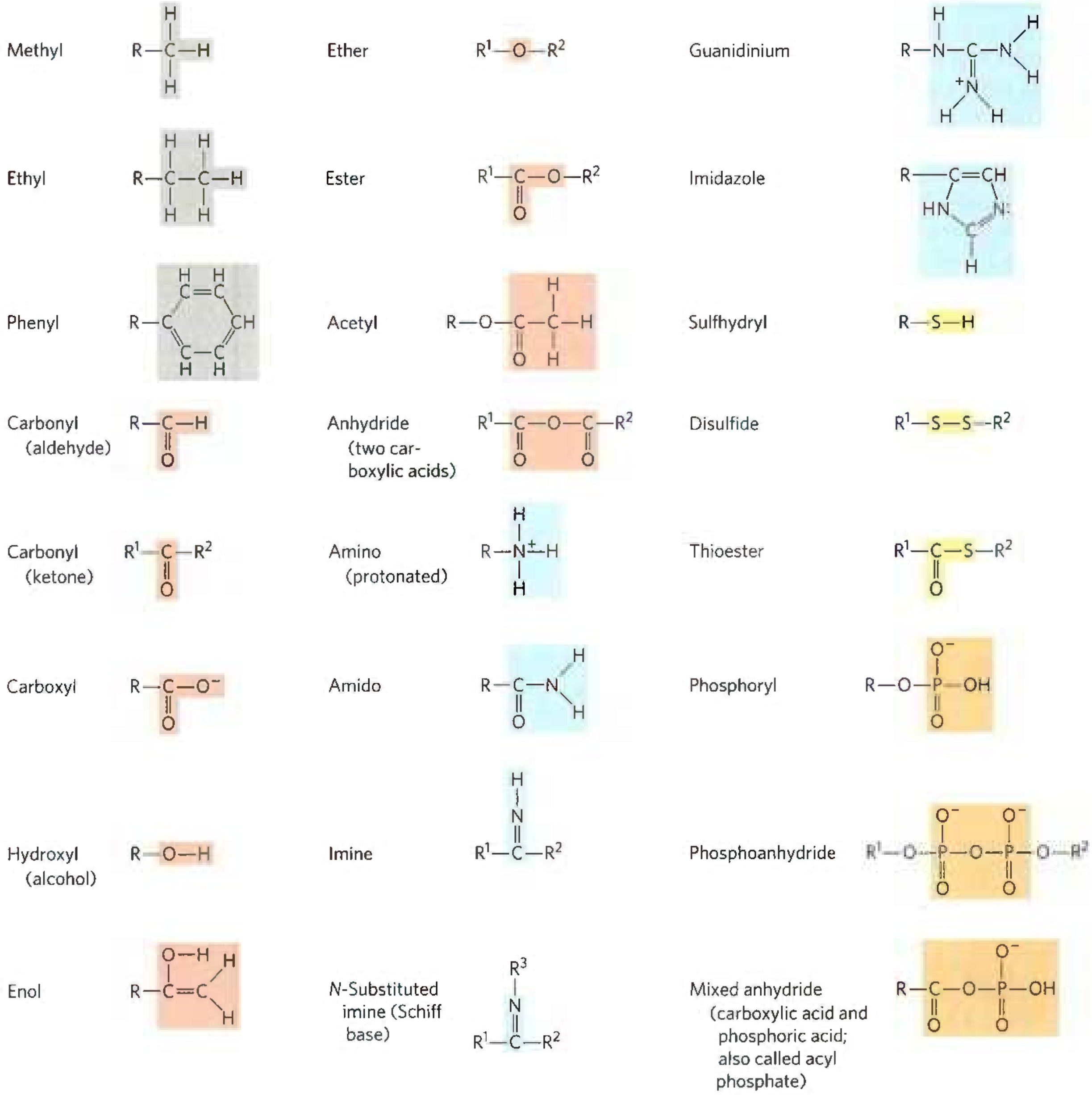

Methyl
Ethyl
Phenyl
Carbonyl (aldehyde)
Carbonyl (ketone)
Carboxyl
Hydroxyl (alcohol)
Enol
Ether
Ester
Acetyl
Anhydride (two carboxylic acids)
Amino (protonated)
Amido
Imine
N-Substituted imine (Schiff base)
Guanidinium
Imidazole
Sulfhydryl
Disulfide
Thioester
Phosphoryl
Phosphoanhydride
Mixed anhydride (carboxylic acid and phosphoric acid; also called acyl phosphate)

FIGURE 1-14 Some common functional groups of biomolecules. Functional groups are screened with a color typically used to represent the element that characterizes the group: gray for C, red for O, blue for N, yellow for S, and orange for P. In this figure and throughout the book, we use R to represent "any substituent." It may be as simple as a hydrogen atom, but typically it is a carbon-containing group. When two or more substituents are shown in a molecule, we designate them R^1, R^2, and so forth.

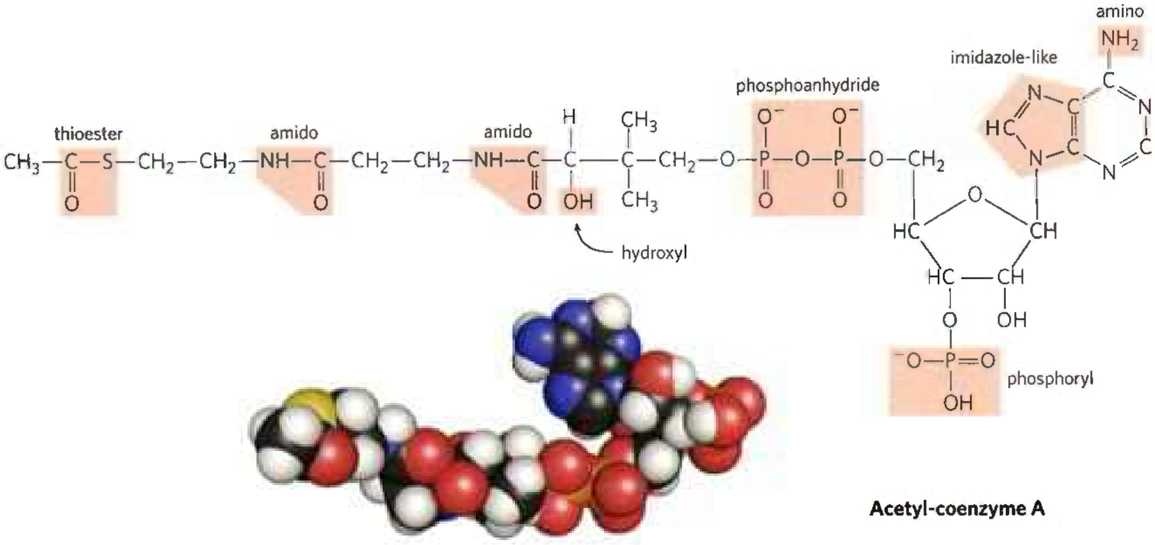


FIGURE 1-15 Several common functional groups in a single biomolecule. Acetyl-coenzyme A (often abbreviated as acetyl-CoA) is a carrier of acetyl groups in some enzymatic reactions. Its functional groups are screened in the structural formula. In the space-filling model, N is blue, C is black, P is orange, O is red, and H is white. The yellow atom at the left is the sulfur of the critical thioester bond between the acetyl moiety and coenzyme A. [Acetyl-CoA structure data from PDB ID 1DM3, Y. Modis and R. K. Wierenga, *J. Mol. Biol.* 297:1171, 2000.]

Proteins, long polymers of amino acids, constitute the largest mass fraction (besides water) of a cell. Some proteins have catalytic activity and function as enzymes; others serve as structural elements, signal receptors, or transporters that carry specific substances into or out of cells. Proteins are perhaps the most versatile of all biomolecules; a catalog of their many functions would be very long. The sum of all the proteins functioning in a given cell is the cell's **proteome**, and **proteomics** is the systematic characterization of this protein complement under a specific set of conditions. The **nucleic acids**, DNA and RNA, are polymers of nucleotides. They store and transmit genetic information, and some RNA molecules have structural and catalytic roles in supramolecular complexes. The **genome** is the entire sequence of a cell's DNA (or in the case of RNA viruses, its RNA), and **genomics** is the characterization of the structure, function, evolution, and mapping of genomes.

The **polysaccharides**, polymers of simple sugars such as glucose, have three major functions: as energy-rich fuel stores, as rigid structural components of cell walls (in plants and bacteria), and as extracellular recognition elements that bind to proteins on other cells. Shorter polymers of sugars (oligosaccharides) attached to proteins or lipids at the cell surface serve as specific cellular signals. A cell's **glycome** is its entire complement of carbohydrate-containing molecules. The **lipids**,

BOX 1-1

Molecular Weight, Molecular Mass, and Their Correct Units

There are two common (and equivalent) ways to describe molecular mass; both are used in this text. The first is *molecular weight*, or *relative molecular mass*, denoted M_r. The molecular weight of a substance is defined as the ratio of the mass of a molecule of that substance to one-twelfth the mass of an atom of carbon-12 (^{12}C). Since M_r is a ratio, it is dimensionless—it has no associated units. The second is *molecular mass*, denoted *m*. This is simply the mass of one molecule, or the molar mass divided by Avogadro's number. The molecular mass, *m*, is expressed in daltons (abbreviated Da). One dalton is equivalent to one-twelfth the mass of an atom of carbon-12; a kilodalton (kDa) is 1,000 daltons; a megadalton (MDa) is 1 million daltons.

Consider, for example, a molecule with a mass 1,000 times that of water. We can say of this molecule either $M_r = 18{,}000$ or $m = 18{,}000$ daltons. We can also describe it as an "18 kDa molecule." However, the expression $M_r = 18{,}000$ daltons is incorrect.

Another convenient unit for describing the mass of a single atom or molecule is the atomic mass unit (formerly amu, now commonly denoted u). One atomic mass unit (1 u) is defined as one-twelfth the mass of an atom of carbon-12. Since the experimentally measured mass of an atom of carbon-12 is 1.9926×10^{-23} g, $1\ u = 1.6606 \times 10^{-24}$ g. The atomic mass unit is convenient for describing the mass of a peak observed by mass spectrometry (see Chapter 3, p. 93).

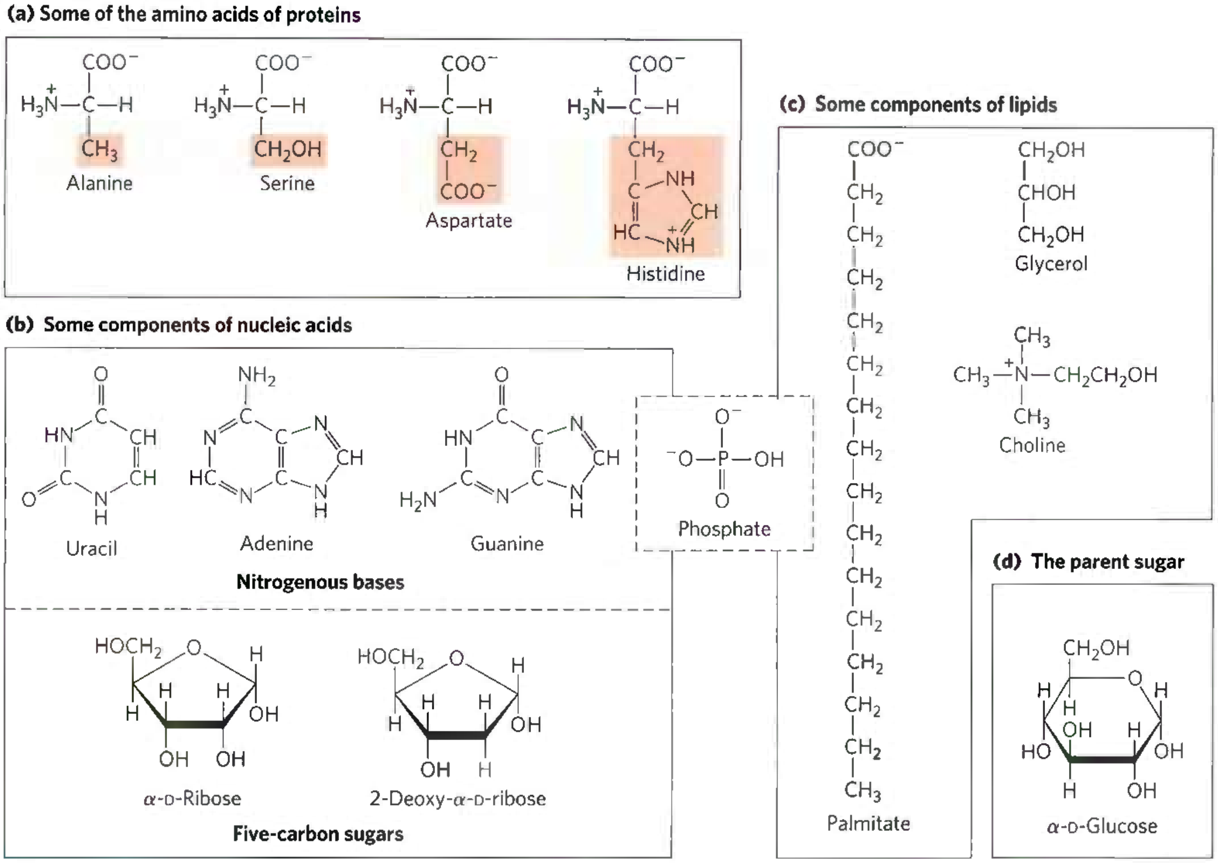

(a) Some of the amino acids of proteins
Alanine
Serine
Aspartate
Histidine
(b) Some components of nucleic acids
Uracil
Adenine
Guanine
Nitrogenous bases
α-D-Ribose
2-Deoxy-α-D-ribose
Five-carbon sugars
Phosphate
(c) Some components of lipids
Palmitate
Glycerol
Choline
(d) The parent sugar
α-D-Glucose

FIGURE 1-16 The organic compounds from which most cellular materials are constructed: the ABCs of biochemistry. Shown here are (a) 4 of the 20 amino acids from which all proteins are built (the side chains are shaded light red); (b) 3 of the 5 nitrogenous bases, the two 5-carbon sugars, and the phosphate ion from which all nucleic acids are built; (c) 4 components of membrane lipids (including phosphate); and (d) D-glucose, the simple sugar from which most carbohydrates are derived.

TABLE 1-1 Molecular Components of an *E. coli* Cell

	Percentage of total weight of cell	Approximate number of different molecular species
Water	70	1
Proteins	15	3,000
Nucleic acids		
DNA	1	1–4
RNA	6	>3,000
Polysaccharides	3	20
Lipids	2	50[a]
Monomeric subunits and intermediates	2	2,600
Inorganic ions	1	20

Source: A. C. Guo et al., *Nucleic Acids Res.* 41:D625, 2013.

[a] If all permutations and combinations of fatty acid substituents are considered, this number is much larger.

water-insoluble hydrocarbon derivatives, serve as structural components of membranes, energy-rich fuel stores, pigments, and intracellular signals. The lipid-containing molecules in a cell constitute its **lipidome**.

Proteins, polynucleotides, and polysaccharides have large numbers of monomeric subunits and thus high molecular weights—in the range of 5,000 to more than 1 million for proteins, up to several *billion* for DNA, and in the millions for polysaccharides such as starch. Individual lipid molecules are much smaller (M_r 750 to 1,500) and are not classified as macromolecules, but they can associate noncovalently into very large structures. Cellular membranes are built of enormous noncovalent aggregates of lipid and protein molecules.

Given their characteristic information-rich subunit sequences, proteins and nucleic acids are often referred to as **informational macromolecules**. Some oligosaccharides, as noted above, also serve as informational molecules.

Three-Dimensional Structure Is Described by Configuration and Conformation

The covalent bonds and functional groups of a biomolecule are, of course, central to its function, but so also is the arrangement of the molecule's constituent atoms in three-dimensional space—its stereochemistry. Carbon-containing compounds commonly exist as

stereoisomers, molecules with the same chemical bonds and same chemical formula but different **configuration**, the fixed spatial arrangement of atoms. Interactions between biomolecules are typically **stereospecific**, requiring specific configurations in the interacting molecules.

Figure 1-17 shows three ways to illustrate the stereochemistry, or configuration, of simple molecules. The perspective diagram specifies stereochemistry unambiguously, but bond angles and center-to-center bond lengths are better represented with ball-and-stick models. In space-filling models, the radius of each "atom" is proportional to its van der Waals radius, and the contours of the model define the space occupied by the molecule (the volume of space from which atoms of other molecules are excluded).

Configuration is conferred by the presence of either (1) double bonds, around which there is little or no freedom of rotation, or (2) chiral centers, around which substituent groups are arranged in a specific orientation. The identifying characteristic of stereoisomers is that they cannot be interconverted without the temporary breaking of one or more covalent bonds. **Figure 1-18a** shows the configurations of maleic acid and its isomer, fumaric acid. These compounds are **geometric isomers**, or **cis-trans isomers**; they differ in the arrangement of their substituent groups with respect to the nonrotating double bond (Latin *cis*, "on this side"—groups on the same side of the double bond; *trans*, "across"—groups on opposite sides).

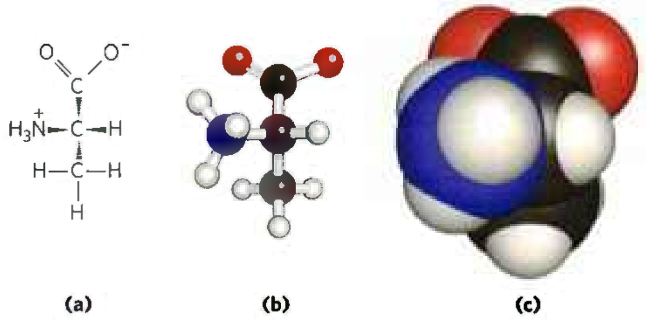
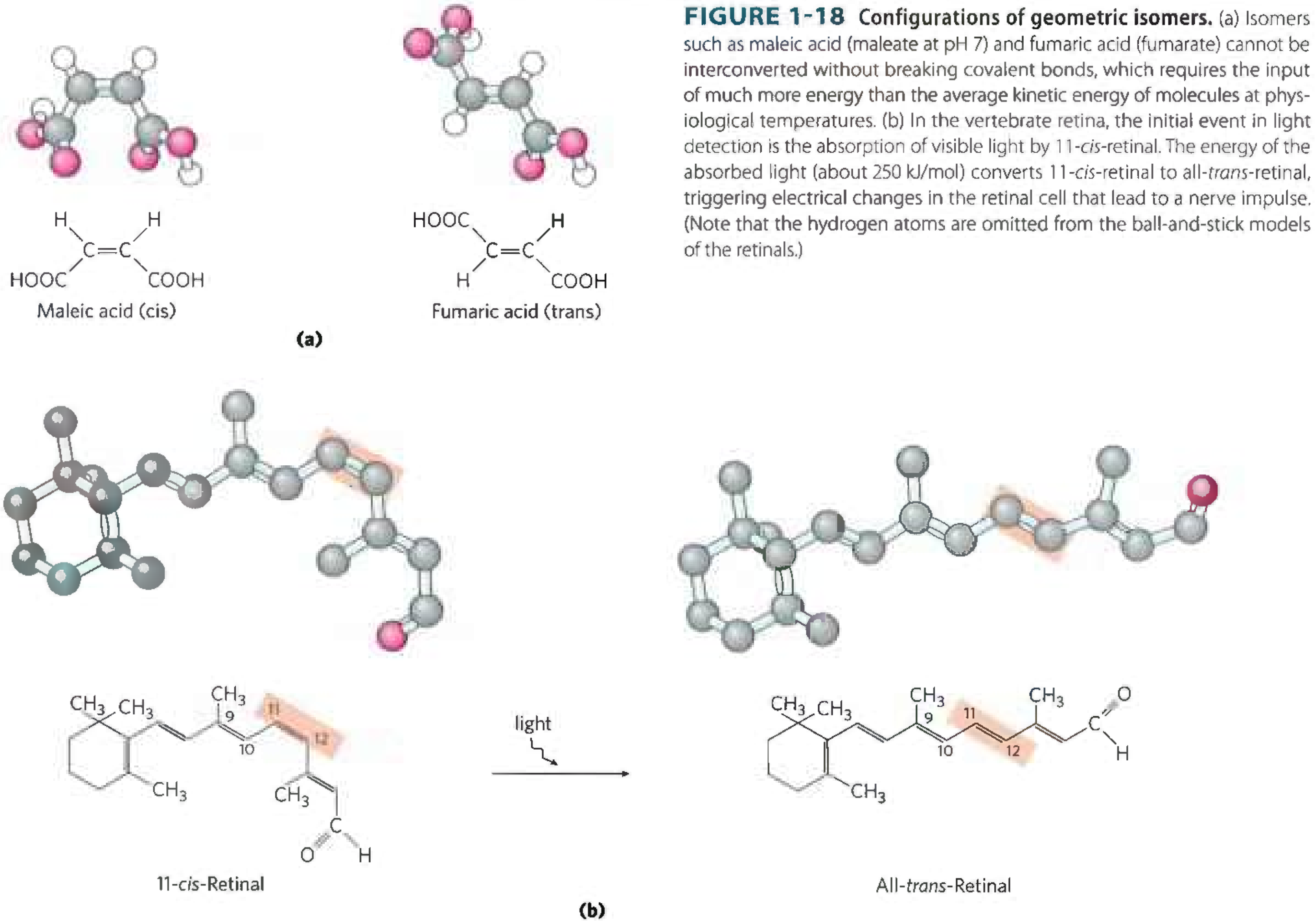


FIGURE 1-17 Representations of molecules. Three ways to represent the structure of the amino acid alanine (shown here in the ionic form found at neutral pH). (a) Structural formula in perspective form: a solid wedge (—) represents a bond in which the atom at the wide end projects out of the plane of the paper, toward the reader; a dashed wedge (—) represents a bond extending behind the plane of the paper. (b) Ball-and-stick model, showing bond angles and relative bond lengths. (c) Space-filling model, in which each atom is shown with its correct relative van der Waals radius.



Maleic acid (cis)

Fumaric acid (trans)

11-*cis*-Retinal

All-*trans*-Retinal

FIGURE 1-18 Configurations of geometric isomers. (a) Isomers such as maleic acid (maleate at pH 7) and fumaric acid (fumarate) cannot be interconverted without breaking covalent bonds, which requires the input of much more energy than the average kinetic energy of molecules at physiological temperatures. (b) In the vertebrate retina, the initial event in light detection is the absorption of visible light by 11-*cis*-retinal. The energy of the absorbed light (about 250 kJ/mol) converts 11-*cis*-retinal to all-*trans*-retinal, triggering electrical changes in the retinal cell that lead to a nerve impulse. (Note that the hydrogen atoms are omitted from the ball-and-stick models of the retinals.)

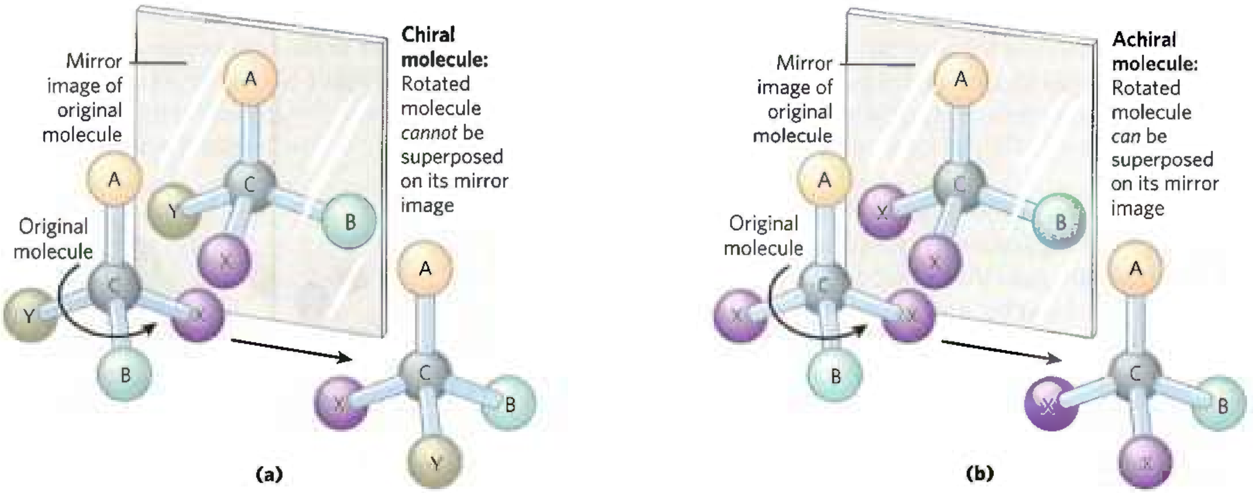

Mirror image of original molecule
Chiral molecule: Rotated molecule *cannot* be superposed on its mirror image
Original molecule
A
B
C
X
Y
(a)
Mirror image of original molecule
Achiral molecule: Rotated molecule *can* be superposed on its mirror image
Original molecule
A
B
C
X
(b)

FIGURE 1-19 Molecular asymmetry: chiral and achiral molecules. (a) When a carbon atom has four different substituent groups (A, B, X, Y), they can be arranged in two ways that represent nonsuperposable mirror images of each other (enantiomers). This asymmetric carbon atom is called a chiral atom or chiral center. (b) When a tetrahedral carbon has only three dissimilar groups (that is, the same group occurs twice), only one configuration is possible and the molecule is symmetric, or achiral. In this case, the molecule is superposable on its mirror image: the molecule on the left can be rotated counterclockwise (when looking down the vertical bond from A to C) to create the molecule in the mirror.

Maleic acid (maleate at the neutral pH of cytoplasm) is the cis isomer, and fumaric acid (fumarate) is the trans isomer; each is a well-defined compound that can be separated from the other, and each has its own unique chemical properties. A binding site (on an enzyme, for example) that is complementary to one of these molecules would not be complementary to the other, which explains why the two compounds have distinct biological roles despite their similar chemical makeup. The visual pigment in the vertebrate eye, rhodopsin, contains retinal, a vitamin A–derived lipid (Fig. 1-18b). In the primary event of vision, light converts one isomer of retinal to another, triggering a neuronal signal to the brain (see Fig. 12-19).

In the second type of stereoisomer, four different substituents bonded to a tetrahedral carbon atom may be arranged in two different ways in space—that is, have two configurations—yielding two stereoisomers that have similar or identical chemical properties but differ in certain physical and biological properties. A carbon atom with four different substituents is said to be asymmetric, and asymmetric carbons are called **chiral centers** (Greek *chiros*, "hand"; some stereoisomers are related structurally as the right hand is to the left hand). A molecule with only one chiral carbon can have two stereoisomers; when two or more (n) chiral carbons are present, there can be 2^n stereoisomers. Stereoisomers that are mirror images of each other are called **enantiomers** (**Fig. 1-19**). Pairs of stereoisomers that are not mirror images of each other are called **diastereomers** (**Fig. 1-20**).

As the biologist, microbiologist, and chemist Louis Pasteur first observed in 1843 (**Box 1-2**), enantiomers have nearly identical chemical reactivities but differ in a characteristic physical property: **optical activity**. In separate solutions, two enantiomers rotate the

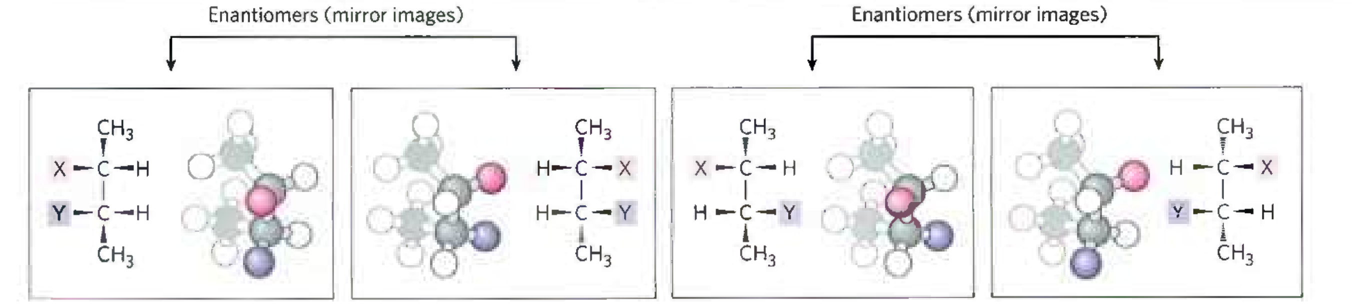

Enantiomers (mirror images)
Enantiomers (mirror images)
CH3
X—C—H
Y—C—H
CH3
CH3
H—C—X
H—C—Y
CH3
CH3
X—C—H
H—C—Y
CH3
CH3
H—C—X
Y—C—H
CH3

FIGURE 1-20 Enantiomers and diastereomers. There are four different stereoisomers of 2,3-disubstituted butane ($n = 2$ asymmetric carbons, hence $2^n = 4$ stereoisomers). Each is shown in a box as a perspective formula and a ball-and-stick model, which has been rotated to show all of the groups. Two pairs of stereoisomers are mirror images of each other, or enantiomers. All other possible pairs are not mirror images, and so are diastereomers. [Information from F. Carroll, *Perspectives on Structure and Mechanism in Organic Chemistry*, p. 63, Brooks/Cole Publishing Co., 1998.]

BOX 1-2

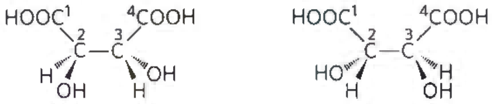
Louis Pasteur and Optical Activity: *In Vino, Veritas*

Louis Pasteur encountered the phenomenon of optical activity in 1843, during his investigation of the crystalline sediment that accumulated in wine casks (a form of tartaric acid called paratartaric acid — also called racemic acid, from Latin *racemus*, "bunch of grapes"). He used fine forceps to separate two types of crystals identical in shape but mirror images of each other. Both types proved to have all the chemical properties of tartaric acid, but in solution one type rotated plane-polarized light to the left (levorotatory), whereas the other rotated it to the right (dextrorotatory). Pasteur later described the experiment and its interpretation:

Louis Pasteur 1822–1895
[Granger, NYC — All rights reserved.]

> In isomeric bodies, the elements and the proportions in which they are combined are the same, only the arrangement of the atoms is different . . . We know, on the one hand, that the molecular arrangements of the two tartaric acids are asymmetric, and, on the other hand, that these arrangements are absolutely identical, excepting that they exhibit asymmetry in opposite directions. Are the atoms of the dextro acid grouped in the form of a right-handed spiral, or are they placed at the apex of an irregular tetrahedron, or are they disposed according to this or that asymmetric arrangement? We do not know.*

Now we do know. In 1951, x-ray crystallographic studies confirmed that the levorotatory and dextrorotatory forms of tartaric acid are mirror images of each other at the molecular level and established the absolute configuration of each (Fig. 1). The same approach has been used to demonstrate that although the amino acid alanine has two stereoisomeric forms (designated D and L), alanine in proteins exists exclusively in one form (the L isomer; see Chapter 3).



(2*R*,3*R*)-Tartaric acid
(dextrorotatory)

(2*S*,3*S*)-Tartaric acid
(levorotatory)

FIGURE 1 Pasteur separated crystals of two stereoisomers of tartaric acid and showed that solutions of the separated forms rotated plane-polarized light to the same extent but in opposite directions. These dextrorotatory and levorotatory forms were later shown to be the (*R*,*R*) and (*S*,*S*) isomers represented here.

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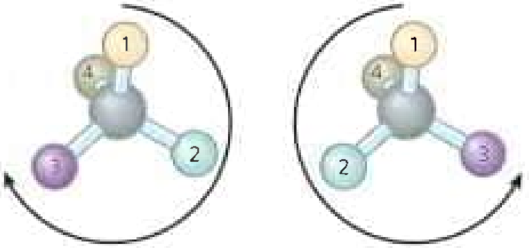
* From Pasteur's lecture to the Société Chimique de Paris in 1883, quoted in R. DuBos, *Louis Pasteur: Free Lance of Science*, p. 95. New York: Charles Scribner's Sons, 1976.

plane of plane-polarized light in opposite directions, but an equimolar solution of the two enantiomers (a **racemic mixture**) shows no optical rotation. Compounds without chiral centers do not rotate the plane of plane-polarized light.

KEY CONVENTION Given the importance of stereochemistry in reactions between biomolecules (see below), biochemists must name and represent the structure of each biomolecule so that its stereochemistry is unambiguous. For compounds with more than one chiral center, the most useful system of nomenclature is the RS system. In this system, each group attached to a chiral carbon is assigned a *priority*. The priorities of some common substituents are

$$—OCH_3 > —OH > —NH_2 > —COOH > —CHO > —CH_2OH > —CH_3 > —H$$

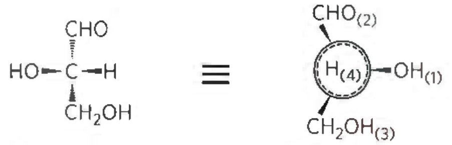
For naming in the RS system, the chiral atom is viewed with the group of lowest priority (4 in the following diagram) pointing away from the viewer. If the priority of the other three groups (1 to 3) decreases in clockwise order, the configuration is (*R*) (Latin *rectus*, "right"); if counterclockwise, the configuration is (*S*) (Latin *sinister*, "left"). In this way, each chiral carbon is designated either (*R*) or (*S*), and the inclusion of these designations in the name of the compound provides an unambiguous description of the stereochemistry at each chiral center.



Clockwise
(*R*)

Counterclockwise
(*S*)

Another naming system for stereoisomers, the D and L system, is described in Chapter 3. A molecule with a single chiral center can be named unambiguously by either system, as shown here. The two naming systems are based on different criteria, so no general correlation can be made between, say, the L isomer and the (*S*) isomer seen in this example.



L-Glyceraldehyde (*S*)-Glyceraldehyde ■

Distinct from configuration is molecular **conformation**, the spatial arrangement of substituent groups that, without breaking any bonds, are free to assume different positions in space because of the freedom of rotation about single bonds. In the simple hydrocarbon ethane, for example, there is nearly complete freedom of rotation around the C—C bond. Many different, interconvertible conformations of ethane are possible, depending on the degree of rotation (**Fig. 1-21**). Two conformations are of special interest: the staggered, which is more stable than all others and thus predominates, and the eclipsed, which is the least stable. We cannot isolate either of these conformational forms, because they are freely interconvertible. However, when one or more of the hydrogen atoms on each carbon is replaced by a functional group that is either very large or electrically charged, freedom of rotation around the C—C bond is hindered. This limits the number of stable conformations of the ethane derivative.

Interactions between Biomolecules Are Stereospecific

P2 When biomolecules interact, the "fit" between them is often stereochemically correct; they are complementary. The three-dimensional structure of biomolecules large and small—the combination of configuration and conformation—is of the utmost importance in their biological interactions: reactant with its enzyme, hormone with its receptor, antigen with its specific antibody, for example (**Fig. 1-22**). The study of biomolecular stereochemistry, with precise physical methods, is an important part of modern research on cell structure and biochemical function.

In living organisms, chiral molecules are usually present in only one of their chiral forms. For example, the amino acids in proteins occur only as their L isomers; glucose occurs only as its D isomer. (The conventions for naming stereoisomers of the amino acids are described in Chapter 3; those for sugars, in Chapter 7. The RS system, described on p. 17, is the most useful for some biomolecules.) In contrast, when a compound with an asymmetric carbon atom is chemically synthesized in the laboratory, the reaction usually produces both possible chiral forms: a mixture of the D and L forms, for example. Living cells produce only one chiral form of a biomolecule because the enzymes that synthesize that molecule are also chiral.

Stereospecificity, the ability to distinguish between stereoisomers, is a property of enzymes and other proteins and a characteristic feature of biochemical interactions. If the binding site on a protein is complementary to one isomer of a chiral compound, it will not be complementary to the other isomer, for the same reason that a left-handed glove does not fit a right hand. Two striking examples of the ability of biological systems to distinguish stereoisomers are shown in **Figure 1-23**.

The common classes of chemical reactions encountered in biochemistry are described in Chapter 13, as an introduction to the reactions of metabolism.

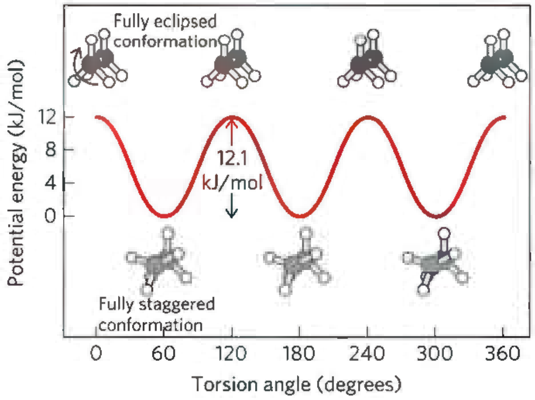


FIGURE 1-21 Conformations. Many conformations of ethane are possible because of freedom of rotation around the C—C bond. In the ball-and-stick model, when the front carbon atom (as viewed by the reader) with its three attached hydrogens is rotated relative to the rear carbon atom, the potential energy of the molecule rises to a maximum in the fully eclipsed conformation (torsion angle 0°, 120°, and so on), then falls to a minimum in the fully staggered conformation (torsion angle 60°, 180°, and so on). Because the energy differences are small enough to allow rapid interconversion of the two forms (millions of times per second), the eclipsed and staggered forms cannot be separately isolated.

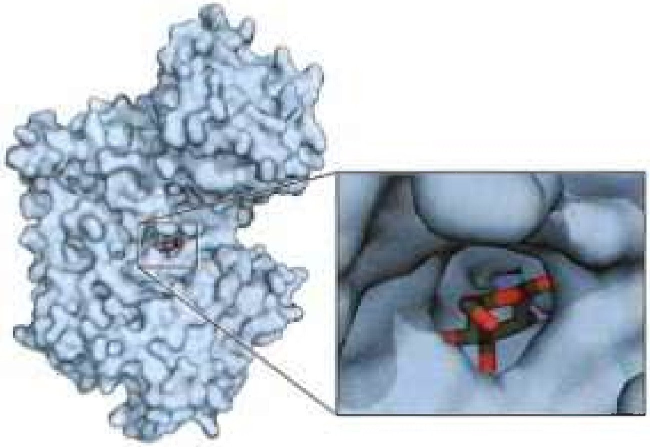
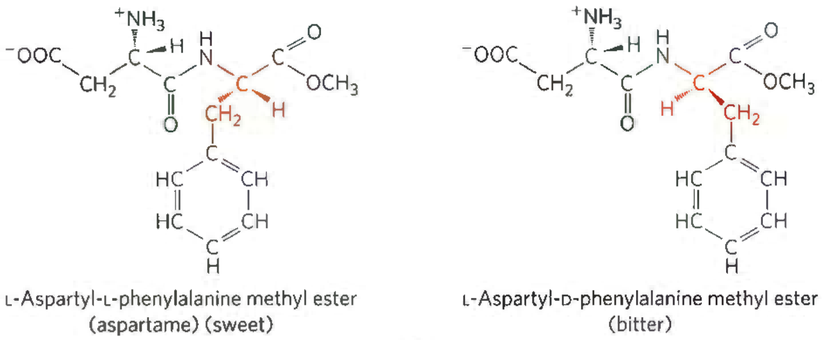


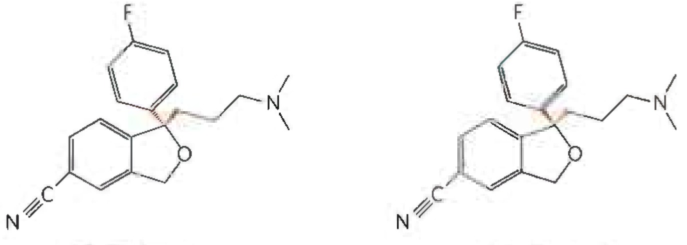
FIGURE 1-22 Complementary fit between a macromolecule and a small molecule. A glucose molecule fits into a pocket on the surface of the enzyme hexokinase and is held in this orientation by several noncovalent interactions between the protein and the sugar. This representation of the hexokinase molecule is produced with software that can calculate the shape of the outer surface of a macromolecule, defined either by the van der Waals radii of all the atoms in the molecule or by the "solvent exclusion volume," the volume that a water molecule cannot penetrate. [Data from PDB ID 3B8A, P. Kuser et al., *Proteins* 72:731, 2008.]



L-Aspartyl-L-phenylalanine methyl ester (aspartame) (sweet)

L-Aspartyl-D-phenylalanine methyl ester (bitter)

(a)



(*S*)-Citalopram (therapeutically active)

(*R*)-Citalopram (therapeutically inactive)

(b)

FIGURE 1-23 Stereoisomers have different effects in humans. (a) Aspartame, the artificial sweetener sold under the trade name NutraSweet, is easily distinguishable by taste receptors from its bitter-tasting stereoisomer, although the two differ only in the configuration at one of the two chiral carbon atoms. (b) The antidepressant medication citalopram (trade name Celexa), a selective serotonin reuptake inhibitor, is a racemic mixture of these two stereoisomers, but only (*S*)-citalopram has the therapeutic effect. A stereochemically pure preparation of (*S*)-citalopram (escitalopram oxalate) is sold under the trade name Lexapro. As you might predict, the effective dose of Lexapro is one-half the effective dose of Celexa.

SUMMARY 1.2 ***Chemical Foundations***

- Because of its bonding versatility, carbon can produce a broad array of carbon–carbon skeletons with a variety of functional groups; these groups give biomolecules their biological and chemical personalities.
- A nearly universal set of several thousand small molecules is found in living cells; the interconversions of these molecules in the central metabolic pathways have been conserved in evolution.
- Proteins and nucleic acids are macromolecules—long, linear polymers of simple monomeric subunits; their sequences contain the information that gives each molecule its three-dimensional structure and its biological functions.
- Molecular configuration can be changed only by breaking and re-forming covalent bonds. For a carbon atom with four different substituents (a chiral carbon), the substituent groups can be arranged in two different ways, generating stereoisomers with distinct properties. Only one stereoisomer is biologically active. Molecular conformation is the position of atoms in space that can be changed by rotation about single bonds, without covalent bonds being broken.
- Interactions between biological molecules are often stereospecific: there is a close fit between complementary structures in the interacting molecules.

1.3 Physical Foundations

Living cells and organisms must perform work to stay alive and to reproduce themselves. The synthetic reactions that occur within cells, like the synthetic processes in any factory, require the input of energy. Energy input is also needed in the motion of a bacterium or an Olympic sprinter, in the flashing of a firefly or the electrical discharge of an eel. And the storage and expression of information require energy, without which structures that are rich in information inevitably become disordered and meaningless.

In the course of evolution, cells have developed highly efficient mechanisms for coupling the energy obtained from sunlight or chemical fuels to the many energy-requiring processes they must carry out. One goal of biochemistry is to understand, in quantitative and chemical terms, the means by which energy is extracted, stored, and channeled into useful work in living cells. We can consider cellular energy conversions—like all other energy conversions—in the context of the laws of thermodynamics. For a more extensive discussion of cellular thermodynamics, see Chapter 13.

Living Organisms Exist in a Dynamic Steady State, Never at Equilibrium with Their Surroundings

The molecules and ions contained within a living organism differ in kind and in concentration from those in the organism's surroundings. A paramecium in a pond, a shark in the ocean, a bacterium in the soil, an apple tree in an orchard—all are different in composition from their surroundings and, once they have reached maturity, maintain a more or less constant composition in the face of a constantly changing environment.

Although the characteristic composition of an organism changes little through time, the population of molecules within the organism is far from static. Small molecules, macromolecules, and supramolecular complexes are continuously synthesized and broken down in chemical reactions that involve a constant flux of mass and energy through the system. The hemoglobin molecules carrying oxygen from your lungs to your brain at this moment were synthesized within the past month; by next month they will have been degraded and entirely replaced by new hemoglobin molecules. The glucose you ingested with your most recent meal is now circulating in your bloodstream; before the day is over these particular glucose molecules will have been converted into something else — carbon dioxide or fat, perhaps — and will have been replaced with a fresh supply of glucose, so that your blood glucose concentration is more or less constant over the whole day. The amounts of hemoglobin and glucose in the blood remain nearly constant because the rate of synthesis or intake of each just balances the rate of its breakdown, consumption, or conversion into some other product. The constancy of concentration is the result of a *dynamic steady state,* a steady state that is far from equilibrium. Maintaining this steady state requires the constant investment of energy; when a cell can no longer obtain energy, it dies and begins to decay toward equilibrium with its surroundings. We consider below exactly what is meant by "steady state" and "equilibrium."

Organisms Transform Energy and Matter from Their Surroundings

For chemical reactions occurring in solution, we can define a **system** as all the constituent reactants and products, the solvent that contains them, and the immediate atmosphere — in short, everything within a defined region of space. The system and its surroundings together constitute the **universe**. If the system exchanges neither matter nor energy with its surroundings, it is said to be **isolated**. If the system exchanges energy but not matter with its surroundings, it is a **closed system**; if it exchanges both energy and matter with its surroundings, it is an **open system**.

A living organism is an open system; it exchanges both matter and energy with its surroundings. Organisms obtain energy from their surroundings in two ways: (1) they take up chemical fuels (such as glucose) from the environment and extract energy by oxidizing them (see Box 1-3, Case 2); or (2) they absorb energy from sunlight.

The first law of thermodynamics describes the principle of the conservation of energy: *in any physical or chemical change, the total amount of energy in the universe remains constant, although the form of the energy may change.* This means that while energy is "used" by a system, it is not "used up"; rather, it is converted from one form into another — from potential energy in chemical bonds, say, into kinetic energy of heat and motion. Cells are consummate transducers of energy, capable of interconverting chemical, electromagnetic, mechanical, and osmotic energy with great efficiency (**Fig. 1-24**).

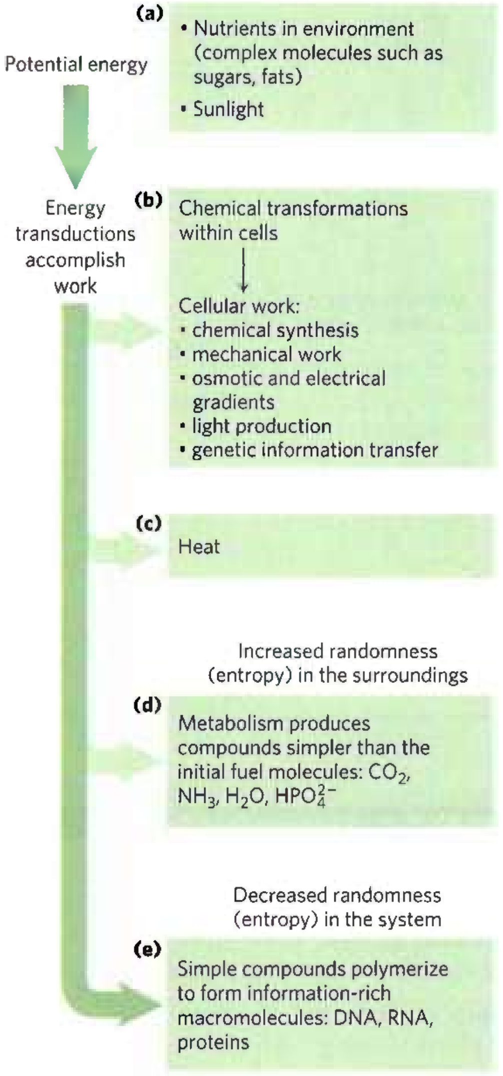

(a)
Potential energy
• Nutrients in environment (complex molecules such as sugars, fats)
• Sunlight
(b)
Energy transductions accomplish work
Chemical transformations within cells
Cellular work:
• chemical synthesis
• mechanical work
• osmotic and electrical gradients
• light production
• genetic information transfer
(c)
Heat
Increased randomness (entropy) in the surroundings
(d)
Metabolism produces compounds simpler than the initial fuel molecules: CO_2, NH_3, H_2O, HPO_4^{2-}
Decreased randomness (entropy) in the system
(e)
Simple compounds polymerize to form information-rich macromolecules: DNA, RNA, proteins

FIGURE 1-24 Some energy transformations in living organisms. As metabolic energy is spent to do cellular work, the randomness of the system plus surroundings (expressed quantitatively as entropy) increases as the potential energy of complex nutrient molecules decreases. (a) Living organisms extract energy from their surroundings; (b) convert some of it into useful forms of energy to produce work; (c) return some energy to the surroundings as heat; and (d) release end-product molecules that are less well organized than the starting fuel, increasing the entropy of the universe. One effect of all these transformations is (e) increased order (decreased randomness) in the system in the form of complex macromolecules.

P3 Nearly all living organisms derive their energy, directly or indirectly, from the radiant energy of sunlight. In the photoautotrophs, light-driven splitting of water during photosynthesis releases its electrons for the reduction of CO_2 and the release of O_2 into the atmosphere:

$$6CO_2 + 6H_2O \xrightarrow{\text{light}} C_6H_{12}O_6 + 6O_2$$

(light-driven reduction of CO_2)

Nonphotosynthetic organisms (chemotrophs) obtain the energy they need by oxidizing the energy-rich products of photosynthesis stored in plants, then passing the electrons thus acquired to atmospheric O_2 to form water, CO_2, and other end products, which are recycled in the environment:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + \text{energy}$$

(energy-yielding oxidation of glucose)

Thus autotrophs and heterotrophs participate in global cycles of O_2 and CO_2, driven ultimately by sunlight, making these two large groups of organisms interdependent. Virtually all energy transductions in cells can be traced to this flow of electrons from one molecule to another, in a "downhill" flow from higher to lower electrochemical potential; as such, this is formally analogous to the flow of electrons in a battery-driven electric circuit. P3 All these reactions involved in electron flow are **oxidation-reduction reactions**: one reactant is oxidized (loses electrons) as another is reduced (gains electrons).

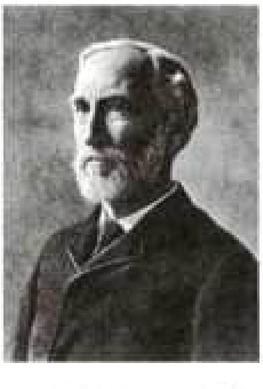
Creating and Maintaining Order Requires Work and Energy

As we've noted, DNA, RNA, and proteins are informational macromolecules; the precise sequence of their monomeric subunits contains information, just as the letters in this sentence do. In addition to using chemical energy to form the covalent bonds between these subunits, the cell must invest energy to order the subunits in their correct sequence. It is extremely improbable that amino acids in a mixture would spontaneously condense into a single type of protein, with a unique sequence. This would represent increased order in a population of molecules; but according to the second law of thermodynamics, the tendency in nature is toward ever-greater disorder in the universe: *randomness in the universe is constantly increasing*. To bring about the synthesis of macromolecules from their monomeric units, free energy must be supplied to the system (in this case, the cell). We discuss the quantitative energetics of oxidation-reduction reactions in Chapter 13.

The randomness or disorder of the components of a chemical system is expressed as **entropy**, ***S*** (**Box 1-3**). Any change in randomness of the system is expressed as entropy change, ΔS, which by convention has a positive value when randomness increases. J. Willard Gibbs, the scientist who developed the theory of energy changes during chemical reactions, showed that the **free energy**, ***G***, of any closed system can be defined in terms of three quantities: **enthalpy**, ***H***, or heat content, roughly reflecting the number and kinds of bonds; entropy, S; and the absolute temperature, T (in Kelvin). The definition of free energy is $G = H - TS$. When a chemical reaction occurs at constant temperature, the **free-energy change**, ΔG, is determined by the enthalpy change, ΔH, reflecting the kinds and numbers of chemical bonds and noncovalent interactions broken and formed, and the entropy change, ΔS, describing the change in the system's randomness:

$$\Delta G = \Delta H - T\Delta S$$

where, by definition, ΔH is negative for a reaction that releases heat, and ΔS is positive for a reaction that increases the system's randomness.



J. Willard Gibbs, 1839–1903
[Science Source.]

A process tends to occur spontaneously only if ΔG is negative (if free energy is *released* in the process). Yet cell function depends largely on molecules, such as proteins and nucleic acids, for which the free energy of formation is positive: the molecules are less stable and more highly ordered than a mixture of their monomeric components. To carry out these thermodynamically unfavorable, energy-requiring (**endergonic**) reactions, cells couple them to other reactions that liberate free energy (**exergonic** reactions), so that the overall process is exergonic: the *sum* of the free-energy changes is negative. The exergonic reaction most commonly employed in this way involves adenosine triphosphate (ATP; **Fig. 1-25**) in which two phosphoanhydride bonds are capable of supplying the free energy to make a coupled endergonic reaction possible. In Section 13.3 we discuss in more detail this role of ATP.

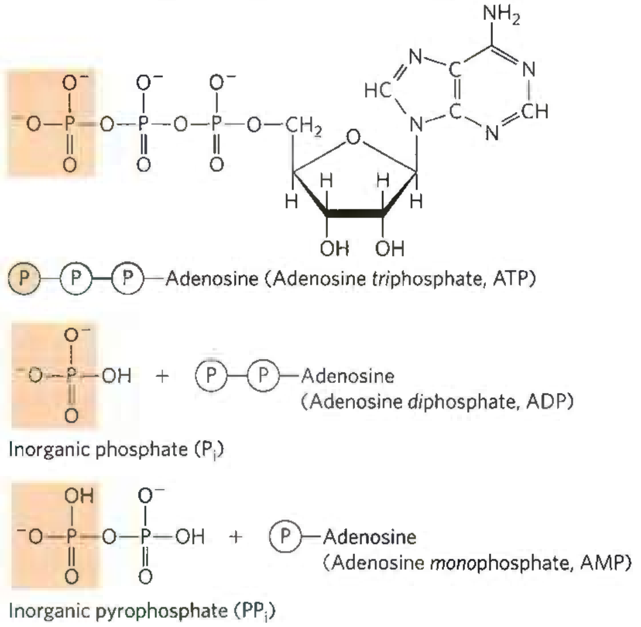

NH2
N
C
N
HC
C
CH
N
N
O⁻
O⁻
O⁻
⁻O—P—O—P—O—P—O—CH2
O
O
O
O
H
H
H
H
OH
OH
P—P—P—Adenosine (Adenosine triphosphate, ATP)
O⁻
⁻O—P—OH
O
+
P—P—Adenosine
(Adenosine diphosphate, ADP)
Inorganic phosphate (Pi)
OH
O⁻
⁻O—P—O—P—OH
O
O
+
P—Adenosine
(Adenosine monophosphate, AMP)
Inorganic pyrophosphate (PPi)

FIGURE 1-25 Adenosine triphosphate (ATP) provides energy. Here, each Ⓟ represents a phosphoryl group. The removal of the terminal phosphoryl group (shaded light red) of ATP, by breakage of a phosphoanhydride bond to generate adenosine diphosphate (ADP) and inorganic phosphate ion (HPO_4^{2-}), is highly exergonic, and this reaction is coupled to many endergonic reactions in the cell (as in the example in Worked Example 1-2). ATP also provides energy for many cellular processes by undergoing cleavage that releases the two terminal phosphates as inorganic pyrophosphate ($H_2P_2O_7^{2-}$), often abbreviated PP_i.

BOX 1-3

Entropy: Things Fall Apart

The term "entropy," which literally means "a change within," was first used in 1851 by Rudolf Clausius, one of the formulators of the second law of thermodynamics. It refers to the randomness or disorder of the components of a chemical system. Entropy is a central concept in biochemistry; life requires continual maintenance of order in the face of nature's tendency to increase randomness. A rigorous quantitative definition of entropy involves statistical and probability considerations. However, its nature can be illustrated qualitatively by three simple examples, each demonstrating one aspect of entropy. The key descriptors of entropy are *randomness* and *disorder,* manifested in different ways.

Case 1: The Teakettle and the Randomization of Heat

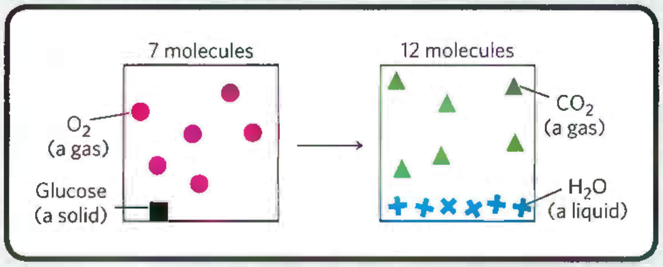
We know that steam generated from boiling water can do useful work. But suppose we turn off the burner under a teakettle full of water at 100 °C (the "system") in the kitchen (the "surroundings") and allow the teakettle to cool. As it cools, no work is done, but heat passes from the teakettle to the surroundings, raising the temperature of the surroundings (the kitchen) by an infinitesimally small amount until complete equilibrium is attained. At this point all parts of the teakettle and the kitchen are at precisely the same temperature. The free energy that was once concentrated in the teakettle of hot water at 100 °C, *potentially* capable of doing work, has disappeared. Its equivalent in heat energy is still present in the teakettle + kitchen (that is, the "universe") but has become completely randomized throughout. This energy is no longer available to do work because there is no temperature differential within the kitchen. Moreover, the increase in entropy of the kitchen (the surroundings) is irreversible. We know from everyday experience that heat never spontaneously passes back from the kitchen into the teakettle to raise the temperature of the water to 100 °C again.

Case 2: The Oxidation of Glucose

Entropy is a state not only of energy but of matter. Aerobic (heterotrophic) organisms extract free energy from glucose obtained from their surroundings by oxidizing the glucose with O_2, also obtained from the surroundings. The end products of this oxidative metabolism, CO_2 and H_2O, are returned to the surroundings. In this process the surroundings undergo an increase in entropy, whereas the organism itself remains in a steady state and undergoes no change in its internal order. Although some entropy arises from the dissipation of heat, entropy also arises from another kind of disorder, illustrated by the equation for the oxidation of glucose:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

We can represent this schematically as



Energy Coupling Links Reactions in Biology

The central issue in *bioenergetics* (the study of energy transformations in living systems) is the means by which energy from fuel metabolism or light capture is coupled to a cell's energy-requiring reactions. With regard to energy coupling, it is useful to consider a simple mechanical example, as shown in **Figure 1-26a**. An object at the top of an inclined plane has a certain amount of potential energy as a result of its elevation. It tends to slide down the plane, losing its potential energy of position as it approaches the ground. When an appropriate string-and-pulley device couples the falling object to another, smaller object, the spontaneous downward motion of the larger can lift the smaller, accomplishing a certain amount of work. The amount of energy available to do work is the **free-energy change**, ΔG; this is always somewhat less than the theoretical amount of energy released, because some energy is dissipated as the heat of friction. The greater the elevation of the larger object, the greater the energy released (ΔG) as the object slides downward and the greater the amount of work that can be accomplished. The larger object can lift the smaller one only because, at the outset, the larger object was *far from its equilibrium position*: it had at some earlier point been elevated above the ground, in a process that itself required the input of energy.

How does this apply in chemical reactions? In closed systems, chemical reactions proceed spontaneously until **equilibrium** is reached. When a system is at equilibrium, the rate of product formation exactly equals the rate at which product is converted to reactant. Thus there is no net change in the concentration of reactants and products. The energy change as the system moves from its initial state to equilibrium, with no changes in temperature or pressure, is given by the free-energy change, ΔG. The magnitude of ΔG depends on the particular chemical reaction *and on how far from equilibrium the system is initially*. Each compound involved in a chemical reaction contains a certain amount of potential energy, related

The atoms contained in 1 molecule of glucose plus 6 molecules of oxygen, a total of 7 molecules, are more randomly dispersed by the oxidation reaction and are now present in a total of 12 molecules ($6CO_2 + 6H_2O$).

Whenever a chemical reaction results in an increase in the number of molecules — or when a solid substance is converted into liquid or gaseous products, which allow more freedom of molecular movement than solids — molecular disorder, and thus entropy, increases.

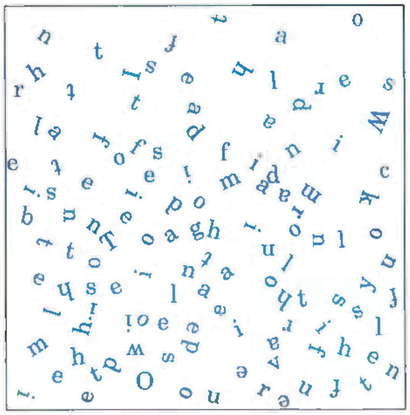
Case 3: Information and Entropy

The following short passage from Shakespeare's *Julius Caesar*, Act IV, Scene 3, is spoken by Brutus, when he realizes that he must face Mark Antony's army. It is an information-rich nonrandom arrangement of 125 letters of the English alphabet:

> There is a tide in the affairs of men,
> Which, taken at the flood, leads on to fortune;
> Omitted, all the voyage of their life
> Is bound in shallows and in miseries.

In addition to what this passage says overtly, it has many hidden meanings. It not only reflects a complex sequence of events in the play, but also echoes the play's ideas on conflict, ambition, and the demands of leadership. Permeated with Shakespeare's understanding of human nature, it is very rich in information.

However, if the 125 letters making up this quotation were allowed to fall into a completely random, chaotic pattern, as shown in the following box, they would have no meaning whatsoever.



In this form, the 125 letters contain little or no information, but they are very rich in entropy. Such considerations have led to the conclusion that information is a form of energy; information has been called "negative entropy." In fact, the branch of mathematics called information theory, which is basic to the programming logic of computers, is closely related to thermodynamic theory. Living organisms are highly ordered, nonrandom structures, immensely rich in information and thus entropy-poor.

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to the kind and number of its bonds. In reactions that occur spontaneously, the products have less free energy than the reactants and thus the reaction releases free energy, which is then available to do work. Such reactions are exergonic; the decline in free energy from reactants to products is expressed as a negative value. Endergonic reactions require an input of energy, and their ΔG values are positive. This coupling of an exergonic reaction to an endergonic reaction is illustrated in Figure 1-26b. As in mechanical processes, only part of the energy released in exergonic chemical reactions can be used to accomplish work. In living systems, some energy is dissipated as heat or is lost to increasing entropy.

K_{eq} and $\Delta G°$ Are Measures of a Reaction's Tendency to Proceed Spontaneously

The tendency of a chemical reaction to go to completion can be expressed as an equilibrium constant. For the reaction in which *a* moles of A react with *b* moles of B to give *c* moles of C and *d* moles of D,

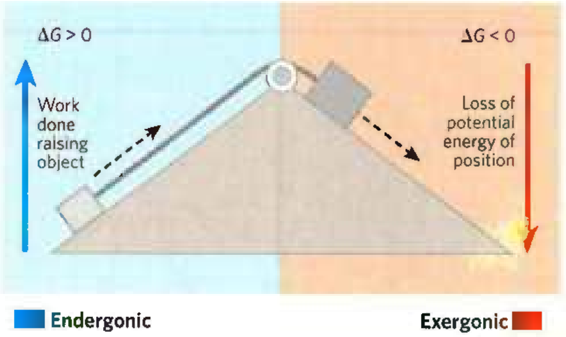
$$aA + bB \longrightarrow cC + dD$$

the equilibrium constant, K_{eq}, is given by

$$K_{eq} = \frac{[C]^c_{eq}[D]^d_{eq}}{[A]^a_{eq}[B]^b_{eq}}$$

where $[A]_{eq}$ is the concentration of A, $[B]_{eq}$ the concentration of B, and so on, *when the system has reached equilibrium*. K_{eq} is dimensionless (that is, has no units of measurement), but, as we explain on page 54, we will include molar units in our calculations to reinforce the point that molar concentrations (represented by the square brackets) must be used in the calculation of equilibrium constants. A large value of K_{eq} means the reaction tends to proceed until the reactants are almost completely converted into the products.

(a) Mechanical example



(b) Chemical example

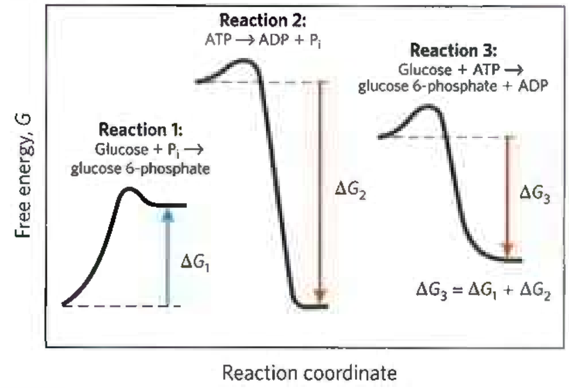


FIGURE 1-26 Energy coupling in mechanical and chemical processes. (a) The downward motion of an object releases potential energy that can do mechanical work. The potential energy made available by spontaneous downward motion, an exergonic process (red), can be coupled to the endergonic upward movement of another object (blue). (b) In reaction 1, the formation of glucose 6-phosphate from glucose and inorganic phosphate (P_i) yields a product of higher energy than the two reactants. For this endergonic reaction, ΔG is positive. In reaction 2, the exergonic breakdown of adenosine triphosphate (ATP) has a large, negative free-energy change (ΔG_2). The third reaction is the sum of reactions 1 and 2, and the free-energy change, ΔG_3, is the arithmetic sum of ΔG_1 and ΔG_2. Because ΔG_3 is negative and relatively large, the overall reaction is exergonic and proceeds spontaneously.

WORKED EXAMPLE 1-1 *Are ATP and ADP at Equilibrium in Cells?*

ATP breakdown yields adenosine diphosphate (ADP) and inorganic phosphate (P_i). The equilibrium constant, K_{eq}, for the reaction is 2×10^5 M:

$$\text{ATP} \longrightarrow \text{ADP} + \text{HPO}_4^{2-}$$

If the measured cellular concentrations are [ATP] = 5 mM, [ADP] = 0.5 mM, and [P_i] = 5 mM, is this reaction at equilibrium in living cells?

SOLUTION: The definition of the equilibrium constant for this reaction is

$$K_{eq} = [\text{ADP}]\,[P_i]/[\text{ATP}]$$

From the measured cellular concentrations given above, we can calculate the **mass-action ratio, *Q*:**

$$\begin{aligned} Q &= [\text{ADP}][P_i]/[\text{ATP}] = (0.5\ \text{mM})(5\ \text{mM})/5\ \text{mM} \\ &= 0.5\ \text{mM} = 5 \times 10^{-4}\ \text{M} \end{aligned}$$

This value is *far* from the equilibrium constant for the reaction (2×10^5 M), so the reaction is *very far* from equilibrium in cells. [ATP] is far higher, and [ADP] is far lower, than is expected at equilibrium. How can a cell hold its [ATP]/[ADP] ratio so far from equilibrium? It does so by continuously extracting energy (from nutrients such as glucose) and using it to make ATP from ADP and P_i.

Gibbs showed that ΔG (the actual free-energy change) for any chemical reaction is a function of the **standard free-energy change, $\Delta G°$**—a constant that is characteristic of each specific reaction—and a term that expresses the initial concentrations of reactants and products:

$$\Delta G = \Delta G° + RT \ln \frac{[\text{C}]_i^c[\text{D}]_i^d}{[\text{A}]_i^a[\text{B}]_i^b} \qquad (1\text{-}1)$$

where $[\text{A}]_i$ is the initial concentration of A, and so forth; R is the gas constant; and T is the absolute temperature.

ΔG is a measure of the distance of a system from its equilibrium position. When a reaction has reached equilibrium, no driving force remains and it can do no work: $\Delta G = 0$. For this special case, $[\text{A}]_i = [\text{A}]_{eq}$, and so on, for all reactants and products, and

$$\frac{[\text{C}]_i^c[\text{D}]_i^d}{[\text{A}]_i^a[\text{B}]_i^b} = \frac{[\text{C}]_{eq}^c[\text{D}]_{eq}^d}{[\text{A}]_{eq}^a[\text{B}]_{eq}^d}$$

Substituting 0 for ΔG and K_{eq} for $[\text{C}]_i^c[\text{D}]_i^d/[\text{A}]_i^a[\text{B}]_i^b$ in Equation 1-1, we obtain the relationship

$$\Delta G° = -RT \ln K_{eq}$$

from which we see that $\Delta G°$ is simply a second way (besides K_{eq}) of expressing the driving force on a reaction. Because K_{eq} is experimentally measurable, we have a way of determining $\Delta G°$, the thermodynamic constant characteristic of each reaction.

The units of $\Delta G°$ and ΔG are joules per mole (or calories per mole). When $K_{eq} >> 1$, $\Delta G°$ is large and negative; when $K_{eq} << 1$, $\Delta G°$ is large and positive. From a table of experimentally determined values of either K_{eq} or $\Delta G°$, we can see at a glance which reactions tend to go to completion and which do not.

One caution about the interpretation of $\Delta G°$: *thermodynamic* constants such as this show where the final equilibrium for a reaction lies but tell us nothing about how fast that equilibrium will be achieved. The rates of reactions are governed by the parameters of *kinetics*, a topic we consider in detail in Chapter 6.

P3 In living organisms, just as in the mechanical example in Figure 1-26a, an exergonic reaction can be coupled to an endergonic reaction to drive otherwise unfavorable reactions. Figure 1-26b, a **reaction coordinate diagram**, illustrates this principle for the conversion of glucose to glucose 6-phosphate, the first step in the pathway for oxidation of glucose. The most direct way to produce glucose 6-phosphate would be

Reaction 1: $\text{Glucose} + \text{P}_i \longrightarrow \text{glucose 6-phosphate}$ (endergonic; ΔG_1 is positive)

This reaction does not occur spontaneously; ΔG_1 is positive. A second, highly exergonic reaction can occur in all cells:

Reaction 2: $\text{ATP} \longrightarrow \text{ADP} + \text{P}_i$ (exergonic; ΔG_2 is negative)

These two chemical reactions share a common intermediate, P_i, which is consumed in reaction 1 and produced in reaction 2. The two reactions can therefore be coupled in the form of a third reaction, which we can write as the sum of reactions 1 and 2, with the common intermediate, P_i, omitted from both sides of the equation:

Reaction 3: $\text{Glucose} + \text{ATP} \longrightarrow \text{glucose 6-phosphate} + \text{ADP}$

Because more energy is released in reaction 2 than is consumed in reaction 1, the free-energy change for reaction 3, ΔG_3, is negative, and the synthesis of glucose 6-phosphate can therefore occur by reaction 3.

WORKED EXAMPLE 1-2 *Standard Free-Energy Changes Are Additive*

Given that the standard free-energy change for the reaction $\text{glucose} + \text{P}_i \longrightarrow \text{glucose 6-phosphate}$ is 13.8 kJ/mol, and the standard free-energy change for the reaction $\text{ATP} \longrightarrow \text{ADP} + \text{P}_i$ is −30.5 kJ/mol, what is the free-energy change for the reaction $\text{glucose} + \text{ATP} \longrightarrow \text{glucose 6-phosphate} + \text{ADP}$?

SOLUTION: We can write the equation for this reaction as the sum of two other reactions:

(1) $\text{Glucose} + \text{P}_i \longrightarrow \text{glucose 6-phosphate} \qquad \Delta G_1^\circ = 13.8\ \text{kJ/mol}$

(2) $\text{ATP} \longrightarrow \text{ADP} + \text{P}_i \qquad \Delta G_2^\circ = -30.5\ \text{kJ/mol}$

Sum: $\text{Glucose} + \text{ATP} \longrightarrow \text{glucose 6-phosphate} + \text{ADP}$

$\Delta G_{\text{Sum}}^\circ = -16.7\ \text{kJ/mol}$

The standard free-energy change for two reactions that sum to a third is simply the sum of the two individual reactions. A negative value for ΔG° (−16.7 kJ/mol) indicates that the reaction will tend to occur spontaneously.

The coupling of exergonic and endergonic reactions through a shared intermediate is central to the energy exchanges in living systems. As we shall see, reactions that break down ATP (such as reaction 2 in Fig. 1-26b) release energy that drives many endergonic processes in cells. ATP breakdown in cells is exergonic because *all living cells maintain a concentration of ATP far above its equilibrium concentration*. It is this disequilibrium that allows ATP to serve as the major carrier of chemical energy in all cells. P3 As we describe in detail in Chapter 13, it is not the mere breakdown of ATP that provides energy to drive endergonic reactions; rather, it is the *transfer of a phosphoryl group* from ATP to another small molecule (glucose in the case above) that conserves some of the chemical potential originally in ATP.

WORKED EXAMPLE 1-3 *Energetic Cost of ATP Synthesis*

If the equilibrium constant, K_{eq}, for the reaction

$$\text{ATP} \longrightarrow \text{ADP} + \text{P}_i$$

is $2.22 \times 10^5\ \text{M}$, calculate the standard free-energy change, ΔG°, for the *synthesis* of ATP from ADP and P_i at 25 °C.

SOLUTION: First calculate ΔG° for the reaction above:

$$\begin{aligned}\Delta G^\circ &= -RT \ln K_{\text{eq}} \\ &= -(8.315\ \text{J/mol}\cdot\text{K})(298\ \text{K})(\ln 2.22 \times 10^5) \\ &= -30.5\ \text{kJ/mol}\end{aligned}$$

This is the standard free-energy change for the *breakdown* of ATP to ADP and P_i. The standard free-energy change for the *reverse* of a reaction has the same absolute value but the opposite sign. The standard free-energy change for the reverse of the above reaction is therefore 30.5 kJ/mol. So, to synthesize 1 mol of ATP under standard conditions (25 °C, 1 M concentrations of ATP, ADP, and P_i), at least 30.5 kJ of energy must be supplied. The actual free-energy change in cells — approximately 50 kJ/mol — is greater than this because the concentrations of ATP, ADP, and P_i in cells are not the standard 1 M (see Worked Example 13-2).

Enzymes Promote Sequences of Chemical Reactions

All biological macromolecules are much less thermodynamically stable than their monomeric subunits, yet they are *kinetically stable*: their *uncatalyzed* breakdown occurs so slowly (over years rather than seconds) that, on a time scale that matters for the organism, these molecules are stable. Virtually every chemical reaction in a cell occurs at a significant rate only because of the presence of **enzymes** — biocatalysts that, like all other catalysts, greatly enhance the rate of specific chemical reactions without being consumed in the process.

The path from reactant(s) to product(s) almost invariably involves an energy barrier, called the activation barrier (**Fig. 1-27**), that must be surmounted for any reaction to proceed. The breaking of existing bonds and formation of new ones generally requires, first, a distortion of the existing bonds to create a **transition state** of higher free energy than either the reactant or the product (see Section 6.2). The highest point in the reaction coordinate diagram represents the transition state, and the difference in energy between the reactant in its ground state and in its transition state is the **activation energy**, $\Delta G^\ddagger$. An enzyme catalyzes a reaction by providing a more comfortable fit for the transition state: a surface that complements the transition state in stereochemistry, polarity, and charge. The binding of

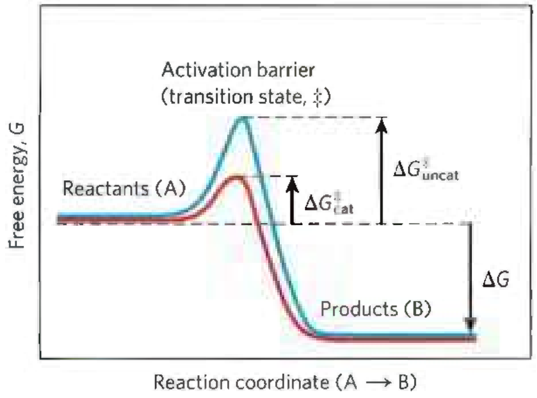


FIGURE 1-27 Energy changes during a chemical reaction. An activation barrier, representing the transition state, must be overcome in the conversion of reactants (A) into products (B), even though the products are more stable than the reactants, as indicated by a large, negative free-energy change (ΔG). The energy required to overcome the activation barrier is the activation energy ($\Delta G^{\ddagger}$). Enzymes catalyze reactions by lowering the activation barrier. They bind the transition-state intermediates tightly, and the binding energy of this interaction effectively reduces the activation energy from $\Delta G^{\ddagger}_{\text{uncat}}$ (blue curve) to $\Delta G^{\ddagger}_{\text{cat}}$ (red curve). (Note that activation energy is *not* related to free-energy change, ΔG.)

enzyme to the transition state is exergonic, and the energy released by this binding reduces the activation energy for the reaction and greatly increases the reaction rate.

A further contribution to catalysis occurs when two or more reactants bind to the enzyme's surface close to each other and with stereospecific orientations that favor their reaction. This increases by orders of magnitude the probability of productive collisions between reactants. As a result of these factors and several others, discussed in Chapter 6, many enzyme-catalyzed reactions proceed at rates 10^6 times faster than the uncatalyzed reactions.

Cellular catalysts are, with some notable exceptions, proteins. (Some RNA molecules have enzymatic activity, as discussed in Chapters 26 and 27.) Again with a few exceptions, each enzyme catalyzes a specific reaction, and each reaction in a cell is catalyzed by a different enzyme. Thousands of different enzymes are therefore required by each cell. The multiplicity of enzymes, their specificity (the ability to discriminate between reactants), and their susceptibility to regulation give cells the capacity to lower activation barriers selectively. This selectivity is crucial for the effective regulation of cellular processes. By allowing specific reactions to proceed at significant rates at particular times, enzymes determine how matter and energy are channeled into cellular activities.

The thousands of enzyme-catalyzed chemical reactions in cells are functionally organized into many sequences of consecutive reactions, called **pathways**, in which the product of one reaction becomes the reactant in the next. Some pathways degrade organic nutrients into simple end products in order to extract chemical energy and convert it into a form useful to the cell; together, these degradative, free-energy-yielding reactions are designated **catabolism**. The energy released by catabolic reactions drives the synthesis of ATP. As a result, the cellular concentration of ATP is held far above its equilibrium concentration, so that ΔG for ATP breakdown is large and negative. Similarly, catabolism results in the production of the reduced electron carriers NADH (nicotinamide adenine dinucleotide) and NADPH (nicotinamide adenine dinucleotide phosphate hydrogen), both of which can donate electrons in processes that generate ATP or drive reductive steps in biosynthetic pathways. They are often referred to collectively as NAD(P)H.

Other pathways start with small precursor molecules and convert them to progressively larger and more complex molecules, including proteins and nucleic acids. Such synthetic pathways, which invariably require the input of energy, are collectively designated **anabolism**.

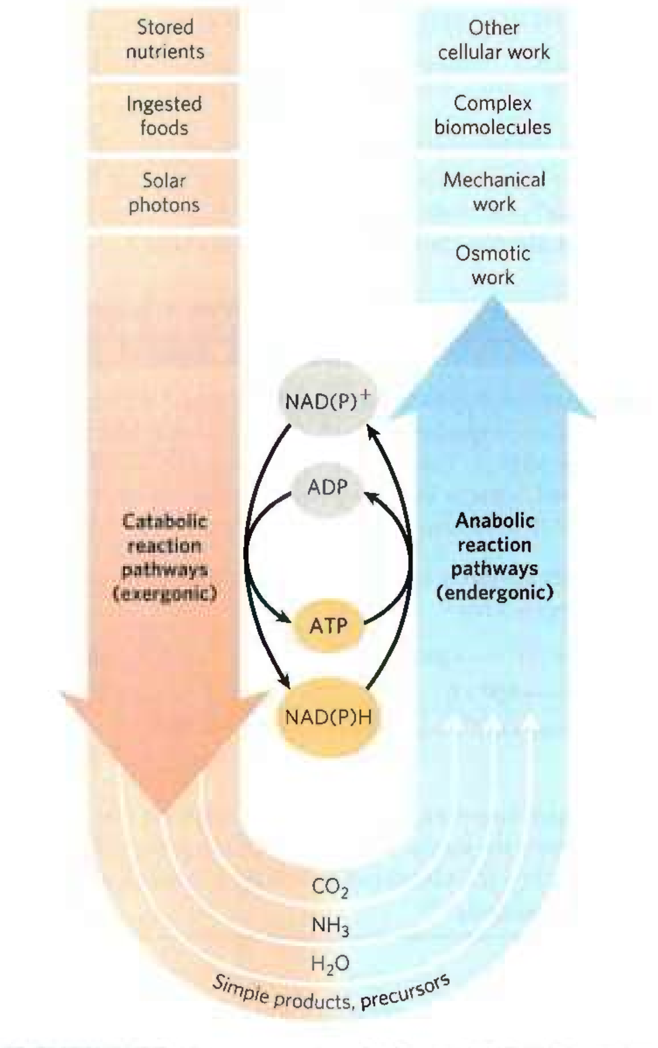


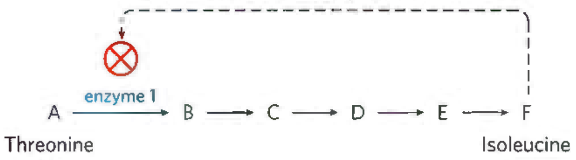
FIGURE 1-28 The central roles of ATP and NAD(P)H in metabolism. ATP is the shared chemical intermediate linking energy-releasing and energy-consuming cellular processes. Its role in the cell is analogous to that of money in an economy: it is "earned/produced" in exergonic reactions and "spent/consumed" in endergonic ones. NADH is an electron-carrying cofactor that collects electrons from oxidative reactions. The closely related NADPH carries electrons in a wide variety of reduction reactions in biosynthesis. Present in relatively low concentrations, these cofactors essential to anabolic reactions must be constantly regenerated by catabolic reactions.

The overall network of enzyme-catalyzed pathways, both catabolic and anabolic, constitutes cellular **metabolism**. ATP (as well as other energetically equivalent nucleoside triphosphates) is the connecting link between the catabolic and anabolic components of this network (shown schematically in **Fig. 1-28**). **P2** The pathways of enzyme-catalyzed reactions that act on the main constituents of cells—proteins, fats, sugars, and nucleic acids—are nearly identical in all living organisms. This remarkable **unity of life** is part of the evidence for a common evolutionary precursor for all living things.

Metabolism Is Regulated to Achieve Balance and Economy

Not only do living cells simultaneously synthesize thousands of different kinds of carbohydrate, fat, protein, and nucleic acid molecules and their simpler subunits, but they do so in the precise proportions required by the cell under any given circumstance. For example, during rapid cell growth the precursors of proteins and nucleic acids must be made in large quantities, whereas in nongrowing cells the requirement for these precursors is much reduced. Key enzymes in each metabolic pathway are regulated so that each type of precursor molecule is produced in a quantity appropriate to the current requirements of the cell.

Consider the pathway in *E. coli* that leads to the synthesis of the amino acid isoleucine, a constituent of proteins. The pathway has five steps catalyzed by five different enzymes (A through F represent the intermediates in the pathway):



If a cell begins to produce more isoleucine than it needs for protein synthesis, the unused isoleucine accumulates, and the increased concentration inhibits the catalytic activity of the first enzyme in the pathway, immediately slowing the production of isoleucine. Such **feedback inhibition** keeps the production and utilization of each metabolic intermediate in balance. (Throughout the book, we use ⊗ to indicate inhibition of an enzymatic reaction.)

Although the concept of discrete pathways is an important tool for organizing our understanding of metabolism, it is an oversimplification. There are thousands of metabolic intermediates in a cell, many of which are part of more than one pathway. **P3** Metabolism would be better represented as a web of interconnected and interdependent pathways. A change in the concentration of any one metabolite would start a ripple effect, influencing the flow of materials through other pathways. The task of understanding these complex interactions among intermediates and pathways in quantitative terms is daunting, but **systems biology**, discussed in Chapter 9, has begun to offer important insights into the overall regulation of metabolism.

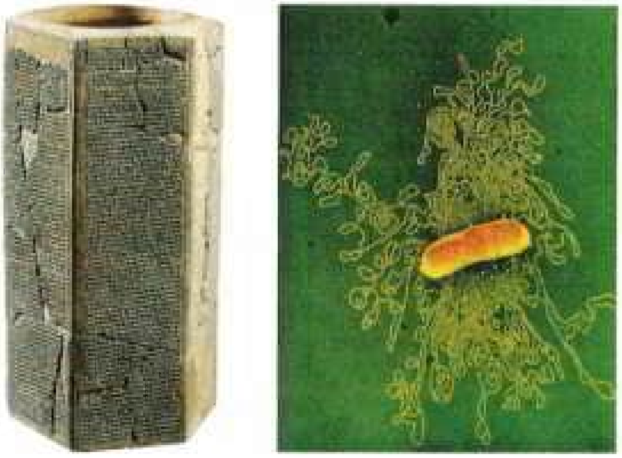
Cells also regulate the synthesis of their own catalysts, the enzymes, in response to increased or diminished need for a metabolic product; this is the substance of Chapter 28. The regulated expression of genes (the translation from information in DNA to active protein in the cell) and the synthesis of enzymes are other layers of metabolic control in the cell. All layers must be taken into account when the overall control of cellular metabolism is described.

SUMMARY 1.3 *Physical Foundations*

- Living cells extract and channel energy to maintain themselves in a dynamic steady state distant from equilibrium.
- Living cells are open systems, exchanging matter and energy with their surroundings. Energy is obtained from sunlight or chemical fuels when the energy from electron flow is converted into the chemical bonds of ATP.
- The tendency for a chemical reaction to proceed toward equilibrium can be expressed as the free-energy change, ΔG. When ΔG of a reaction is negative, the reaction is exergonic and tends to go toward completion; when ΔG is positive, the reaction is endergonic and tends to go in the reverse direction. When two reactions can be summed to yield a third reaction, the ΔG for this overall reaction is the sum of the ΔG values for the two separate reactions.
- The reactions converting ATP to P_i and ADP are highly exergonic (large negative ΔG). Many endergonic cellular reactions are driven by coupling them, through a common intermediate, to these highly exergonic reactions.
- The standard free-energy change for a reaction, $\Delta G°$, is a physical constant that is related to the equilibrium constant by the equation $\Delta G° = -RT \ln K_{eq}$.
- Most cellular reactions proceed at useful rates only because enzymes are present to catalyze them. Enzymes act in part by stabilizing the transition state, reducing the activation energy, $\Delta G^{\ddagger}$, and increasing the reaction rate by many orders of magnitude. The catalytic activity of enzymes in cells is regulated.
- Metabolism is the sum of many interconnected reaction sequences that interconvert cellular metabolites. Each sequence is regulated to provide what the cell needs at a given time and to expend energy only when necessary.

1.4 Genetic Foundations

P4 Perhaps the most remarkable property of living cells and organisms is their ability to reproduce themselves for countless generations with nearly perfect fidelity.



(a) (b)

FIGURE 1-29 Two ancient scripts. (a) The Prism of Sennacherib, inscribed in about 700 BCE, describes in characters of the Assyrian language some historical events during the reign of King Sennacherib. The Prism contains about 20,000 characters, weighs about 50 kg, and has survived almost intact for about 2,700 years. (b) The single DNA molecule of the bacterium *E. coli*, leaking out of a disrupted cell, is hundreds of times longer than the cell itself and contains all the encoded information necessary to specify the cell's structure and functions. The bacterial DNA contains about 4.6 million characters (nucleotides), weighs less than 10^{-10} g, and has undergone only relatively minor changes during the past several million years. (The yellow spots and dark specks in this colorized electron micrograph are artifacts of the preparation.) [(a) Erich Lessing/Art Resource, New York. (b) Dr. Gopal Murti/Science Source.]

This continuity of inherited traits implies constancy, over millions of years, in the structure of the molecules that contain the genetic information. Very few historical records of civilization, even those etched in copper or carved in stone (**Fig. 1-29**), have survived for a thousand years. But there is good evidence that the genetic instructions in living organisms have remained nearly unchanged over very much longer periods; many bacteria have nearly the same size, shape, and internal structure as bacteria that lived almost four billion years ago. This continuity of structure and composition is the result of continuity in the structure of the genetic material.

Among the seminal discoveries in biology in the twentieth century were the chemical nature and the three-dimensional structure of the genetic material, **deoxyribonucleic acid, DNA**. P2 The sequence of the monomeric subunits, the nucleotides (strictly, deoxyribonucleotides, as discussed below), in this linear polymer encodes the instructions for forming all other cellular components and provides a template for the production of identical DNA molecules to be distributed to progeny when a cell divides.

Genetic Continuity Is Vested in Single DNA Molecules

DNA is a long, thin, organic polymer, the rare molecule that is constructed on the atomic scale in one dimension (width) and the human scale in another (length: a molecule of DNA can be many centimeters long). A human sperm or egg, carrying the accumulated hereditary information of billions of years of evolution, transmits this inheritance in the form of DNA molecules, in which the linear sequence of covalently linked nucleotide subunits encodes the genetic message.

Usually when we describe the properties of a chemical species, we describe the average behavior of a very large number of identical molecules. While it is difficult to predict the behavior of any single molecule in a collection of, say, a picomole (about 6×10^{11} molecules) of a compound, the *average* behavior of the molecules is predictable because so many molecules enter into the average. Cellular DNA is a remarkable exception. P4 The DNA that is the entire genetic material of an *E. coli cell* is a *single molecule* containing 4.64 million nucleotide pairs. That single molecule must be replicated perfectly in every detail if an *E. coli* cell is to give rise to identical progeny by cell division; there is no room for averaging in this process! The same is true for all cells. A human sperm brings to the egg that it fertilizes just one molecule of DNA in each of its 23 different chromosomes, to combine with just one DNA molecule in each corresponding chromosome in the egg. The result of this union is highly predictable: an embryo with all of its ~20,000 genes, constructed of 3 billion nucleotide pairs, intact. An amazing chemical feat!

WORKED EXAMPLE 1-4 *Fidelity of DNA Replication*

Calculate the number of times the DNA of a modern *E. coli* cell has been copied accurately since its earliest bacterial precursor cell arose about 3.5 billion years ago. Assume for simplicity that over this time period, *E. coli* has undergone, on average, one cell division every 12 hours (this is an overestimate for modern bacteria, but probably an underestimate for ancient bacteria).

SOLUTION:

$$(1\,\text{generation}/12\,\text{h})(24\,\text{h}/\text{day})(365\,\text{days/yr})(3.5 \times 10^9\,\text{yr}) = 2.6 \times 10^{12}\,\text{generations}$$

A single page of this book contains about 5,000 characters, so the entire book contains about 5 million characters. The chromosome of *E. coli* also contains about 5 million characters (nucleotide pairs). Imagine making a handwritten copy of this book and passing on the copy to a classmate, who copies it by hand and passes this second copy to a third classmate, who makes a third copy, and so on. How closely would each successive copy of the book resemble the original? Now, imagine the textbook that would result from hand-copying this one a few trillion times!

The Structure of DNA Allows Its Replication and Repair with Near-Perfect Fidelity

P4 The capacity of living cells to preserve their genetic material and to duplicate it for the next generation results from the structural complementarity between the two strands of the DNA molecule (**Fig. 1-30**). The basic

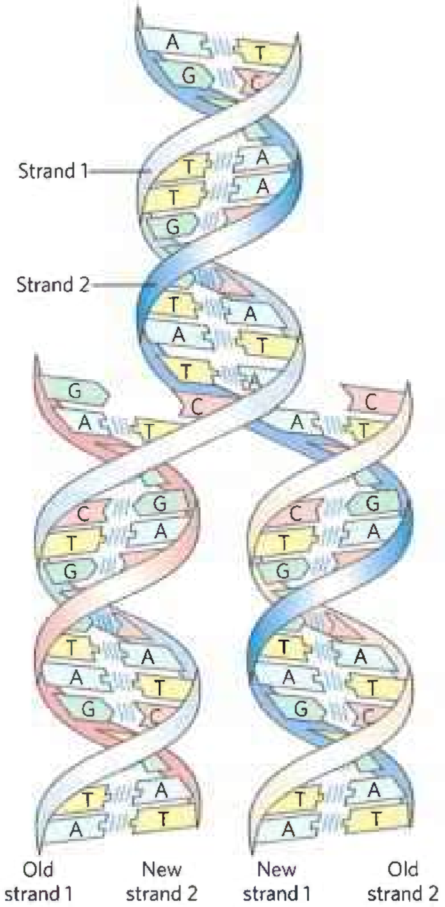


FIGURE 1-30 Complementarity between the two strands of DNA. DNA is a linear polymer of covalently joined deoxyribonucleotides of four types: deoxyadenylate (A), deoxyguanylate (G), deoxycytidylate (C), and deoxythymidylate (T). Each nucleotide, with its unique three-dimensional structure, can associate very specifically but noncovalently with one other nucleotide in the complementary chain: A always associates with T, and G with C. Thus, in the double-stranded DNA molecule, the entire sequence of nucleotides in one strand is *complementary* to the sequence in the other. The two strands, held together by hydrogen bonds (represented here by vertical light blue lines) between each pair of complementary nucleotides, twist about each other to form the DNA double helix. In DNA replication, the two strands (blue) separate and two new strands (pink) are synthesized, each with a sequence complementary to one of the original strands. The result is two double-helical molecules, each identical to the original DNA.

unit of DNA is a linear polymer of four different monomeric subunits, **deoxyribonucleotides**, arranged in a precise linear sequence. It is this linear sequence that encodes the genetic information. Two of these polymeric strands are twisted about each other to form the DNA double helix, in which each deoxyribonucleotide in one strand pairs specifically with a complementary deoxyribonucleotide in the opposite strand. **P4** Before a cell divides, the two DNA strands separate locally and each serves as a template for the synthesis of a new, complementary strand, generating two identical double-helical molecules, one for each daughter cell. If either strand is damaged at any time, continuity of information is assured by the information present in the other strand, which can act as a template for repair of the damage.

The Linear Sequence in DNA Encodes Proteins with Three-Dimensional Structures

P4 The information in DNA is encoded in its linear (one-dimensional) sequence of deoxyribonucleotide subunits, but the expression of this information results in a three-dimensional cell. This change from one to three dimensions occurs in two phases. A linear sequence of deoxyribonucleotides in DNA codes (through an intermediary, RNA) for the production of a protein with a corresponding linear sequence of amino acids (**Fig. 1-31**). The protein folds into a particular three-dimensional shape, determined by its amino acid sequence and stabilized primarily by noncovalent interactions. Although the final shape of the folded protein is dictated by its amino acid sequence, the folding of many proteins is aided by "molecular chaperones" (see Fig. 4-28). The precise three-dimensional structure, or **native conformation**, of the protein is crucial to its function.

Once in its native conformation, a protein may associate noncovalently with other macromolecules (other proteins, nucleic acids, or lipids) to form supramolecular

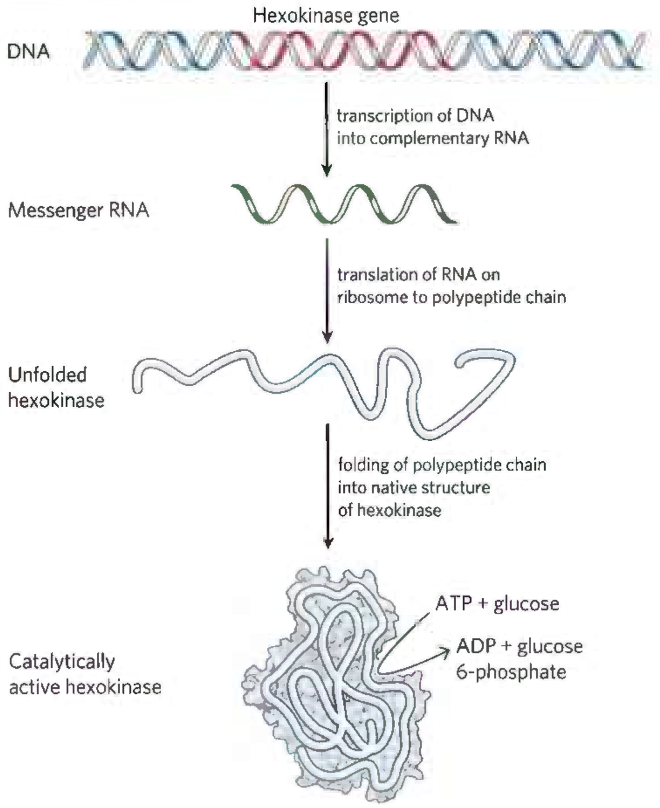

Hexokinase gene
DNA
transcription of DNA into complementary RNA
Messenger RNA
translation of RNA on ribosome to polypeptide chain
Unfolded hexokinase
folding of polypeptide chain into native structure of hexokinase
Catalytically active hexokinase
ATP + glucose
ADP + glucose 6-phosphate

FIGURE 1-31 DNA to RNA to protein to enzyme (hexokinase). The linear sequence of deoxyribonucleotides in the DNA (the gene) that encodes the protein hexokinase is first transcribed into a ribonucleic acid (RNA) molecule with the complementary ribonucleotide sequence. The RNA sequence (messenger RNA) is then translated into the linear protein chain of hexokinase, which folds into its native three-dimensional shape, most likely aided by molecular chaperones. Once in its native form, hexokinase acquires its catalytic activity: it can catalyze the phosphorylation of glucose, using ATP as the phosphoryl group donor.

complexes such as chromosomes, ribosomes, and membranes. The individual molecules of these complexes have specific, high-affinity binding sites for each other, and within the cell they spontaneously self-assemble into functional complexes.

Although the amino acid sequences of proteins carry all necessary information for achieving the proteins' native conformation, accurate folding and self-assembly also require the right cellular environment—pH, ionic strength, metal ion concentrations, and so forth. Thus, DNA sequence alone is not enough to form and maintain a fully functioning cell.

SUMMARY 1.4 *Genetic Foundations*

- Genetic information is encoded in the linear sequence of four types of deoxyribonucleotides in DNA.
- Despite the enormous size of DNA, the sequence of its nucleotides is very precise, and the maintenance of this precise sequence over very long times is the basis for genetic continuity in organisms.
- The double-helical DNA molecule contains an internal template for its own replication and repair.
- The linear sequence of amino acids in a protein, which is encoded in the DNA of the gene for that protein, produces a protein's unique three-dimensional structure—a process that is also dependent on environmental conditions.
- Individual macromolecules with specific affinity for other macromolecules self-assemble into supramolecular complexes.

1.5 Evolutionary Foundations

> Nothing in biology makes sense except in the light of evolution.
>
> —Theodosius Dobzhansky, in *The American Biology Teacher*, March 1973

Great progress in biochemistry and molecular biology in recent decades has amply confirmed the validity of Dobzhansky's striking generalization. **P5** The remarkable similarity of metabolic pathways and gene sequences across the three domains of life argues strongly that all modern organisms are derived from a common evolutionary progenitor by a series of small changes (mutations), each of which conferred a selective advantage to some organism in some ecological niche.

Changes in the Hereditary Instructions Allow Evolution

Despite the near-perfect fidelity of genetic replication, infrequent unrepaired mistakes in the DNA replication process lead to changes in the nucleotide sequence of DNA, producing a genetic **mutation** and changing the instructions for a cellular component. Incorrectly repaired damage to one of the DNA strands has the same effect. Mutations in the DNA handed down to offspring—that is, mutations carried in the reproductive cells—may be harmful or even lethal to the new organism or cell; they may, for example, cause the synthesis of a defective enzyme that is not able to catalyze an essential metabolic reaction. Occasionally, however, a mutation *better* equips an organism or cell to survive in its environment (**Fig. 1-32**). The mutant enzyme might have acquired a slightly different specificity, for example, so that it is now able to use some compound that the cell was previously unable to metabolize. If a population of cells were to find itself in an environment where that compound was the only or the most abundant available source of fuel, the mutant cell would have a selective advantage over the other, unmutated (**wild-type**) cells in the population. The mutant cell and its progeny would survive and prosper in the new environment, whereas wild-type cells would starve and be eliminated. This is what Charles Darwin meant by natural selection—what is sometimes summarized as "survival of the fittest."

Occasionally, a second copy of a whole gene is introduced into the chromosome as a result of defective replication of the chromosome. The second copy is superfluous, and mutations in this gene will not be deleterious; it becomes a means by which the cell may evolve, by producing a new gene with a new function while retaining the original gene and gene function. Seen in this light, the DNA molecules of modern organisms are historical documents, records of the long journey from the earliest cells to modern organisms. The historical accounts in DNA are not complete, however; in the course of evolution, many mutations must have been erased or written over. But DNA molecules are the best source of biological history we have. **P5** The frequency of errors in DNA replication represents a balance between too many errors, which would yield nonviable daughter cells, and too few errors, which would prevent the genetic variation that allows survival of mutant cells in new ecological niches.

Several billion years of natural selection have refined cellular systems to take maximum advantage of the chemical and physical properties of available raw materials. Chance genetic mutations occurring in individuals in a population, combined with natural selection, have resulted in the evolution of the enormous variety of species we see today, each adapted to its particular ecological niche.

Biomolecules First Arose by Chemical Evolution

In our account thus far, we have passed over the first chapter of the story of evolution: the appearance of the first living cell. Apart from their occurrence in living organisms, organic compounds, including the basic biomolecules such as amino acids and carbohydrates, are found in only trace amounts in the Earth's crust,

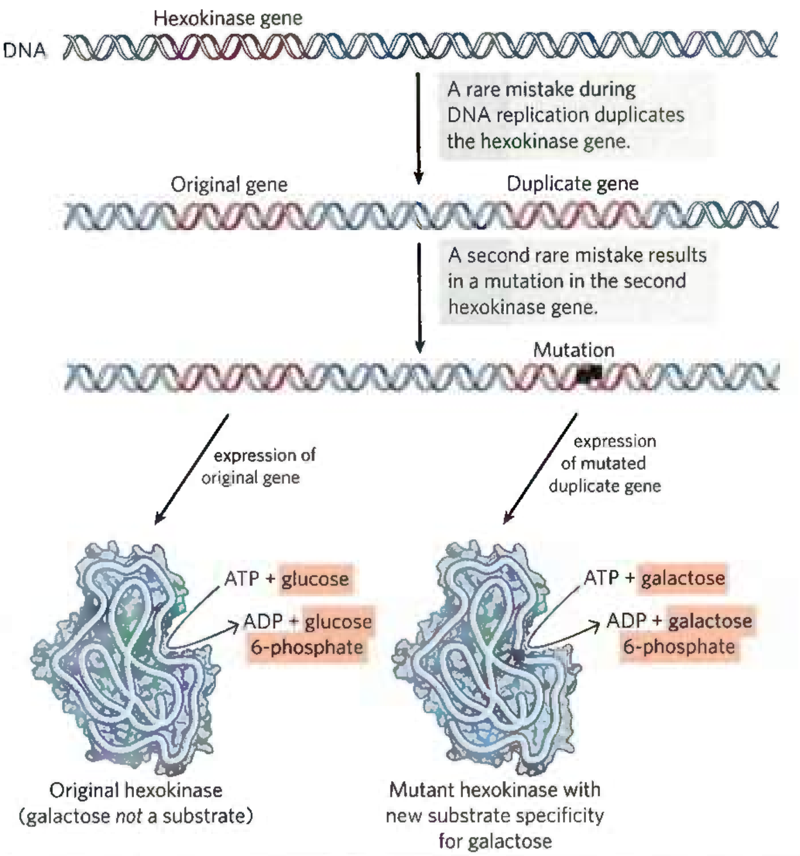

Hexokinase gene
DNA
A rare mistake during DNA replication duplicates the hexokinase gene.
Original gene
Duplicate gene
A second rare mistake results in a mutation in the second hexokinase gene.
Mutation
expression of original gene
expression of mutated duplicate gene
ATP + glucose
ADP + glucose 6-phosphate
ATP + galactose
ADP + galactose 6-phosphate
Original hexokinase (galactose *not* a substrate)
Mutant hexokinase with new substrate specificity for galactose

FIGURE 1-32 Gene duplication and mutation: one path to generate new enzymatic activities. In this example, the single hexokinase gene in a hypothetical organism might occasionally, by accident, be copied twice during DNA replication, such that the organism has two full copies of the gene, one of which is superfluous. Over many generations, as the DNA with two hexokinase genes is repeatedly duplicated, rare mistakes occur, leading to changes in the nucleotide sequence of the superfluous gene and thus of the protein that it encodes. In a few very rare cases, the altered protein produced from this mutant gene can bind a new substrate—galactose in our hypothetical case. The cell containing the mutant gene has acquired a new capability (metabolism of galactose), which may allow the cell to survive in an ecological niche that provides galactose but not glucose. If no gene duplication precedes mutation, the original function of the gene product is lost.

the sea, and the atmosphere. How did the first living organisms acquire their characteristic organic building blocks? According to one hypothesis, these compounds were created by the effects of powerful environmental forces—ultraviolet irradiation, lightning, or volcanic eruptions—on the gases in the prebiotic Earth's atmosphere and on inorganic solutes in superheated thermal vents deep in the ocean.

This hypothesis was tested in a classic experiment on the abiotic (nonbiological) origin of organic biomolecules carried out in 1953 by biochemist Stanley Miller in the laboratory of the physical chemist Harold Urey. Miller subjected gaseous mixtures such as those presumed to exist on the prebiotic Earth, including NH_3, CH_4, H_2O, and H_2, to electrical sparks produced across a pair of electrodes (to simulate lightning) for periods of a week or more, then analyzed the contents of the closed reaction vessel (**Fig. 1-33**). The gas phase of the resulting mixture contained CO and CO_2 as well as the starting materials. The water phase contained a variety of organic compounds, including some amino acids, hydroxy acids, aldehydes, and hydrogen cyanide (HCN). This experiment established the possibility of abiotic production of biomolecules in relatively short times under relatively mild conditions. When Miller's carefully stored samples were rediscovered in 2010 and examined with much more sensitive and discriminating techniques (high-performance liquid chromatography and mass spectrometry), his original observations were confirmed and greatly broadened. Previously unpublished experiments by Miller that included H_2S in the gas mixture (mimicking the "smoking" volcanic plumes at the sea bottom; **Fig. 1-34**) showed the formation of 23 amino acids and 7 organosulfur compounds, as well as a large number of other simple compounds that might have served as building blocks in prebiotic evolution.

More-refined laboratory experiments have provided good evidence that many of the chemical components of living cells can form under these conditions. Polymers of the nucleic acid **RNA (ribonucleic acid)** can act as catalysts in biologically significant reactions (see Chapters 26 and 27), and RNA probably played a crucial role in prebiotic evolution, both as catalyst and as information repository.

RNA or Related Precursors May Have Been the First Genes and Catalysts

In modern organisms, nucleic acids encode the genetic information that specifies the structure of enzymes, and enzymes catalyze the replication and repair of nucleic acids. The mutual dependence of these two classes of biomolecules brings up the perplexing question: Which came first, DNA or protein?

The answer may be that they appeared about the same time, and RNA preceded them both.

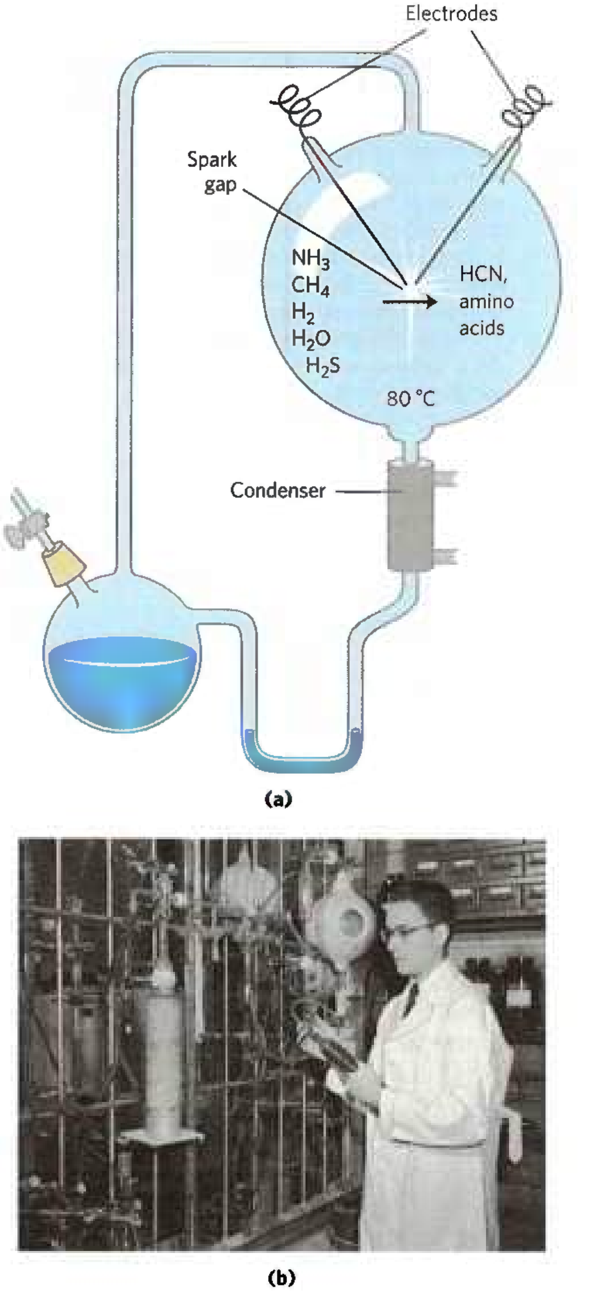


FIGURE 1-33 Abiotic production of biomolecules. (a) Spark-discharge apparatus of the type used by Miller and Urey in experiments demonstrating abiotic formation of organic compounds under primitive atmospheric conditions. After subjection of the gaseous contents of the system to electrical sparks, products were collected by condensation. Biomolecules such as amino acids were among the products. (b) Stanley L. Miller (1930–2007) using his spark-discharge apparatus. [(b) Bettmann/Getty Images.]

FIGURE 1-34 Black smokers. Hydrothermal vents in the sea floor emit superheated water rich in dissolved minerals. Black "smoke" is formed when the vented solution meets cold seawater and dissolved sulfides precipitate. Diverse life forms, including a variety of archaea and some remarkably complex multicellular organisms, are found in the immediate vicinity of such vents, which may have been the sites of early biogenesis. [NOAA/Science Source]

The discovery that RNA molecules can act as catalysts in their own formation suggests that RNA or a similar molecule may have been the first gene *and* the first catalyst. According to this scenario (**Fig. 1-35**), one of the earliest stages of biological evolution was the chance formation of an RNA molecule that could catalyze the formation of other RNA molecules of the same sequence—a self-replicating, self-perpetuating RNA. The concentration of a self-replicating RNA molecule would increase exponentially, as one molecule formed several, several formed many, and so on. The fidelity of self-replication was presumably less than perfect, so the process would generate variants of the RNA, some of which might be even better able to self-replicate. In the competition for nucleotides, the most efficient of the self-replicating sequences would win, and less efficient replicators would fade from the population.

The division of function between DNA (genetic information storage) and protein (catalysis) was, according to the "RNA world" hypothesis, a later development. New variants of self-replicating RNA molecules developed that had the additional ability to catalyze the condensation of amino acids into peptides. Occasionally, the peptide(s) thus formed would reinforce the self-replicating ability of the RNA, and the pair—RNA molecule and helping peptide—could undergo further modifications in sequence, generating increasingly efficient self-replicating systems. The remarkable discovery that in the protein-synthesizing machinery of modern cells (ribosomes), RNA molecules, not proteins, catalyze the formation of peptide bonds is consistent with the RNA world hypothesis.

Some time after the evolution of this primitive protein-synthesizing system, there was a further development: DNA molecules with sequences complementary to the self-replicating RNA molecules took over the function of conserving the "genetic" information, and RNA molecules evolved to play roles in protein synthesis. (We explain in Chapter 8 why DNA is a more stable molecule than RNA and thus a better repository of inheritable information.) Proteins proved to be versatile catalysts

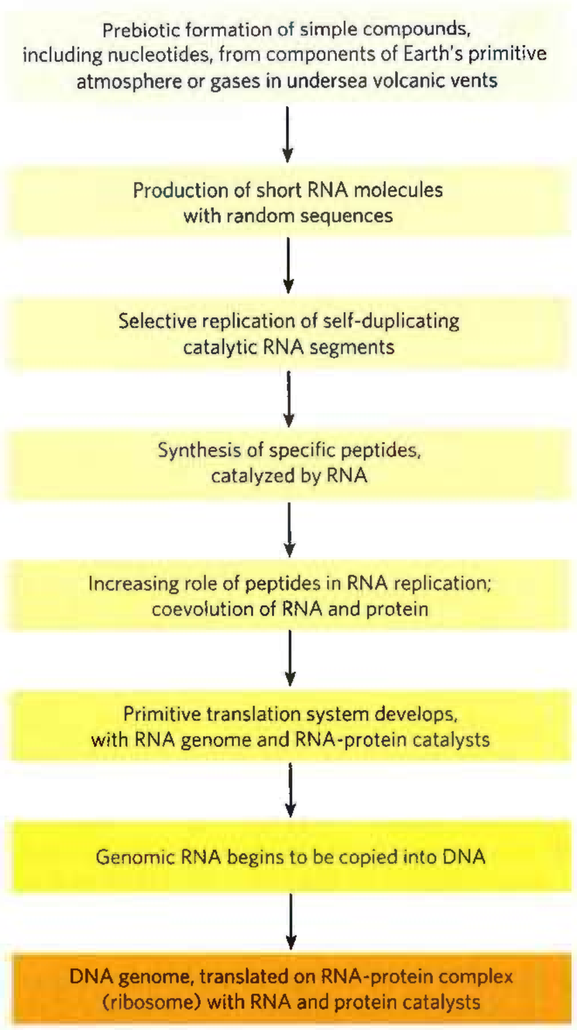

Prebiotic formation of simple compounds, including nucleotides, from components of Earth's primitive atmosphere or gases in undersea volcanic vents
Production of short RNA molecules with random sequences
Selective replication of self-duplicating catalytic RNA segments
Synthesis of specific peptides, catalyzed by RNA
Increasing role of peptides in RNA replication; coevolution of RNA and protein
Primitive translation system develops, with RNA genome and RNA-protein catalysts
Genomic RNA begins to be copied into DNA
DNA genome, translated on RNA-protein complex (ribosome) with RNA and protein catalysts

FIGURE 1-35 A possible "RNA world" scenario.

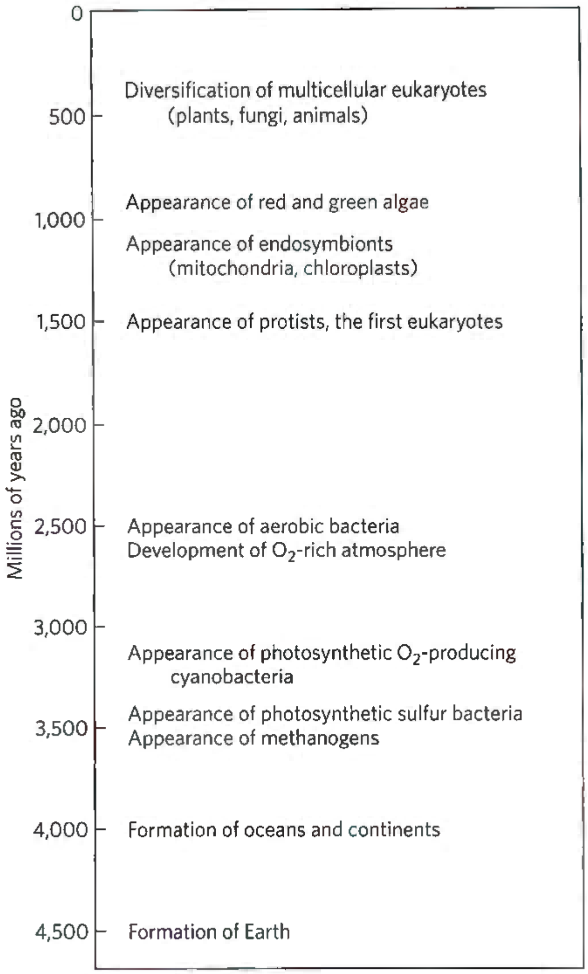


FIGURE 1-36 Landmarks in the evolution of life on Earth.

and, over time, took over most of that function. Lipidlike compounds in the primordial mixture formed relatively impermeable layers around self-replicating collections of molecules. The concentration of proteins and nucleic acids within these lipid enclosures favored the molecular interactions required in self-replication.

The RNA world scenario is intellectually satisfying, but it leaves unanswered a vexing question: Where did the nucleotides needed to make the initial RNA molecules come from? An alternative to this scenario supposes that simple metabolic pathways evolved first, perhaps at the hot vents in the ocean floor. A set of linked chemical reactions there might have produced precursors, including nucleotides, before the advent of lipid membranes or RNA. Without more experimental evidence, neither of these hypotheses can be disproved.

Biological Evolution Began More Than Three and a Half Billion Years Ago

Earth was formed about 4.6 billion years ago, and the first evidence of life dates to more than 3.5 billion years ago (see the timeline in **Figure 1-36**). In 1996, scientists working in Greenland found chemical evidence of life ("fossil molecules") from as far back as 3.85 billion years ago, forms of carbon embedded in rock that seem to have a distinctly biological origin. Somewhere on Earth during its first billion years the first simple organism arose, capable of replicating its own structure from a template (RNA?) that was the first genetic material. Because the terrestrial atmosphere at the dawn of life was nearly devoid of oxygen, and because there were few microorganisms to scavenge organic compounds formed by natural processes, these compounds were relatively stable. Given this stability and eons of time, the improbable became inevitable: lipid vesicles containing organic compounds and self-replicating RNA gave rise to the first cells, or protocells, and those protocells with the greatest capacity for self-replication became more numerous. The process of biological evolution had begun.

The First Cell Probably Used Inorganic Fuels

The earliest cells arose in a reducing atmosphere (there was no oxygen) and probably obtained energy from inorganic fuels such as ferrous sulfide and ferrous carbonate, both abundant on the early Earth. For example, the reaction

$$FeS + H_2S \longrightarrow FeS_2 + H_2$$

yields enough energy to drive the synthesis of ATP or similar compounds. The organic compounds these early cells required may have arisen by the nonbiological actions of lightning or of heat from volcanoes or thermal vents in the sea on components of the early atmosphere such as CO, CO_2, N_2, NH_3, and CH_4. An alternative source of organic compounds has been proposed: extraterrestrial space. Space missions in 2006 (the NASA Stardust space probe) and 2014 (the European Space Agency lander Philae) found particles of comet dust containing the simple amino acid glycine and 20 other organic compounds capable of reacting to form biomolecules.

Early unicellular organisms gradually acquired the ability to derive energy from compounds in their environment and to use that energy to synthesize more of their own precursor molecules, thereby becoming less dependent on outside sources. A very significant evolutionary event was the development of pigments capable of capturing the energy of light from the sun, which could be used to reduce, or "fix," CO_2 to form more complex, organic compounds. The original electron donor for these **photosynthetic** processes was probably H_2S, yielding elemental sulfur or sulfate (SO_4^{2-}) as the byproduct. Some hydrothermal vents in the sea bottom (black smokers; Fig. 1-36) emit significant amounts of H_2, which is another possible electron donor in the metabolism of the earliest organisms. Later cells developed the enzymatic capacity to use H_2O as the electron donor in photosynthetic reactions, producing O_2 as waste. Cyanobacteria are the modern descendants of these early photosynthetic oxygen-producers.

Because the atmosphere of Earth in the earliest stages of biological evolution was nearly devoid of oxygen, the earliest cells were anaerobic. Under these conditions, chemotrophs could oxidize organic compounds to CO_2 by passing electrons not to O_2 but to acceptors such as SO_4^{2-}, in this case yielding H_2S as the product. With the rise of O_2-producing photosynthetic bacteria, the atmosphere became progressively richer in oxygen—a powerful oxidant and a deadly poison to anaerobes. Responding to the evolutionary pressure of what evolutionary theorist and biologist Lynn Margulis and science writer Dorion Sagan called the "oxygen holocaust," some lineages of microorganisms gave rise to aerobes that obtained energy by passing electrons from fuel molecules to oxygen. Because the transfer of electrons from organic molecules to O_2 releases a great deal of energy, aerobic organisms had an energetic advantage over their anaerobic counterparts when both competed in an environment containing oxygen. This advantage translated into the predominance of aerobic organisms in O_2-rich environments.

Modern bacteria and archaea inhabit almost every ecological niche in the biosphere, and there are organisms capable of using virtually every type of organic compound as a source of carbon and energy. Photosynthetic microbes in both fresh and marine waters trap solar energy and use it to generate carbohydrates and all other cell constituents, which are in turn used as food by other forms of life. The process of evolution continues—and, in rapidly reproducing bacterial cells, on a time scale that allows us to witness it in the laboratory.

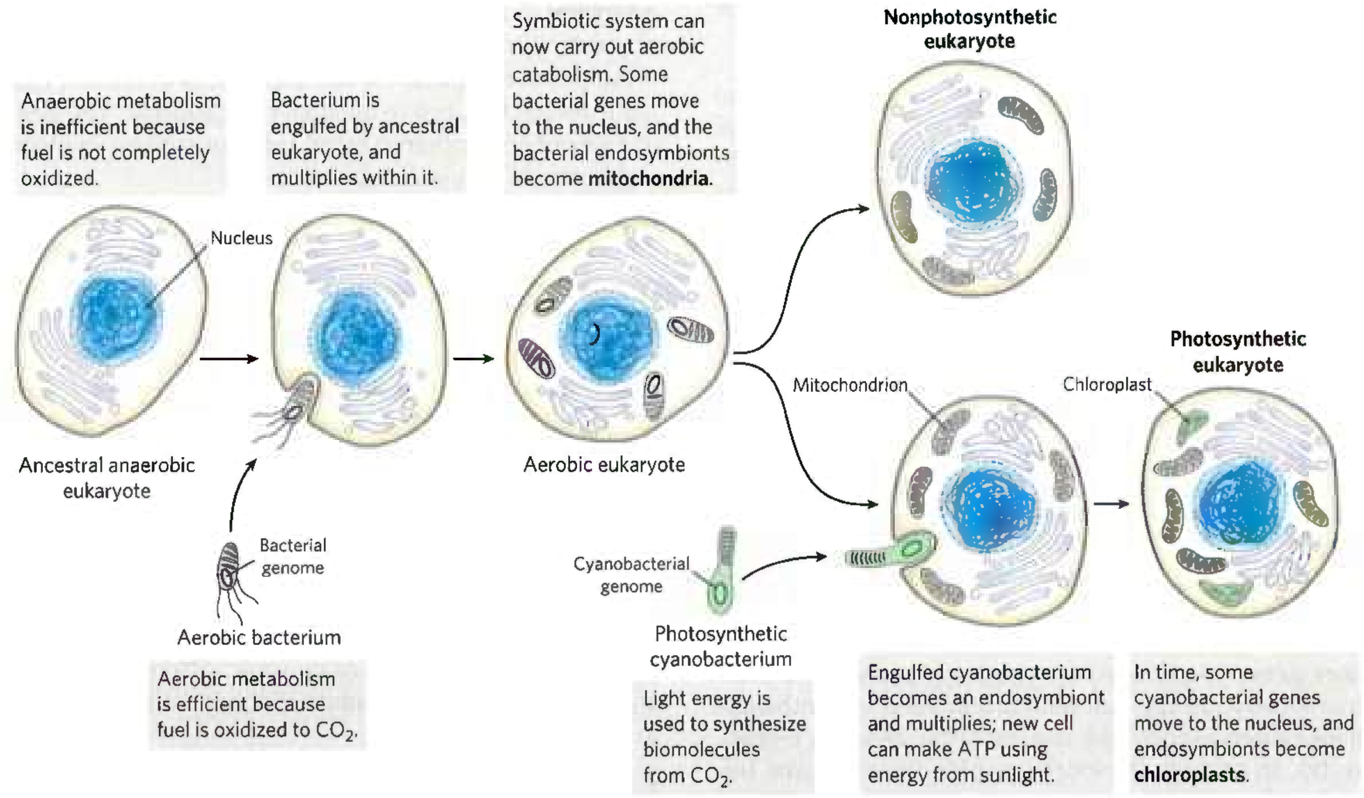
Eukaryotic Cells Evolved from Simpler Precursors in Several Stages

Starting about 1.5 billion years ago, the fossil record begins to show evidence of larger and more complex organisms, probably the earliest eukaryotic cells (see Fig. 1-37). Details of the evolutionary path from nonnucleated to nucleated cells cannot be deduced from the fossil record alone, but morphological and biochemical comparisons of modern organisms have suggested a sequence of events consistent with the fossil evidence.

Three major changes must have occurred. First, as cells acquired more DNA, the mechanisms required to fold it compactly into discrete complexes with specific proteins and to divide it equally between daughter cells at cell division became more elaborate. Specialized proteins were required to stabilize folded DNA and to pull the resulting DNA-protein complexes (chromosomes) apart during cell division. This was the evolution of the chromosome. Second, as cells became larger, a system of intracellular membranes developed, including a double membrane surrounding the DNA. This membrane segregated the nuclear process of RNA synthesis on a DNA template from the cytoplasmic process of protein synthesis on ribosomes. This was the evolution of the nucleus, a defining feature of eukaryotes. Third, early eukaryotic cells, which were incapable of photosynthesis or aerobic metabolism, enveloped aerobic bacteria or photosynthetic bacteria to form **endosymbiotic** associations that eventually became permanent (**Fig. 1-37**). Some aerobic bacteria evolved into the mitochondria of modern eukaryotes, and some photosynthetic cyanobacteria became the plastids, such as the chloroplasts of green algae, the likely ancestors of modern plant cells.

At some later stage of evolution, unicellular organisms found it advantageous to cluster together, thereby acquiring greater motility, efficiency, or reproductive success than their free-living single-celled competitors. Further evolution of such clustered organisms led to permanent associations among individual cells and eventually to specialization within the colony—to cellular differentiation.

The advantages of cellular specialization led to the evolution of increasingly complex and highly differentiated organisms, in which some cells carried out the



Anaerobic metabolism is inefficient because fuel is not completely oxidized.

Bacterium is engulfed by ancestral eukaryote, and multiplies within it.

Symbiotic system can now carry out aerobic catabolism. Some bacterial genes move to the nucleus, and the bacterial endosymbionts become **mitochondria.**

Aerobic metabolism is efficient because fuel is oxidized to CO_2.

Light energy is used to synthesize biomolecules from CO_2.

Engulfed cyanobacterium becomes an endosymbiont and multiplies; new cell can make ATP using energy from sunlight.

In time, some cyanobacterial genes move to the nucleus, and endosymbionts become **chloroplasts.**

FIGURE 1-37 Evolution of eukaryotes through endosymbiosis. The earliest eukaryote, an anaerobe, acquired endosymbiotic purple bacteria, which carried with them their capacity for aerobic catabolism and became, over time, mitochondria. When photosynthetic cyanobacteria subsequently became endosymbionts of some aerobic eukaryotes, these cells became the photosynthetic precursors of modern green algae and plants.

sensory functions; others the digestive, photosynthetic, or reproductive functions; and so forth. Many modern multicellular organisms contain hundreds of different cell types, each specialized for a function that supports the entire organism. Fundamental mechanisms that evolved early have been further refined and embellished through evolution. The same basic structures and mechanisms that underlie the beating motion of cilia in *Paramecium* and of flagella in *Chlamydomonas* are employed by the highly differentiated vertebrate sperm cell, for example.

Molecular Anatomy Reveals Evolutionary Relationships

Now that genomes can be sequenced relatively quickly and inexpensively, biochemists have an enormously rich, ever-increasing treasury of information on the molecular anatomy of cells that they can use to analyze evolutionary relationships and refine evolutionary theory. Thus far, the molecular phylogeny derived from gene sequences is consistent with, but in many cases more precise than, the classical phylogeny based on macroscopic structures. **P5** Although organisms have continuously diverged at the level of gross anatomy, at the molecular level the basic unity of life is readily apparent; molecular structures and mechanisms are remarkably similar from the simplest to the most complex organisms. These similarities are most easily seen at the level of sequences, either the DNA sequences that encode proteins or the protein sequences themselves.

When two genes share readily detectable sequence similarities (nucleotide sequence in DNA or amino acid sequence in the proteins they encode), their sequences are said to be homologous and the proteins they encode are **homologs**. In the course of evolution, new structures, processes, or regulatory mechanisms are acquired, reflections of the changing genomes of the evolving organisms. The genome of a simple eukaryote such as yeast should have genes related to formation of the nuclear membrane, genes not present in bacteria or archaea. The genome of an insect should contain genes that encode proteins involved in specifying a characteristic segmented body plan, genes not present in yeast. The genomes of all vertebrate animals should share genes that specify the development of a spinal column, and those of mammals should have unique genes necessary for the development of the placenta, a characteristic of mammals—and so on. Comparisons of the whole genomes of species in each phylum are leading to the identification of genes critical to fundamental evolutionary changes in body plan and development.

Functional Genomics Shows the Allocations of Genes to Specific Cellular Processes

When the sequence of a genome is fully determined and each gene is assigned a function, molecular geneticists can group genes according to the processes (DNA synthesis, protein synthesis, generation of ATP, and so forth) in which they function and thus find what fraction of the genome is allocated to each of a cell's activities. The largest category of genes in *E. coli*, *A. thaliana*, and *H. sapiens* consists of those of (as yet) unknown function, which make up more than 40% of the genes in each species. The genes encoding the transporters that move ions and small molecules across plasma membranes make up a significant proportion of the genes in all three species, more in the bacterium and plant than in the mammal (10% of the ~4,400 genes of *E. coli*, ~8% of the ~27,000 genes of *A. thaliana*, and ~4% of the ~20,000 genes of *H. sapiens*). Genes that encode the proteins and RNA required for protein synthesis make up 3% to 4% of the *E. coli* genome; but in the more complex cells of *A. thaliana*, more genes are needed for targeting proteins to their final location in the cell than are needed to synthesize those proteins (about 6% and 2% of the genome, respectively). In general, the more complex the organism, the greater the proportion of its genome that encodes genes involved in the *regulation* of cellular processes and the smaller the proportion dedicated to basic processes, or "housekeeping" functions, such as ATP generation and protein synthesis. The **housekeeping genes** typically are expressed under all conditions and are not subject to much regulation.

Genomic Comparisons Have Increasing Importance in Medicine

Large-scale studies in which the entire genomic sequence has been determined for hundreds or thousands of people with cancer, type 2 diabetes, schizophrenia, or other diseases or conditions have allowed the identification of many genes in which mutations correlate with a medical condition. Typically, sequence differences are found in a number of different genes, each of which makes a partial contribution to the predisposition to a given condition or disease. Each of those genes codes for a protein that, in principle, might become the target for drugs to treat that condition. We may expect that for some genetic diseases, palliatives will be replaced by cures, and that for disease susceptibilities associated with particular genetic markers, forewarning and perhaps increased preventive measures will prevail. Today's "medical history" may be replaced by a "medical forecast." ■

SUMMARY 1.5 *Evolutionary Foundations*

- Occasional inheritable mutations yield organisms that are better suited for survival and reproduction in an ecological niche, and their progeny come to dominate the population in that niche. This process of mutation and selection is the basis for the Darwinian evolution that led from the first cell to all modern organisms. The large number of genes shared by all living organisms explains organisms' fundamental similarities.
- The components for the first cell may have been produced near hydrothermal vents at the bottom of the sea or by the action of lightning and high temperature on simple atmospheric molecules such as CO_2 and NH_3.
- The earliest cells may have been formed by the enclosure of a self-replicating RNA molecule within a membrane-like lipid layer. The catalytic and genetic roles played by the early RNA genome were, over time, taken over by proteins and DNA, respectively.
- Hydrothermal vents may have provided the oxidizable fuels (iron compounds) for the first organisms.
- Eukaryotic cells acquired the capacity for photosynthesis and oxidative phosphorylation from endosymbiotic bacteria. In multicellular organisms, differentiated cell types specialize in one or more of the functions essential to the organism's survival.
- Detailed phylogenetic relationships can be determined from gene or protein sequence similarities between organisms.
- From knowledge of the roles of proteins encoded in the genome, scientists can approximate the proportion of the genome dedicated to a specific process, such as membrane transport or protein synthesis.
- Knowledge of the complete genomic sequences of organisms from different branches of the phylogenetic tree provides insights into evolution and offers great opportunities in medicine.

KEY TERMS

All terms are defined in the glossary.

biochemistry	2
metabolite	2
nucleus	2
genome	2
eukaryotes	2
bacteria	3
archaea	3
cytoskeleton	6
stereoisomers	15
configuration	15
chiral center	16
conformation	18
entropy, S	21
enthalpy, H	21
free-energy change, ΔG	21
endergonic reaction	21
exergonic reaction	21
equilibrium	22
standard free-energy change, ΔG°	24
activation energy, $\Delta G^\ddagger$	25
catabolism	26
anabolism	26
metabolism	27
systems biology	27
mutation	30
housekeeping genes	36

PROBLEMS

For all numerical problems, keep in mind that answers should be expressed with the correct number of significant figures. (In solving end-of-chapter problems, you may wish to refer to the tables on the inside of the back cover.) Brief solutions are provided in Appendix B; expanded solutions are published in the *Absolute Ultimate Study Guide to Accompany Principles of Biochemistry*.

1. The Size of Cells and Their Components A typical eukaryotic cell has a cellular diameter of 50 μm.

(a) If you used an electron microscope to magnify this cell 10,000-fold, how big would the cell appear?
(b) If this cell were a liver cell (hepatocyte) with the same cellular diameter, how many mitochondria could the cell contain? Assume the cell is spherical; that the cell contains no other cellular components; and that each mitochondrion is spherical, with a diameter of 1.5 μm. (The volume of a sphere is $\frac{4}{3}\pi r^3$.)
(c) Glucose is the major energy-yielding nutrient for most cells. Assuming a cellular concentration of 1 mM glucose (that is, 1 millimole/L), calculate how many molecules of glucose would be present in the spherical eukaryotic cell. (Avogadro's number, the number of molecules in 1 mol of a nonionized substance, is 6.02×10^{23}.)

2. Components of *E. coli* *E. coli* cells are rod-shaped, about 2 μm long, and 0.8 μm in diameter. *E. coli* has a protective envelope 10 nm thick. The volume of a cylinder is $\pi r^2 h$, where h is the height of the cylinder.

(a) What percentage of the total volume of the bacterium does the cell envelope occupy?
(b) *E. coli* is capable of growing and multiplying rapidly because it contains some 15,000 spherical ribosomes (diameter 18 nm), which carry out protein synthesis. What percentage of the cell volume do the ribosomes occupy?
(c) The molecular weight of an *E. coli* DNA molecule is about 3.1×10^9 g/mol. The average molecular weight of a nucleotide pair is 660 g/mol, and each nucleotide pair contributes 0.34 nm to the length of DNA. Calculate the length of an *E. coli* DNA molecule. Compare the length of the DNA molecule with the cell dimensions. Now, consider the photomicrograph showing the single DNA molecule of the bacterium *E. coli* leaking out of a disrupted cell (Fig. 1-31b). How does the DNA molecule fit into the cell?

3. Isolating Ribosomes through Differential Centrifugation Assume you have a crude lysate sample that you obtained from mechanically homogenizing *E. coli* cells. You centrifuged the supernatant from the sample at a medium speed (20,000 *g*) for 20 min, collected the supernatant, and then centrifuged the supernatant at high speed (80,000 *g*) for 1 h. What procedure should you follow to isolate the ribosomes from this sample?

4. The High Rate of Bacterial Metabolism Bacterial cells have a much higher rate of metabolism than animal cells. Under ideal conditions, some bacteria double in size and divide every 20 min, whereas most animal cells under rapid growth conditions require 24 hours. The high rate of bacterial metabolism requires a high ratio of surface area to cell volume.

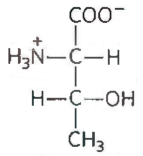
(a) How does the surface-to-volume ratio affect the maximum rate of metabolism?
(b) Calculate the surface-to-volume ratio for the spherical bacterium *Neisseria gonorrhoeae* (diameter 0.5 μm), responsible for the disease gonorrhea. The surface area of a sphere is $4\pi r^2$.
(c) How many times greater is the surface-to-volume ratio of *Neisseria gonorrhoeae* compared to that of a globular amoeba, a large eukaryotic cell (diameter 150 μm)?

5. Fast Axonal Transport Neurons have long thin processes called axons, structures specialized for conducting signals throughout the organism's nervous system. The axons that originate in a person's spinal cord and terminate in the muscles of the toes can be as long as 2 m. Small membrane-enclosed vesicles carrying materials essential to axonal function move along microtubules of the cytoskeleton, from the cell body to the tips of the axons. If the average velocity of a vesicle is 1 μm/s, how long does it take a vesicle to move from a cell body in the spinal cord to the axonal tip in the toes?

6. Comparing Synthetic versus Natural Vitamin C Some purveyors of health foods claim that vitamins obtained from natural sources are more healthful than those obtained by chemical synthesis. For example, some claim that pure L-ascorbic acid (vitamin C) extracted from rose hips is better than pure L-ascorbic acid manufactured in a chemical plant. Are the vitamins from the two sources different? Can the body distinguish a vitamin's source? Explain your answer.

7. Fischer Projections of L- and D-Threonine

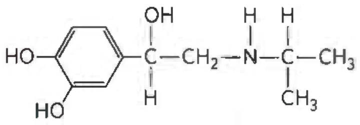
(a) Identify the functional groups in the Fischer projection of L-threonine.



L-Threonine

(b) Draw the Fischer projection structure of D-threonine.
(c) How many chiral centers does D-threonine have?

8. Drug Activity and Stereochemistry The quantitative differences in biological activity between the two enantiomers of a compound are sometimes quite large. For example, the D isomer of the drug isoproterenol, used to treat mild asthma, is 50 to 80 times more effective as a bronchodilator than the L isomer. Identify the chiral center in isoproterenol. Why do the two enantiomers have such radically different bioactivity?

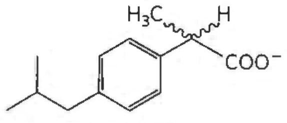


Isoproterenol

9. Separating Biomolecules In studying a particular biomolecule (a protein, nucleic acid, carbohydrate, or lipid) in the laboratory, the biochemist first needs to separate it from other biomolecules in the sample—that is, to *purify* it. Specific purification techniques are described later in this book. However, by looking at the monomeric subunits of a biomolecule, you can determine the characteristics of the molecule that will allow you to separate it from other molecules. For example, how would you separate **(a)** amino acids from fatty acids and **(b)** nucleotides from glucose?

10. Possibility of Silicon-Based Life Carbon and silicon are in the same group on the periodic table, and both can form up to four single bonds. As such, many science fiction stories have been based on the premise of silicon-based life. Consider what you know about carbon's bonding versatility (refer to a beginning inorganic chemistry resource for silicon's bonding properties, if needed). What property of carbon makes it especially suitable for the chemistry of living organisms? What characteristics of silicon make it *less* well adapted than carbon as the central organizing element for life?

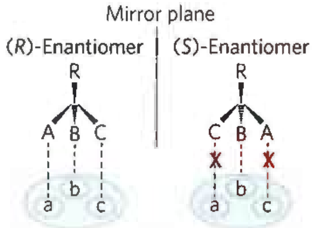
11. Stereochemistry and Drug Activity of Ibuprofen Ibuprofen is an over-the-counter drug that blocks the formation of a class of prostaglandins that cause inflammation and pain.



Ibuprofen

Ibuprofen is available as a racemic mixture of (*R*)-ibuprofen and (*S*)-ibuprofen. In living organisms, an isomerase catalyzes the chiral inversion of the (*R*)-enantiomer to the (*S*)-enantiomer. The reverse reaction does not occur at an appreciable rate. The accompanying figure represents the two enantiomers relative to the binding sites a, b, and c in the isomerase enzyme that converts the (*R*)-enantiomer to the (*S*)-enantiomer. All three sites recognize the corresponding functional groups of the (*R*)-enantiomer of ibuprofen. However, sites a and c do not recognize the corresponding functional groups of the (*S*)-enantiomer of ibuprofen.

(a) What substituents represent A, B, and C in the (*R*)-enantiomer and in the (*S*)-enantiomer?

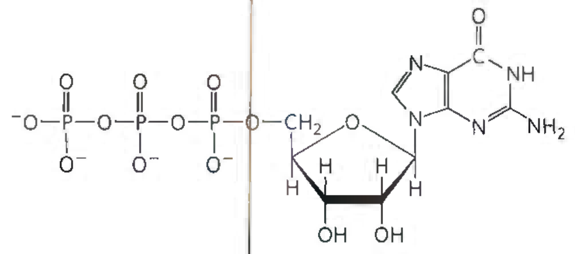


The (*S*)-enantiomer of ibuprofen is 100 times more efficacious for pain relief than is the (*R*)-enantiomer. Drug companies sometimes make enantiomerically pure versions of drugs that were previously sold as racemic mixes, such as esomeprazole (Nexium) and escitalopram (Lexapro).

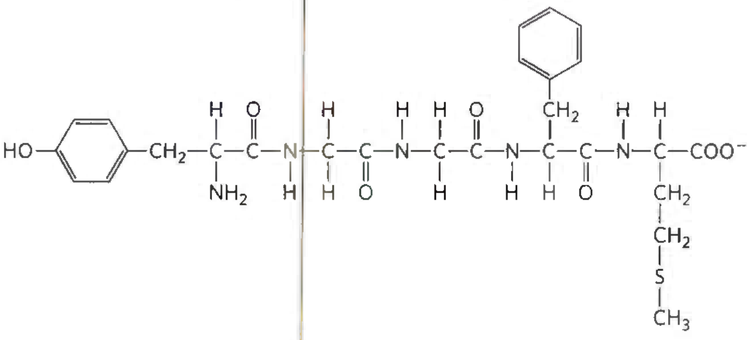
(b) Given that (*S*)-ibuprofen is more effective, why do drug companies not sell enantiomerically pure (*S*)-ibuprofen?

12. Components of Complex Biomolecules Three important biomolecules are depicted in their ionized forms at physiological pH. Identify the chemical constituents that are part of each molecule.

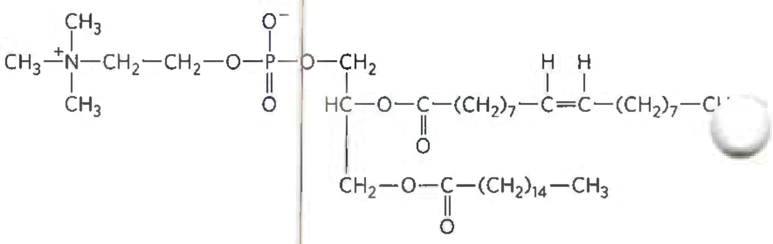
(a) Guanosine triphosphate (GTP), an energy-rich nucleotide that serves as a precursor to RNA:



(b) Methionine enkephalin, the brain's own opiate:



(c) Phosphatidylcholine, a component of many membranes:



13. Experimental Determination of the Structure of a Biomolecule Researchers isolated an unknown substance, X, from rabbit muscle. They determined its structure from the following observations and experiments. Qualitative analysis showed that X was composed entirely of C, H, and O. A weighed sample of X was completely oxidized, and the H_2O and CO_2 produced were measured; this quantitative analysis revealed that X contained 40.00% C, 6.71% H, and 53.29% O by weight. The molecular mass of X, determined by mass spectrometry, was 90.00 u (atomic mass units; see Box 1-1). Infrared spectroscopy showed that X contained one double bond. X dissolved readily in water to give an acidic solution that demonstrated optical activity when tested in a polarimeter.

(a) Determine the empirical and molecular formula of X.

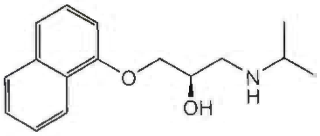
(b) Draw the possible structures of X that fit the molecular formula and contain one double bond. Consider *only* linear or branched structures and disregard cyclic structures. Note that oxygen makes very poor bonds to itself.

(c) What is the structural significance of the observed optical activity? Which structures in (b) are consistent with the observation?

(d) What is the structural significance of the observation that a solution of X was acidic? Which structures in (b) are consistent with the observation?

(e) What is the structure of X? Is more than one structure consistent with all the data?

14. Naming Stereoisomers with One Chiral Carbon Using the RS System Propranolol is a chiral compound. (*R*)-Propranolol is used as a contraceptive; (*S*)-propranolol is used to treat hypertension. The structure of one of the propranolol isomers is shown.



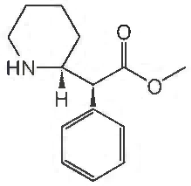
(a) Identify the chiral carbon in propranolol.

(b) Does the structure show the (*R*) isomer or the (*S*) isomer?

(c) Draw the other isomer of propranolol.

15. Naming Stereoisomers with Two Chiral Carbons Using the RS System The (*R,R*) isomer of methylphenidate (Ritalin) is used to treat attention deficit hyperactivity disorder (ADHD). The (*S,S*) isomer is an antidepressant.

(a) Identify the two chiral carbons in the methylphenidate structure shown here.



(b) Does the structure show the (*R,R*) isomer or the (*S,S*) isomer?

(c) Draw the other isomer of methylphenidate.

16. State of Bacterial Spores A bacterial spore is metabolically inert and may remain so for years. Spores contain no measurable ATP, exclude water, and consume no oxygen. However, when a spore is transferred into an appropriate liquid medium, it germinates, makes ATP, and begins cell division within an hour. Is the spore dead, or is it alive? Explain your answer.

17. Activation Energy of a Combustion Reaction Firewood is chemically unstable compared with its oxidation products, CO_2 and H_2O.

$$\text{Firewood} + O_2 \rightarrow CO_2 + H_2O$$

(a) What can one say about the standard free-energy change for this reaction?
(b) Why doesn't firewood stacked beside the fireplace undergo spontaneous combustion to its much more stable products?
(c) How can the activation energy be supplied to this reaction?
(d) Suppose you have an enzyme (firewoodase) that catalyzes the rapid conversion of firewood to CO_2 and H_2O at room temperature. How does the enzyme accomplish that in thermodynamic terms?

18. Consequence of Nucleotide Substitutions Suppose deoxycytidine (C) in one strand of DNA is mistakenly replaced with deoxythymidine (T) during cell division. What is the consequence for the cell if the deoxynucleotide change is not repaired?

19. Mutation and Protein Function Suppose that the gene for a protein 500 amino acids in length undergoes a mutation. If the mutation causes the synthesis of a mutant protein in which just one of the 500 amino acids is incorrect, the protein may lose *all* of its biological function. How can this *small* change in a protein's sequence inactivate it?

20. Gene Duplication and Evolution Suppose that a rare DNA replication error results in the duplication of a single gene, giving the daughter cell two copies of the same gene.

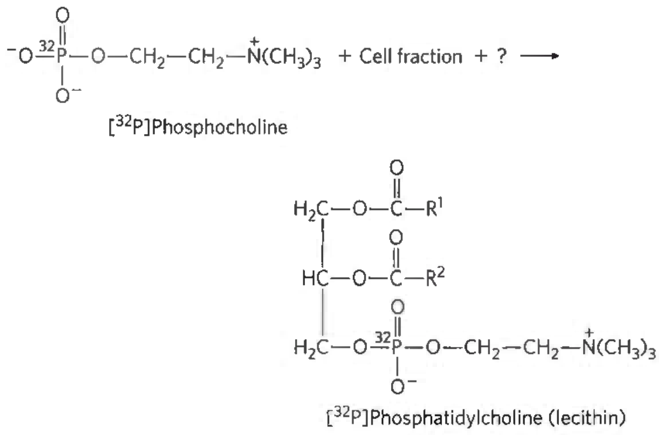
(a) How does this change favor the acquisition of a new function by the daughter cell?
(b) In the vascular plant *Arabidopsis thaliana*, 50% to 60% of the genome consists of duplicate content. How might this confer a selective advantage?

21. Cryptobiotic Tardigrades and Life Tardigrades, also called water bears or moss piglets, are small animals that can grow to about 0.5 mm in length. Terrestrial tardigrades (pictured here) typically live in the moist environments of mosses and lichens. Some of these species are capable of surviving extreme conditions. Some tardigrades can enter a reversible state called **cryptobiosis**, in which metabolism completely stops until conditions become hospitable. In this state, various tardigrade species have withstood dehydration, extreme temperatures from −200 °C to +150° C, pressures from 6,000 atm to a vacuum, anoxic conditions, and the radiation of space. Do tardigrades in cryptobiosis meet the definition of life? Why or why not?

Eye of Science/Science Source

22. Effects of Ionizing Radiation on Bacteria Treatment of a bacterial culture (*E. coli*) with ionizing radiation resulted in the survival of only a tiny fraction of the cells. The survivors proved to be more resistant to radiation than the starting cells were. When exposed to even higher levels of radiation, a tiny fraction of these resistant cells survived with even greater resistance to radiation. Repetition of this protocol with progressively higher levels of radiation yielded a strain of *E. coli* that was far more resistant to radiation than the starting strain. What changes might be occurring with each successive round of radiation and selection?

23. Data Analysis Problem In 1956, E. P. Kennedy and S. B. Weiss published their study of membrane lipid phosphatidylcholine (lecithin) synthesis in rat liver. Their hypothesis was that phosphocholine joined with some cellular component to yield lecithin. In an earlier experiment, incubating [^{32}P]-labeled phosphocholine at physiological temperature (37 °C) with broken cells from rat liver yielded labeled lecithin. This became their assay for the enzymes involved in lecithin synthesis.



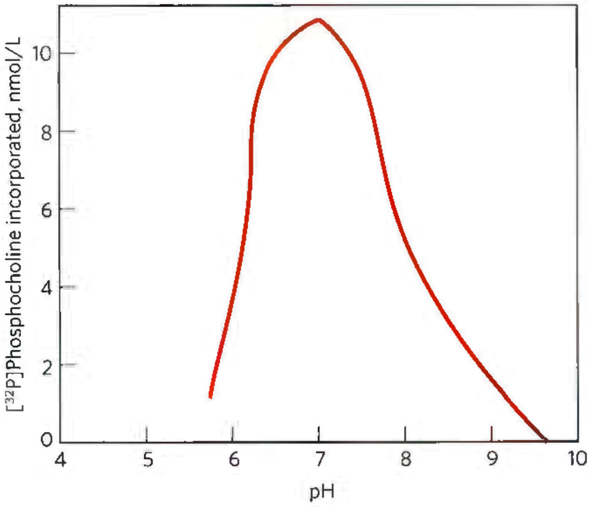
The researchers centrifuged the broken cell preparation to separate the membranes from the soluble proteins. They tested three preparations: whole extract, membranes, and soluble proteins. Table 1 summarizes the results.

TABLE 1 Cell Fraction Requirement for Incorporation of [^{32}P]-Phosphocholine into Lecithin

Tube number	Preparation	[^{32}P]-Phosphocholine incorporated into lecithin
1	Whole extract	6.3 μmol
2	Membranes	18.5 μmol
3	Soluble proteins	2.6 μmol

(a) Was the enzyme responsible for this reaction a soluble protein from the cytoplasm or a membrane-bound enzyme? Why?

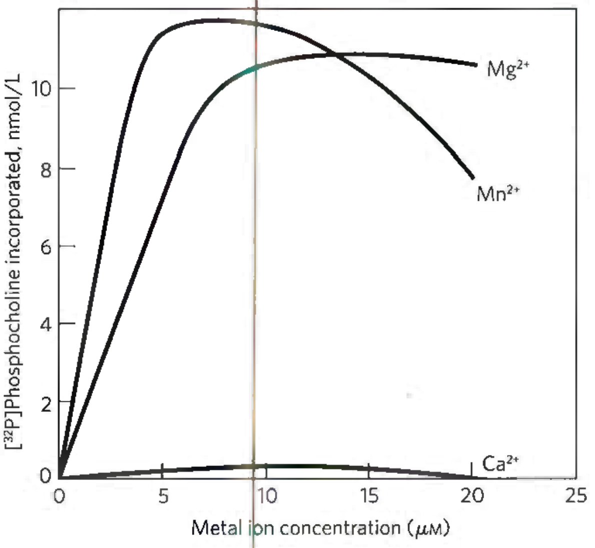
Having determined the location of the enzyme, the researchers investigated the effect of pH on enzyme activity. They carried out their standard assay in solutions buffered at different pH values between 6 and 9. The graph shows the results. The enzyme activity is the amount, in nanomoles per liter, of [^{32}P]-phosphocholine incorporated into lecithin.



(b) What is the optimal pH for this enzyme?

(c) How much more active is the enzyme at pH 8 than at pH 6?

Reactions with phosphorylated intermediates commonly require a divalent metal ion. The researchers tested Ca^{2+}, Mn^{2+}, and Mg^{2+} to determine if a divalent metal ion was important in this reaction. The graph shows the results.



(d) What is the metal ion dependence?

The researchers reasoned that the reaction might require energy. To test the hypothesis, they incubated rat liver membranes and [^{32}P]-phosphocholine with different nucleotides. Because the ATP sold in 1956 was not as highly purified as modern commercial preparations, the researchers used two different ATP sources, lot 116 and lot 122. Table 2 gives the results.

TABLE 2 Requirement of Nucleotides for Lecithin Synthesis from Phosphocholine

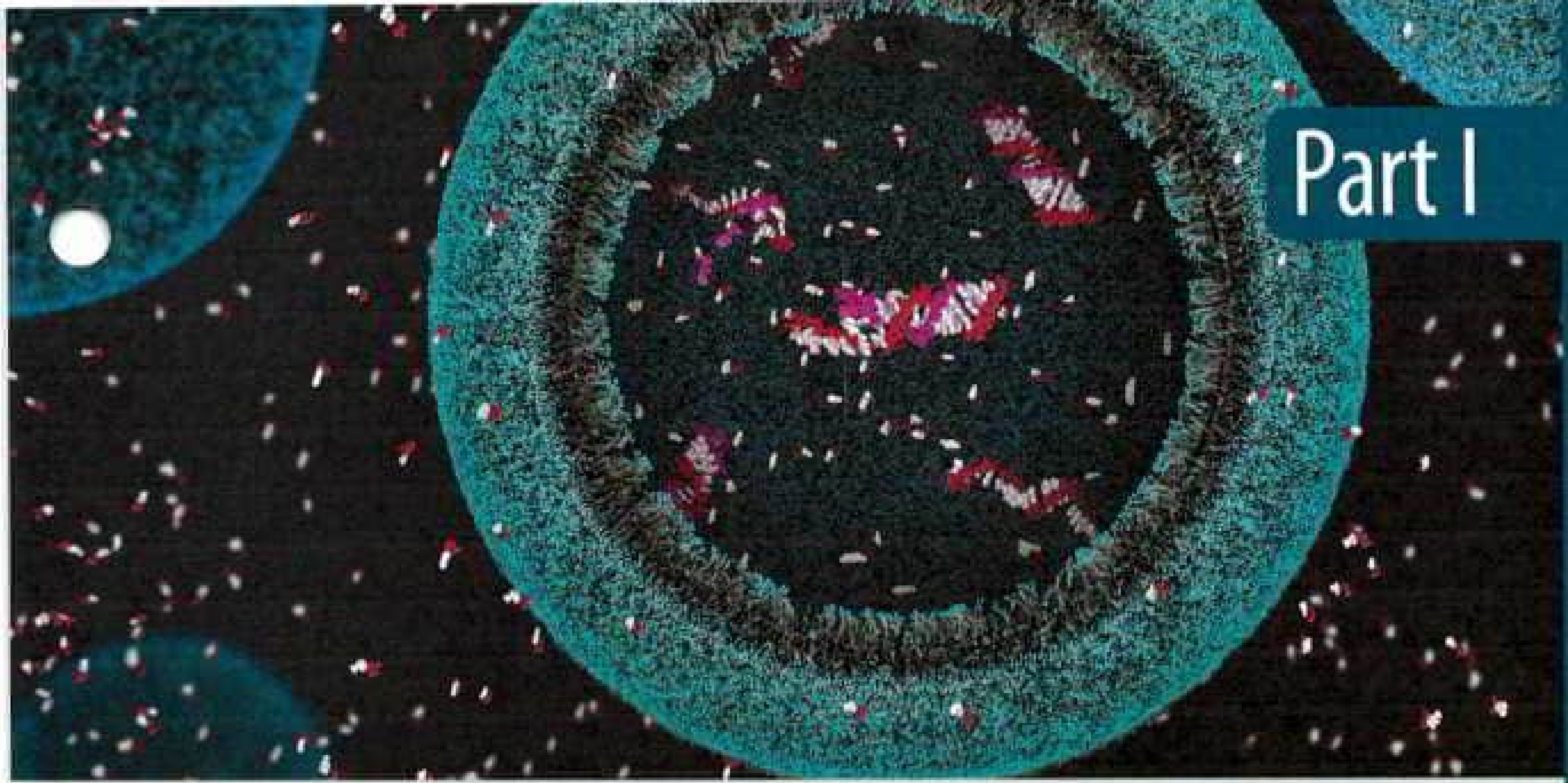
Tube number	Nucleotide added	^{32}P incorporated into lecithin
1	5 μmol ATP from lot 116	5.1 μmol
2	5 μmol ATP from lot 122	0.2 μmol
3	5 μmol ATP from lot 122 + 0.5 μmol GDP	0.4 μmol
4	5 μmol ATP from lot 122 + 0.5 μmol CTP	15.0 μmol
5	5 μmol ATP from lot 122 + 0.1 μmol CTP	10.0 μmol
6	5 μmol ATP from lot 122 + 0.5 μmol UTP	0.4 μmol
7	0.5 μmol CTP with no ATP	8.0 μmol

(e) What is your interpretation of the results in Table 2?

(f) Write the equation for the reaction the researchers studied. Include all required components, including the cell fraction, metal ion, and nucleotide cofactor.

Reference

Kennedy, E. P. and S. B. Weiss. 1956. The function of cytidine coenzymes in the biosynthesis of phospholipids. *J. Biol. Chem.* 193–214.



Part I

STRUCTURE AND CATALYSIS

PART OUTLINE

2 Water, the Solvent of Life *43*

3 Amino Acids, Peptides, and Proteins *70*

4 The Three-Dimensional Structure of Proteins *106*

5 Protein Function *147*

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7 Carbohydrates and Glycobiology *229*

8 Nucleotides and Nucleic Acids *263*

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10 Lipids *341*

11 Biological Membranes and Transport *367*

12 Biochemical Signaling *408*

Biochemistry uses the techniques and insights of chemistry to understand the amazing properties and activities of living organisms. This requires at the outset that the student acquire the vocabulary and language of biochemistry, which are provided in Part I.

The chapters of Part I are devoted to the structure and function of the major classes of cellular constituents: water (Chapter 2), amino acids and proteins (Chapters 3 through 6), sugars and polysaccharides (Chapter 7), nucleotides and nucleic acids (Chapter 8), fatty acids and lipids (Chapter 10), and, finally, membranes and membrane signaling proteins (Chapters 11 and 12). We also discuss, in the context of structure and function, the technologies used to study each class of biomolecules. One whole chapter (Chapter 9) is devoted entirely to biotechnologies associated with cloning and genomics.

We begin, in Chapter 2, with water, because its properties affect the structure and function of all other cellular constituents. For each class of organic molecules, we first consider the covalent chemistry of the monomeric units (amino acids, monosaccharides, nucleotides, and fatty acids) and then describe the structure of the macromolecules and supramolecular complexes derived from them. An overarching theme is that the polymeric macromolecules in living systems, though large, are highly ordered chemical entities, with specific sequences of monomeric subunits giving rise to discrete structures and functions. This fundamental theme can be broken down into three interrelated principles: (1) the unique structure of each macromolecule determines its function; (2) noncovalent interactions play a critical role in the structure and thus the function of macromolecules; and (3) the monomeric subunits in polymeric macromolecules occur in specific sequences, representing a form of information on which the ordered living state depends.

The relationship between structure and function is especially evident in proteins, which exhibit an extraordinary diversity of functions. One particular polymeric sequence of amino acids produces a strong, fibrous structure found in hair and wool; another produces a protein that transports oxygen in the blood; a third binds other proteins and catalyzes cleavage of the bonds between their amino acids. Similarly, the special functions of polysaccharides, nucleic acids, and lipids can be understood as resulting directly from their chemical structure, with their characteristic monomeric subunits precisely linked to form functional polymers. Sugars linked together become energy stores, structural fibers, and points of specific molecular recognition; nucleotides strung together in DNA or RNA provide the blueprint for an entire organism; and aggregated lipids form membranes. Chapter 12 unifies the discussion of biomolecule function, describing how specific signaling systems regulate the activities of biomolecules—within a cell, within an organ, and among organs—to keep an organism in homeostasis. Failure to maintain homeostasis results in failed function—that is, disease.

As we move from monomeric units to larger and larger polymers, the chemical focus shifts from covalent bonds to noncovalent interactions. Covalent bonds, at the monomeric and macromolecular level, place constraints on the shapes assumed by large biomolecules. It is the numerous noncovalent interactions, however, that dictate the stable, native conformations of large molecules while permitting the flexibility necessary for their biological function. As we shall see, noncovalent interactions are essential to the catalytic power of enzymes, the critical interaction of complementary base pairs in nucleic acids, and the arrangement and properties of lipids in membranes. The principle that sequences of monomeric subunits are rich in information emerges most fully in the discussion of nucleic acids (Chapter 8). However, proteins and some short polymers of sugars (oligosaccharides) are also information-rich molecules. The amino acid sequence is a form of information that directs the folding of the protein into its unique three-dimensional structure and ultimately determines the function of the protein. Some oligosaccharides also have unique sequences and three-dimensional structures that are recognized by other macromolecules.

Each class of molecules has a similar structural hierarchy: subunits of fixed structure are connected by bonds of limited flexibility to form macromolecules with three-dimensional structures determined by noncovalent interactions. These macromolecules then interact to form the supramolecular structures and organelles that allow a cell to carry out its many metabolic functions. Together, the molecules described in Part I are the stuff of life.

Chapter 2

WATER, THE SOLVENT OF LIFE

2.1 Weak Interactions in Aqueous Systems *43*

2.2 Ionization of Water, Weak Acids, and Weak Bases *53*

2.3 Buffering against pH Changes in Biological Systems *59*

Water is the most abundant substance in living systems, making up 70% or more of the weight of most organisms. The first living organisms on Earth doubtless arose in an aqueous environment, and the course of evolution has been shaped by the properties of the aqueous medium in which life began.

This chapter begins with descriptions of the physical and chemical properties of water, to which all aspects of cell structure and function are adapted. The attractive forces between water molecules and the slight tendency of water to ionize are of crucial importance to the structure and function of biomolecules. We review the topic of ionization in terms of equilibrium constants, pH, and titration curves, and we consider how aqueous solutions of weak acids or bases and their salts act as buffers against pH changes in biological systems. The water molecule and its ionization products, H^+ and OH^-, profoundly influence the structure, self-assembly, and properties of all cellular components, including proteins, nucleic acids, and lipids. The noncovalent interactions responsible for the strength and specificity of "recognition" among biomolecules are decisively influenced by water's properties as a solvent, including its ability to form hydrogen bonds with itself and with solutes.

This chapter emphasizes the following principles:

P1 **The solvent properties of water shaped the evolution of living things.** Most small intermediates of metabolism, as well as nucleic acids and proteins, are soluble in water. Lipid bilayers, the likely forerunners of biological membranes, form spontaneously in water and are stabilized by their interaction with it. Although hydrogen bonds, ionic interactions, and the hydrophobic effect are individually weak, their combined effects powerfully influence the three-dimensional shape and stability of biological molecules and structures.

P2 **The ionization behavior of water and of weak acids and bases dissolved in water can be represented by one or more equilibrium constants.** Most biomolecules are ionizable; their structure and function depend on their ionization state, which is characterized by equilibrium constants.

P3 **An aqueous solution of a weak acid and its salt makes a buffer that resists changes in pH in response to added acid or base.** Biological systems are buffered to maintain a narrow pH range, in which their macromolecules retain their functional structure, which depends on their ionization state. Conditions that produce blood pH outside the range of 7.3 to 7.5 are life-threatening in humans.

P4 **Enzymes, which catalyze all of the processes inside a cell, have evolved to function optimally at near-neutral (physiological) pH.** However, enzymes that function in intracellular compartments of low or high pH show their greatest activity and stability at those pH values.

2.1 Weak Interactions in Aqueous Systems

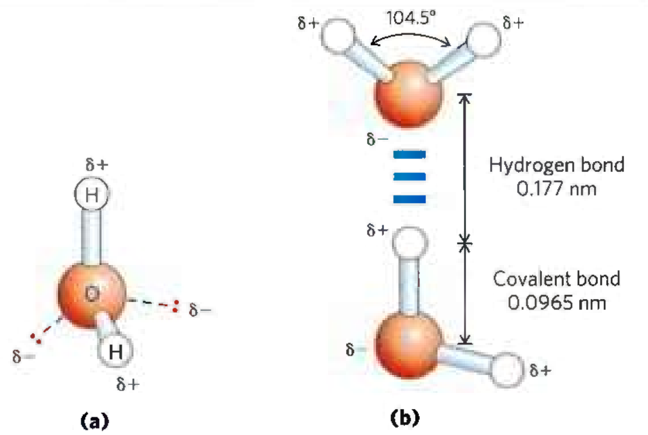
Hydrogen bonds between water molecules provide the cohesive forces that make water a liquid at room temperature and a crystalline solid (ice) with a highly ordered arrangement of molecules at cold temperatures. P1 Polar biomolecules dissolve readily in water because they can replace water-water interactions with energetically favorable water-solute interactions. In contrast, nonpolar biomolecules are poorly soluble in water because they interfere with water-water interactions but are unable to form water-solute interactions. In aqueous solutions, nonpolar molecules tend to cluster together. P1 Hydrogen bonds and ionic, hydrophobic (from the Greek, meaning "water-fearing"), and van der Waals interactions are individually weak, but collectively they have a very significant influence on the three-dimensional structures of proteins, nucleic acids, polysaccharides, and membrane lipids.

Hydrogen Bonding Gives Water Its Unusual Properties

Water has a higher melting point, boiling point, and heat of vaporization than most other common solvents. These unusual properties are a consequence of attractions between adjacent water molecules that give liquid water great internal cohesion. A look at the electron structure of the H_2O molecule reveals the cause of these intermolecular attractions.

Each hydrogen atom of a water molecule shares an electron pair with the central oxygen atom. The geometry of the molecule is dictated by the shapes of the outer electron orbitals of the oxygen atom, which are similar to the sp^3 bonding orbitals of carbon (see Fig. 1-13). These orbitals describe a rough tetrahedron, with a hydrogen atom at each of two corners and nonbonding orbitals at the other two corners (**Fig. 2-1a**). The H—O—H bond angle is 104.5°, slightly less than the 109.5° of a perfect tetrahedron because of crowding by the nonbonding orbitals of the oxygen atom.

The oxygen nucleus attracts electrons more strongly than does the hydrogen nucleus (a proton); that is, oxygen is more electronegative. This means that the shared electrons are more often in the vicinity of the oxygen atom than of the hydrogen. The result of this unequal electron sharing is two electric dipoles in the water molecule, one along each of the H—O bonds; each hydrogen atom bears a partial positive charge (δ+), and the oxygen atom bears a partial negative charge equal in magnitude to the sum of the two partial positives ($2\delta^-$). As a result, there is an electrostatic attraction between the oxygen atom of one water molecule and the hydrogen of another (Fig. 2-1b), called a **hydrogen bond**. Throughout this book, we represent hydrogen bonds with three parallel blue lines, as in Figure 2-1b.



(a) (b)

FIGURE 2-1 Structure of the water molecule. (a) The dipolar nature of the H_2O molecule is shown in a ball-and-stick model; the dashed lines represent the nonbonding orbitals. There is a nearly tetrahedral arrangement of the outer-shell electron pairs around the oxygen atom; the two hydrogen atoms have localized partial positive charges (δ+), and the oxygen atom has a partial negative charge (δ−). (b) Two H_2O molecules are joined by a hydrogen bond (designated here, and throughout this book, by three blue lines) between the oxygen atom of the upper molecule and a hydrogen atom of the lower one. Hydrogen bonds are longer and weaker than covalent O—H bonds.

Hydrogen bonds are relatively weak. Those in liquid water have a **bond dissociation energy** (the energy required to break a bond) of about 23 kJ/mol, compared with 470 kJ/mol for the covalent O—H bond in water or 350 kJ/mol for a covalent C—C bond. The hydrogen bond is about 10% covalent, due to overlaps in the bonding orbitals, and about 90% electrostatic. At room temperature, the thermal energy of an aqueous solution (the kinetic energy of motion of the individual atoms and molecules) is of the same order of magnitude as that required to break hydrogen bonds. When water is heated, the increase in temperature reflects the faster motion of individual water molecules. At any given time, most of the molecules in liquid water are hydrogen-bonded, but the lifetime of each hydrogen bond is just 1 to 20 picoseconds (1 ps = 10^{-12} s); when one hydrogen bond breaks, another hydrogen bond forms, with the same partner or a new one, within 0.1 ps. The apt phrase "flickering clusters" has been applied to the short-lived groups of water molecules interlinked by hydrogen bonds in liquid water. The sum of all the hydrogen bonds between H_2O molecules confers great internal cohesion on liquid water. Extended networks of hydrogen-bonded water molecules also form bridges between solutes (proteins and nucleic acids, for example) that allow the larger molecules to interact with each other over distances of several nanometers without physically touching.

The nearly tetrahedral arrangement of the orbitals about the oxygen atom (Fig. 2-1a) allows each water molecule to form hydrogen bonds with as many as four neighboring water molecules. In liquid water at room temperature and atmospheric pressure, however, water molecules are disorganized and in continuous motion, so that each molecule forms hydrogen bonds with an average of only 3.4 other molecules. In ice, on the other hand, each water molecule is fixed in space and forms hydrogen bonds with a full complement of four other water molecules to yield a regular lattice structure (**Fig. 2-2**). Hydrogen bonds account for the relatively high melting point of water, because much thermal energy is required to break a sufficient proportion of hydrogen bonds to destabilize the crystal lattice of ice. When ice melts or water evaporates, heat is taken up by the system:

$$H_2O(\text{solid}) \longrightarrow H_2O(\text{liquid}) \qquad \Delta H = +5.9 \text{ kJ/mol}$$

$$H_2O(\text{liquid}) \longrightarrow H_2O(\text{gas}) \qquad \Delta H = +44.0 \text{ kJ/mol}$$

During melting or evaporation, the entropy of the aqueous system increases as the highly ordered arrays of water molecules in ice relax into the less orderly

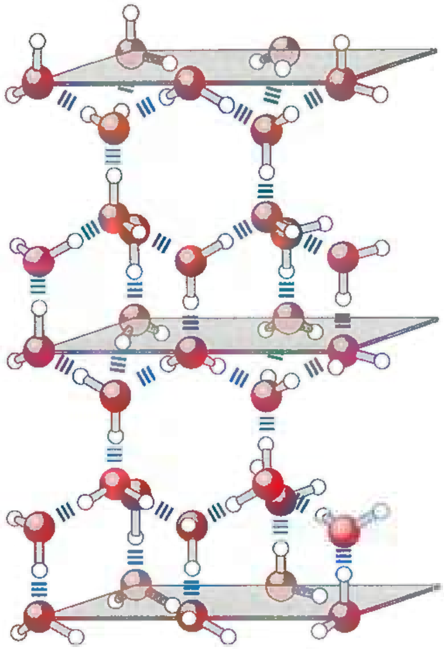


FIGURE 2-2 Hydrogen bonding in ice. In ice, each water molecule forms four hydrogen bonds, the maximum possible for a water molecule, creating a regular crystal lattice. By contrast, in liquid water at room temperature and atmospheric pressure, each water molecule hydrogen-bonds with an average of 3.4 other water molecules. This crystal lattice structure makes ice less dense than liquid water, and thus ice floats on liquid water.

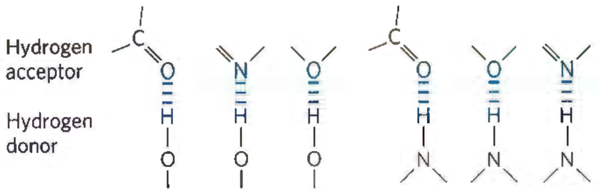


FIGURE 2-3 Common hydrogen bonds in biological systems. The hydrogen acceptor is usually oxygen or nitrogen; the hydrogen donor is another electronegative atom.

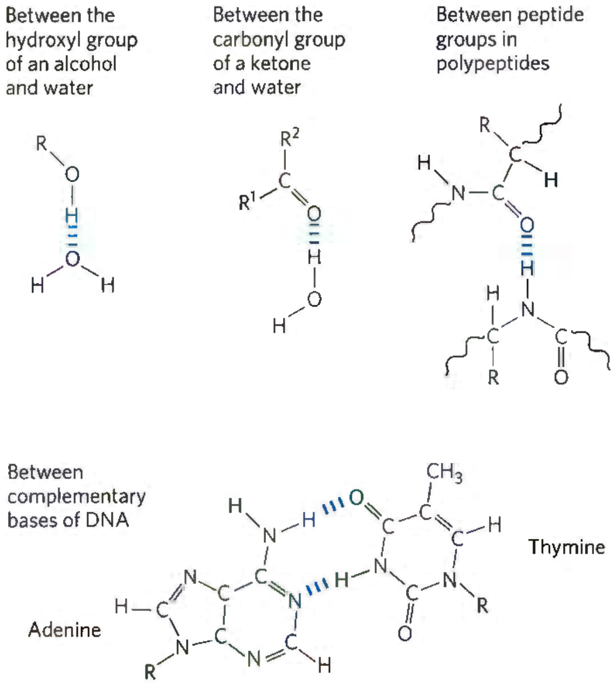

Between the hydroxyl group of an alcohol and water
Between the carbonyl group of a ketone and water
Between peptide groups in polypeptides
Between complementary bases of DNA
Adenine
Thymine
R
R^1
R^2
CH_3
C
N
O
H

FIGURE 2-4 Some biologically important hydrogen bonds.

hydrogen-bonded arrays in liquid water or into the wholly disordered gaseous state. At room temperature, both the melting of ice and the evaporation of water occur spontaneously; the tendency of the water molecules to associate through hydrogen bonds is outweighed by the energetic push toward randomness. Recall that the free-energy change (ΔG) must have a negative value for a process to occur spontaneously: $\Delta G = \Delta H - T\Delta S$, where ΔG represents the driving force, ΔH the enthalpy change from making and breaking bonds, and ΔS the change in randomness. Because ΔH is positive for melting and evaporation, it is clearly the increase in entropy (ΔS) that makes ΔG negative and drives these changes.

Water Forms Hydrogen Bonds with Polar Solutes

Hydrogen bonds are not unique to water. They readily form between an electronegative atom (the hydrogen acceptor, usually oxygen or nitrogen) and a hydrogen atom covalently bonded to another electronegative atom (the hydrogen donor) in the same or another molecule (**Fig. 2-3**). Hydrogen atoms covalently bonded to carbon atoms do not participate in hydrogen bonding, because carbon is only slightly more electronegative than hydrogen and thus the C—H bond is only very weakly polar. The distinction explains why butane ($CH_3(CH_2)_2CH_3$) has a boiling point of only –0.5 °C, whereas butanol ($CH_3(CH_2)_2CH_2OH$) has a relatively high boiling point of 117 °C. Butanol has a polar hydroxyl group and thus can form intermolecular hydrogen bonds. Uncharged but polar biomolecules such as sugars dissolve readily in water because of the stabilizing effect of hydrogen bonds between the hydroxyl groups or carbonyl oxygen of the sugar and the polar water molecules. **P1** Alcohols, aldehydes, ketones, and compounds containing N—H bonds all form hydrogen bonds with water molecules (**Fig. 2-4**) and tend to be soluble in water.

Hydrogen bonds are strongest when the bonded molecules are oriented to maximize electrostatic interaction, which occurs when the hydrogen atom and the two atoms that share it are in a straight line—that is, when the acceptor atom is in line with the covalent bond between the donor atom and H (**Fig. 2-5**). This arrangement puts the positive charge of the hydrogen ion directly between the two partial negative charges. Hydrogen bonds are thus highly directional and capable of holding two hydrogen-bonded molecules or groups in a specific geometric arrangement. As we shall see, this property of hydrogen bonds confers very precise three-dimensional structures on protein and nucleic acid molecules, which have many intramolecular hydrogen bonds.

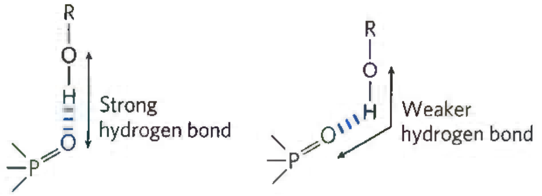


FIGURE 2-5 Directionality of the hydrogen bond. The attraction between the partial electric charges is greatest when the three atoms involved in the bond (in this case O, H, and O) lie in a straight line. When the hydrogen-bonded moieties are structurally constrained (when they are parts of a single protein molecule, for example), this ideal geometry may not be possible and the resulting hydrogen bond is weaker.

Water Interacts Electrostatically with Charged Solutes

Water is a polar solvent. It readily dissolves most biomolecules, which are generally charged or polar compounds (**Table 2-1**); compounds that dissolve easily in water are **hydrophilic** (from the Greek, meaning "water-loving"). In contrast, nonpolar solvents such as chloroform and benzene are poor solvents for polar biomolecules but easily dissolve those that are **hydrophobic**—nonpolar molecules such as lipids and waxes. **Amphipathic** compounds contain regions that are polar (or charged) and regions that are nonpolar. Their behavior in aqueous solution is discussed on p. 48.

Water dissolves salts such as NaCl by hydrating and stabilizing the Na^+ and Cl^- ions, weakening the electrostatic interactions between them and thus counteracting their tendency to associate in a crystalline lattice (**Fig. 2-6**). Water also readily dissolves charged biomolecules, including compounds with functional groups such as ionized carboxylic acids ($—COO^-$), protonated amines ($—NH_3^+$), and phosphate esters or anhydrides. Water replaces the solute-solute hydrogen bonds linking these biomolecules to each other with solute-water hydrogen bonds, thus screening the electrostatic interactions between solute molecules.

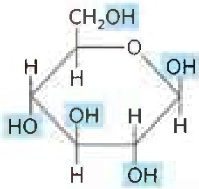
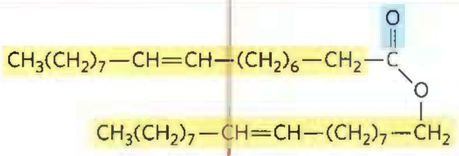
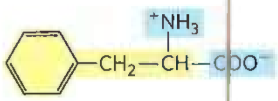
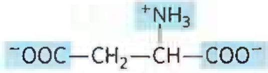
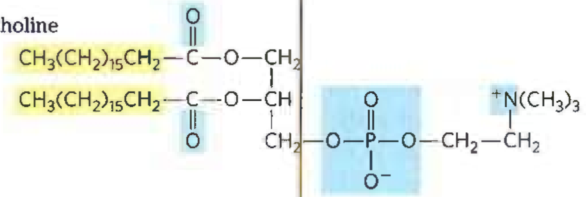
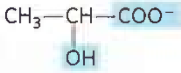
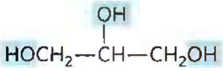
Ionic interactions between dissolved ions are much stronger in less polar environments, because there is less screening of charges by the nonpolar solvent. Water is effective in screening the electrostatic interactions between dissolved ions because it has a high dielectric constant, a physical property that reflects the number of dipoles in a solvent. The strength, or force (F), of ionic interactions in a solution depends on the magnitude of the charges (Q), the distance between the charged groups (r), and the dielectric constant (ε, which is dimensionless) of the solvent in which the interactions occur:

$$F = \frac{Q_1 Q_2}{\varepsilon r^2}$$

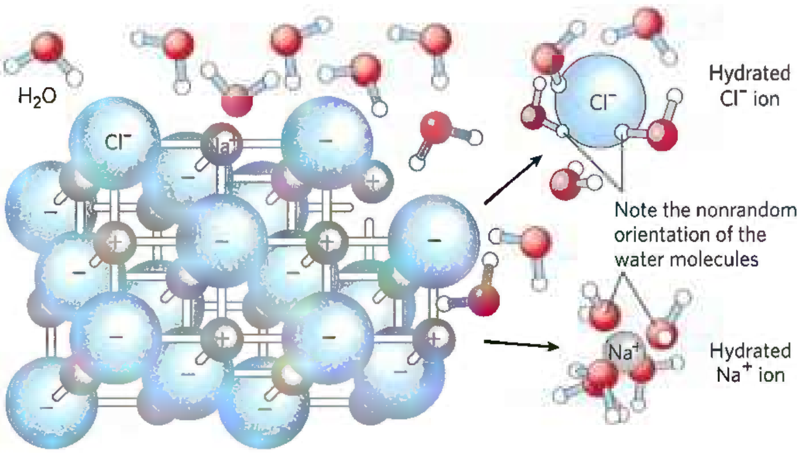
For water at 25 °C, ε is 78.5, and for the very nonpolar solvent benzene, ε is 4.6. The dependence on r^2 is such that ionic attractions or repulsions operate only over short distances—in the range of 10 to 40 nm (depending on the electrolyte concentration) when the solvent is water. In biomolecules, it is not the dielectric constant for the bulk solvent, but the highly localized dielectric constant, as in a hydrophobic pocket of a protein, that determines the interaction of two polar moieties.

As a salt such as NaCl dissolves, the Na^+ and Cl^- ions leaving the crystal lattice acquire far greater freedom of motion (Fig. 2-6). The resulting increase in entropy (randomness) of the system is largely responsible for the ease of dissolving salts such as NaCl in water. In thermodynamic terms, formation of the solution occurs with a favorable free-energy change: $\Delta G = \Delta H - T\Delta S$, where ΔH has a small positive value and $T\Delta S$ a large positive value; thus ΔG is negative.

TABLE 2-1 Some Examples of Polar, Nonpolar, and Amphipathic Biomolecules (Shown as Ionic Forms at pH 7)

Polar		**Nonpolar**	
Glucose		Typical wax	
		Amphipathic	
Glycine	$\mathrm{^{+}NH_3{-}CH_2{-}COO^-}$	Phenylalanine	
Aspartate	 $\mathrm{^{-}OOC{-}CH_2{-}\overset{^{+}NH_3}{CH}{-}COO^-}$	Phosphatidylcholine	
Lactate	 $\mathrm{CH_3{-}\underset{OH}{CH}{-}COO^-}$		
Glycerol	 $\mathrm{HOCH_2{-}\overset{OH}{CH}{-}CH_2OH}$		

▭ Polar groups ▭ Nonpolar groups



Note the nonrandom orientation of the water molecules

FIGURE 2-6 Water as solvent. Water dissolves many crystalline salts by hydrating their component ions. The NaCl crystal lattice is disrupted as water molecules cluster about the Cl^- and Na^+ ions. The ionic charges are partially neutralized, and the electrostatic attractions necessary for lattice formation are weakened.

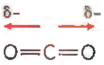
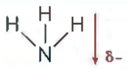
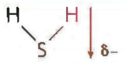
Nonpolar Gases Are Poorly Soluble in Water

The biologically important gases CO_2, O_2, and N_2 are nonpolar molecules. In O_2 and N_2, electrons are shared equally by both atoms. In CO_2, each C═O bond is polar, but the two dipoles are oppositely directed and cancel each other (**Table 2-2**). The movement of molecules from the disordered gas phase into aqueous solution constrains their motion and the motion of water molecules and therefore represents a decrease in entropy. The nonpolar nature of these gases and the decrease in entropy when they enter solution combine to make them very poorly soluble in water. Some organisms have water-soluble "carrier proteins" (hemoglobin and myoglobin, for example) that facilitate the transport of O_2. Carbon dioxide forms carbonic acid (H_2CO_3) in aqueous solution and is transported as the HCO_3^- (bicarbonate) ion, either free—bicarbonate is very soluble in water (~100 g/L at 25 °C)—or bound to hemoglobin. Three other gases, NH_3, NO, and H_2S, also have biological roles in some organisms; these gases are polar and dissolve readily in water.

Nonpolar Compounds Force Energetically Unfavorable Changes in the Structure of Water

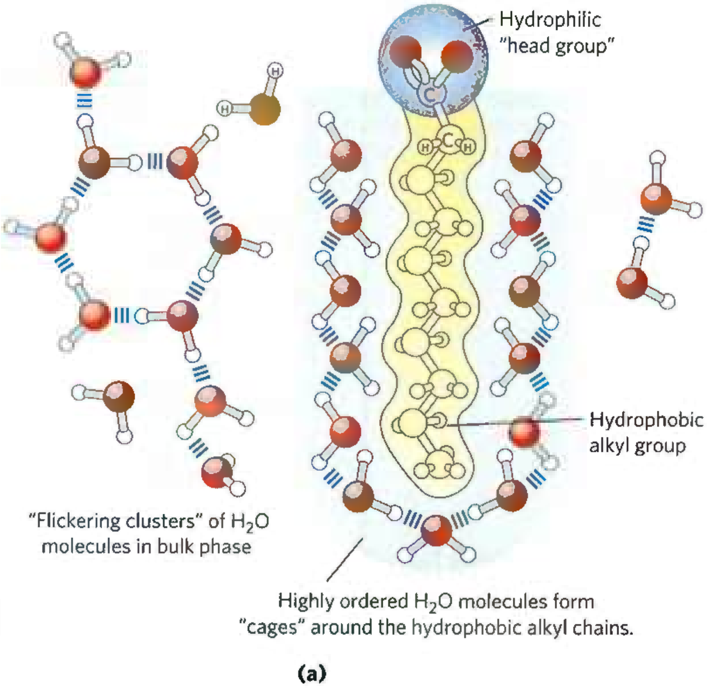
When water is mixed with benzene or hexane, two phases form; neither liquid is soluble in the other. Nonpolar compounds such as benzene and hexane are hydrophobic—they are unable to undergo energetically favorable interactions with water molecules, and they interfere with the hydrogen bonding among water molecules. All molecules or ions in aqueous solution interfere with the hydrogen bonding of some water molecules in their immediate vicinity, but polar or charged solutes (such as NaCl) compensate for lost water-water hydrogen bonds by forming new solute-water interactions. The net change in enthalpy (ΔH) for dissolving these solutes is generally small. Hydrophobic solutes, however, offer no such compensation, and their addition to water may therefore result in a small gain of enthalpy; the breaking of hydrogen bonds between water molecules takes up energy from the system, requiring the input of energy from the surroundings. In addition to requiring this input of energy, dissolving hydrophobic compounds in water produces a measurable decrease in entropy. Water

TABLE 2-2 Solubilities of Some Gases in Water

Gas	Structure[a]	Polarity	Solubility in water (g/L)[b]
Nitrogen	N≡N	Nonpolar	0.018 (40 °C)
Oxygen	O═O	Nonpolar	0.035 (50 °C)
Carbon dioxide		Nonpolar	0.97 (45 °C)
Ammonia		Polar	900 (10 °C)
Hydrogen sulfide		Polar	1,860 (40 °C)

[a] The arrows represent electric dipoles; there is a partial negative charge (δ−) at the head of the arrow, a partial positive charge (δ+; not shown here) at the tail.

[b] Note that polar molecules dissolve far better even at low temperatures than do nonpolar molecules at relatively high temperatures.


Hydrophilic "head group"
Hydrophobic alkyl group
"Flickering clusters" of H_2O molecules in bulk phase
Highly ordered H_2O molecules form "cages" around the hydrophobic alkyl chains.
(a)

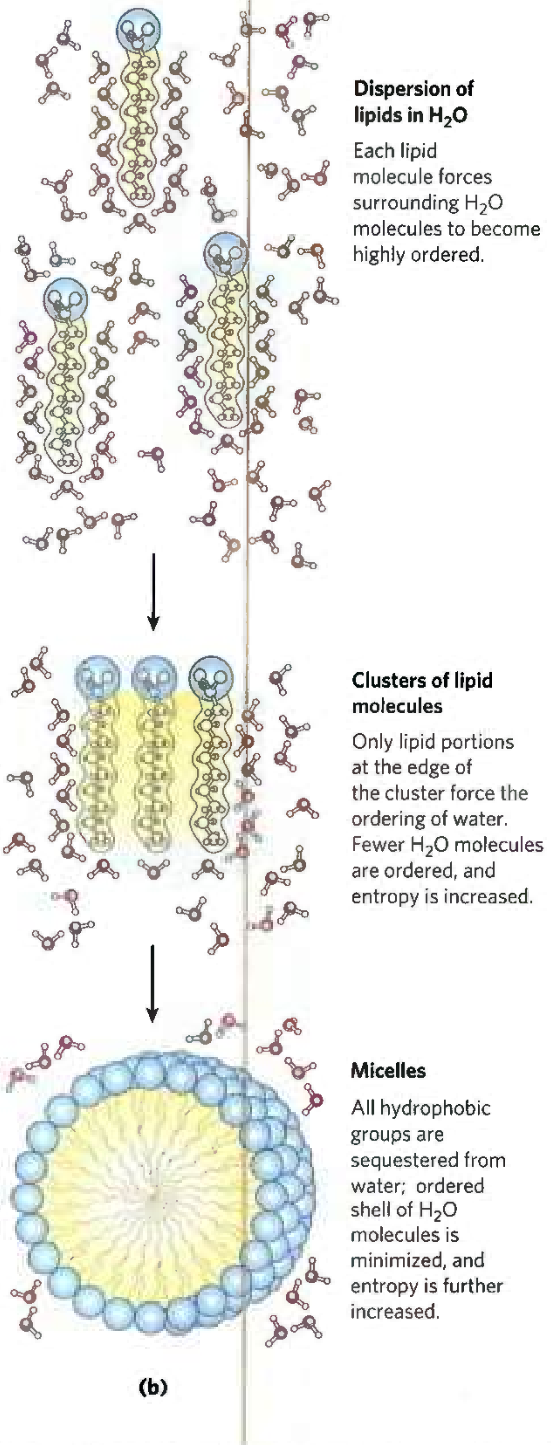

Dispersion of lipids in H_2O
Each lipid molecule forces surrounding H_2O molecules to become highly ordered.
Clusters of lipid molecules
Only lipid portions at the edge of the cluster force the ordering of water. Fewer H_2O molecules are ordered, and entropy is increased.
Micelles
All hydrophobic groups are sequestered from water; ordered shell of H_2O molecules is minimized, and entropy is further increased.
(b)

FIGURE 2-7 Amphipathic compounds in aqueous solution form structures that increase entropy. (a) Long-chain fatty acids have very hydrophobic alkyl chains, each of which is surrounded by a layer of highly ordered water molecules. (b) By clustering together in micelles, the fatty acid molecules expose the smallest possible hydrophobic surface area to the water, and fewer water molecules are required in the shell of ordered water. The entropy gained by freeing immobilized water molecules stabilizes the micelle.

molecules in the immediate vicinity of a nonpolar solute are constrained in their possible orientations, as they form a highly ordered cagelike shell around each solute molecule to maximize solvent-solvent hydrogen bonding. These water molecules are not as highly oriented as those in **clathrates**, crystalline compounds of nonpolar solutes and water, but the effect is the same in both cases: the ordering of water molecules reduces entropy. The number of ordered water molecules, and therefore the magnitude of the entropy decrease, is proportional to the surface area of the hydrophobic solute enclosed within the cage of water molecules. The free-energy change for dissolving a nonpolar solute in water is thus unfavorable: $\Delta G = \Delta H - T\Delta S$, where ΔH has a positive value, ΔS has a negative value, and ΔG is positive.

When an amphipathic compound (Table 2-1) is mixed with water, the polar, hydrophilic region interacts favorably with the water and tends to dissolve, but the nonpolar, hydrophobic region tends to avoid contact with the water (**Fig. 2-7a**). The nonpolar regions of the molecules cluster together to present the smallest hydrophobic area to the aqueous solvent, and the polar regions are arranged to maximize their interaction with each other and with the solvent (Fig. 2-7b), a phenomenon called the **hydrophobic effect**. These stable structures of amphipathic compounds in water, called **micelles**, may contain hundreds or thousands of molecules. By clustering together, nonpolar regions of the molecules achieve the greatest thermodynamic stability by minimizing the number of ordered water molecules required to surround hydrophobic portions of the solute molecules, increasing the entropy of the system. A special case of this hydrophobic effect is the formation of lipid bilayers in biological membranes (see Fig. 11-1).

Many biomolecules are amphipathic; proteins, pigments, certain vitamins, and the sterols and phospholipids of membranes all have both polar and nonpolar surface regions. Structures composed of these molecules are stabilized by the hydrophobic effect, which favors aggregation of the nonpolar regions. **P1** The hydrophobic effect on

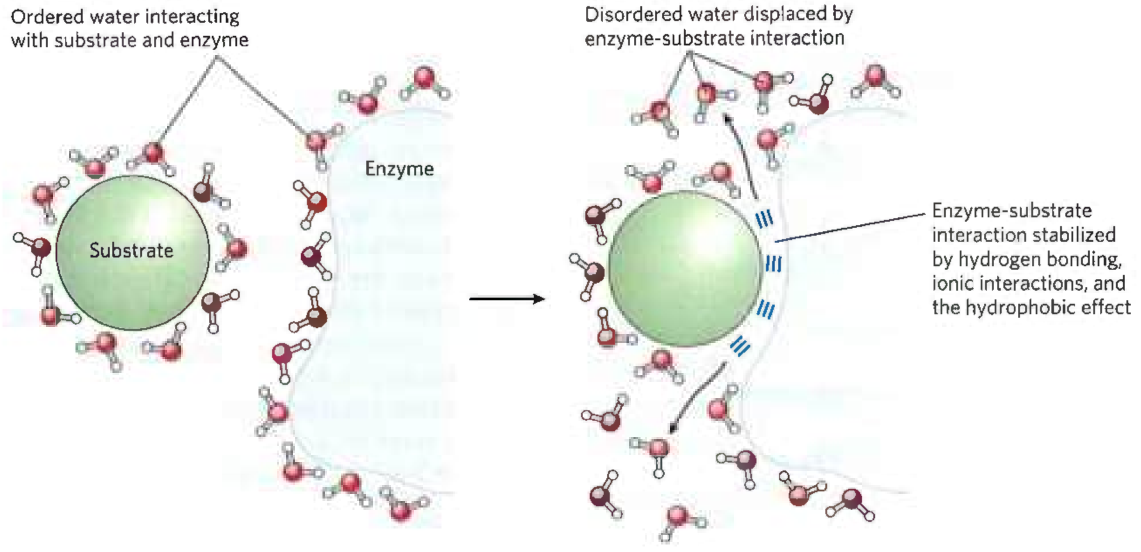

Ordered water interacting with substrate and enzyme
Substrate
Enzyme
Disordered water displaced by enzyme-substrate interaction
Enzyme-substrate interaction stabilized by hydrogen bonding, ionic interactions, and the hydrophobic effect

FIGURE 2-8 Release of ordered water favors formation of an enzyme-substrate complex. While separate, both enzyme and substrate force neighboring water molecules into an ordered shell. Binding of substrate to enzyme releases some of the ordered water, and the resulting increase in entropy provides a thermodynamic push toward formation of the enzyme-substrate complex.

interactions among lipids, and between lipids and proteins, is the most important determinant of structure in biological membranes. The aggregation of nonpolar amino acids in protein interiors, driven by the hydrophobic effect, also stabilizes the three-dimensional structures of proteins.

Hydrogen bonding between water and polar solutes also causes an ordering of water molecules, but the energetic effect is less significant than with nonpolar solutes. Disruption of ordered water molecules is part of the driving force for binding of a polar substrate (reactant) to the complementary polar surface of an enzyme: entropy increases as the enzyme displaces ordered water from the substrate and as the substrate displaces ordered water from the enzyme surface (**Fig. 2-8**). This topic is discussed in greater depth in Chapter 6 (p. 185).

van der Waals Interactions Are Weak Interatomic Attractions

When two uncharged atoms are brought very close together, their surrounding electron clouds influence each other. Random variations in the positions of the electrons around one nucleus may create a transient electric dipole, which induces a transient, opposite electric dipole in the nearby atom. The two dipoles weakly attract each other, bringing the two nuclei closer. These weak attractions are called **van der Waals interactions** (also known as London dispersion forces). As the two nuclei draw closer together, their electron clouds begin to repel each other. At the point where the net attraction is maximal, the nuclei are said to be in van der Waals contact. Each atom has a characteristic **van der Waals radius**, a measure of how close that atom will allow another to approach (**Table 2-3**). In the space-filling molecular models shown throughout this book, the atoms are depicted in sizes proportional to their van der Waals radii.

Weak Interactions Are Crucial to Macromolecular Structure and Function

> I believe that as the methods of structural chemistry are further applied to physiological problems, it will be found that the significance of the hydrogen bond for physiology is greater than that of any other single structural feature.
>
> —Linus Pauling,
> *The Nature of the Chemical Bond*, 1939

The noncovalent interactions we have described—hydrogen bonds and ionic, hydrophobic, and van der Waals interactions (**Table 2-4**)—are much weaker than covalent bonds. An input of about 350 kJ of energy is required to break a mole of (6×10^{23}) C—C single bonds,

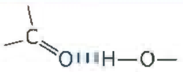
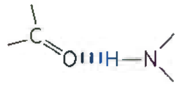
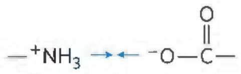
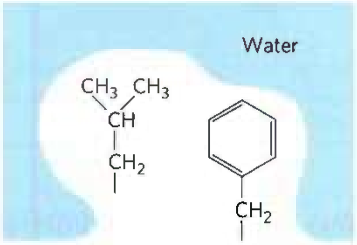
TABLE 2-3 van der Waals Radii and Covalent (Single-Bond) Radii of Some Elements

Element	van der Waals radius (nm)	Covalent radius for single bond (nm)
H	0.11	0.030
O	0.15	0.066
N	0.15	0.070
C	0.17	0.077
S	0.18	0.104
P	0.19	0.110
I	0.21	0.133

Sources: For van der Waals radii, R. Chauvin, *J. Phys. Chem.* 96:9194, 1992. For covalent radii, L. Pauling, *Nature of the Chemical Bond*, 3rd edn, Cornell University Press, 1960.

Note: van der Waals radii describe the space-filling dimensions of atoms. When two atoms are joined covalently, the atomic radii at the point of bonding are shorter than the van der Waals radii, because the joined atoms are pulled together by the shared electron pair. The distance between nuclei in a van der Waals interaction or a covalent bond is about equal to the sum of the van der Waals or covalent radii, respectively, for the two atoms. Thus, the length of a carbon-carbon single bond is about 0.077 nm + 0.077 nm = 0.154 nm.

TABLE 2-4 Four Types of Noncovalent ("Weak") Interactions among Biomolecules in Aqueous Solvent

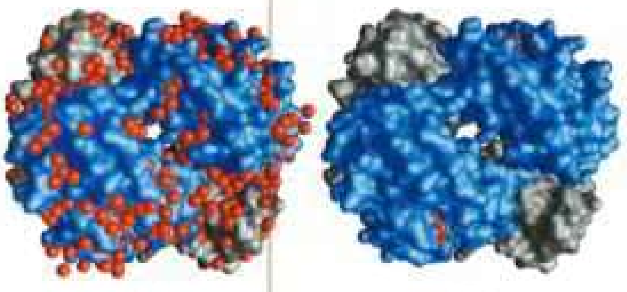
Hydrogen bonds Between neutral groups	
Between peptide bonds	
Ionic interactions Attraction	
Repulsion	$-{}^{+}NH_3 \longleftrightarrow H_3N^{+}-$
Hydrophobic effect	
van der Waals interactions	Any two atoms in close proximity

and about 410 kJ is needed to break a mole of C—H bonds, but as little as 4 kJ is sufficient to disrupt a mole of typical van der Waals interactions. Interactions driven by the hydrophobic effect are also much weaker than covalent bonds, although they are substantially strengthened by a highly polar solvent (a concentrated salt solution, for example). Ionic interactions and hydrogen bonds are variable in strength, depending on the polarity of the solvent and the alignment of the hydrogen-bonded atoms, but they are always significantly weaker than covalent bonds. In aqueous solvent at 25 °C, the available thermal energy can be of the same order of magnitude as the strength of these weak interactions, and the interaction between solute and solvent (water) molecules is nearly as favorable as solute-solute interactions. Consequently, hydrogen bonds and ionic, hydrophobic, and van der Waals interactions are continually forming and breaking.

P1 Although these four types of interactions are individually weak relative to covalent bonds, the cumulative effect of many such interactions can be very significant. For example, the noncovalent binding of an enzyme to its substrate may involve several hydrogen bonds and one or more ionic interactions, as well as the hydrophobic effect and van der Waals interactions. The formation of each of these associations contributes to a net decrease in the free energy of the system. We can calculate the stability of a noncovalent interaction, such as the hydrogen bonding of a small molecule to its macromolecular partner, from the binding energy, the reduction in the energy of the system when binding occurs. Stability, as measured by the equilibrium constant (discussed in Section 2.2) of the binding reaction, varies *exponentially* with binding energy. To dissociate two biomolecules (such as an enzyme and its bound substrate) that are associated noncovalently through multiple weak interactions, all these interactions must be disrupted at the same time. Because the interactions fluctuate randomly, such simultaneous disruptions are very unlikely. Therefore, numerous weak interactions bestow much greater molecular stability than would be expected intuitively from a simple summation of small binding energies.

Macromolecules such as proteins, DNA, and RNA contain so many sites of potential hydrogen bonding or ionic, van der Waals, or hydrophobic clustering that the cumulative effect can be enormous. **P1** For macromolecules, the most stable (that is, the native) structure is usually that in which these weak interactions are maximized. The folding of a single polypeptide or polynucleotide chain into its three-dimensional shape is determined by this principle. The binding of an antigen to a specific antibody depends on the cumulative effects of many weak interactions. The energy released when an enzyme binds noncovalently to its substrate is the main source of the enzyme's catalytic power. The binding of a hormone or a neurotransmitter to its cellular receptor protein is the result of multiple weak interactions. One consequence of the large size of enzymes and receptors (relative to their substrates or ligands) is that their extensive surfaces provide many opportunities for weak interactions. At the molecular level, the complementarity between interacting biomolecules reflects the complementarity and weak interactions between polar and charged groups and the proximity of hydrophobic patches on the surfaces of the molecules.

When the structure of a protein such as hemoglobin is determined by x-ray crystallography (see Fig. 4-30), water molecules are often found to be bound so tightly that they are part of the crystal structure (**Fig. 2-9**); the



(a) (b)

FIGURE 2-9 Water binding in hemoglobin. The crystal structure of hemoglobin, shown (a) with bound water molecules (red spheres) and (b) without the water molecules. The water molecules are so firmly bound to the protein that they affect the x-ray diffraction pattern as though they were fixed parts of the protein. The two α subunits of hemoglobin are shown in gray, the two β subunits in blue. Each subunit has a bound heme group (red stick structure), visible only in the β subunits in this view. The structure and function of hemoglobin are discussed in detail in Chapter 5. [Data from PDB ID 1A3N, J. R. H. Tame and B. Vallone, *Acta Crystallogr.* D 56:805, 2000.]

same is true for water in crystals of RNA or DNA. These bound water molecules, which can also be detected in aqueous solutions by nuclear magnetic resonance (see Fig. 4-31), have properties that are distinctly different from those of the "bulk" water of the solvent. For example, the bound water molecules are not osmotically active (see below). For many proteins, tightly bound water molecules are essential to their function. In a key reaction in photosynthesis, for example, protons flow across a biological membrane as light drives the flow of electrons through a series of electron-carrying proteins (see Fig. 20-17). One of these proteins, cytochrome *f*, has a chain of five bound water molecules (**Fig. 2-10**) that may provide a path for protons to move through the membrane by a process known as proton hopping (described later in this chapter).

Concentrated Solutes Produce Osmotic Pressure

Solutes of all kinds alter certain physical properties of the solvent, water: its vapor pressure, its boiling point, its melting point (freezing point), and its osmotic pressure. These are called **colligative properties** (*colligative* meaning "tied together"), because the effect of solutes on all four properties has the same basis: the concentration of water is lower in solutions than in pure water. The effect of solute concentration on the colligative properties of water is independent of the chemical properties of the solute; it depends only on the *number* of solute particles (molecules or ions) in a given amount of water. For example, a compound such as NaCl, which dissociates in solution, has an effect on osmotic pressure that is twice that of an equal number of moles of a nondissociating solute such as glucose.

Water molecules tend to move from a region of higher water concentration to one of lower water concentration, in accordance with the tendency in nature for a system to become disordered. When two different aqueous solutions are separated by a semipermeable membrane (one that allows the passage of water but not solute molecules), water molecules diffusing from the region of higher water concentration to the region of lower water concentration produce osmotic pressure (**Fig. 2-11**). Osmotic pressure, Π, measured as the force necessary to resist water movement, is approximated by the van't Hoff equation

$$\Pi = icRT$$

in which R is the gas constant and T is the absolute temperature. The symbol i is the van't Hoff factor, a measure of the extent to which the solute dissociates into two

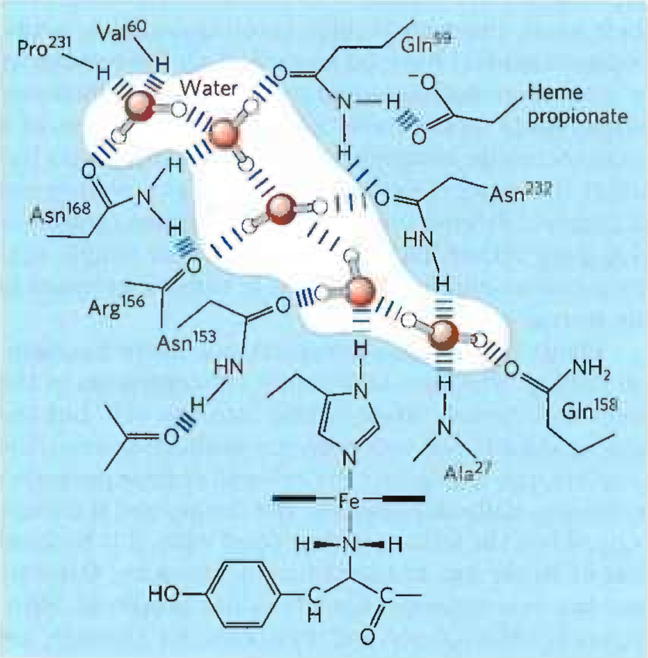
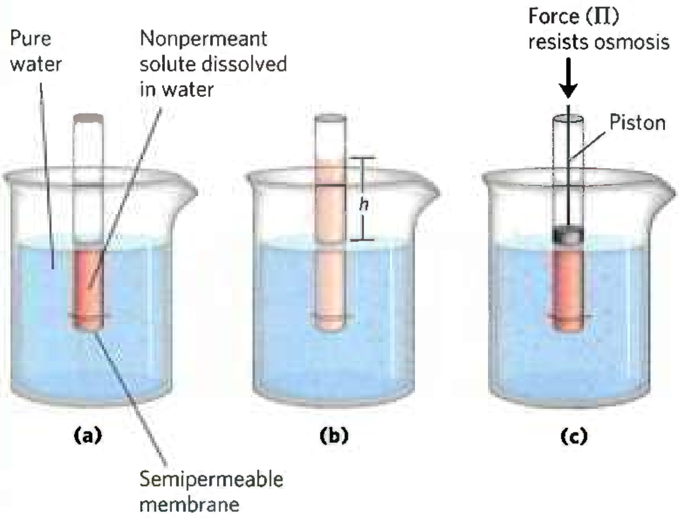


FIGURE 2-10 Water chain in cytochrome *f*. Water is bound in a proton channel of the membrane protein cytochrome *f*, which is part of the energy-trapping machinery of photosynthesis in chloroplasts. Five water molecules are hydrogen-bonded to each other and to functional groups of the protein: the peptide backbone atoms of valine, proline, arginine, and alanine residues, and the side chains of three asparagine and two glutamine residues. The protein has a bound heme, its iron ion facilitating electron flow during photosynthesis. Electron flow is coupled to the movement of protons across the membrane, which probably involves "proton hopping" through this chain of bound water molecules. [Information from P. Nicholls, *Cell. Mol. Life Sci.* 57:987, 2000, Fig. 6a (redrawn from PDB ID 1HCZ, S. E. Martinez et al., *Prot. Sci.* 5:1081, 1996).]



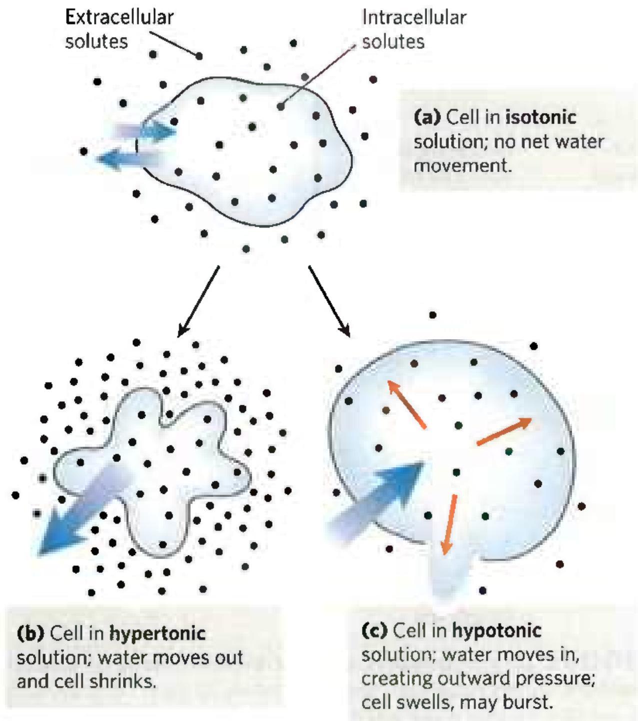
Pure water
Nonpermeant solute dissolved in water
Force (Π) resists osmosis
Piston
h
(a)
(b)
(c)
Semipermeable membrane

FIGURE 2-11 Osmosis and the measurement of osmotic pressure. (a) The initial state. The tube contains an aqueous solution, the beaker contains pure water, and the semipermeable membrane allows the passage of water but not solute. Water flows from the beaker into the tube to equalize its concentration across the membrane. (b) The final state. Water has moved into the solution of the nonpermeant compound, diluting it and raising the column of solution within the tube. At equilibrium, the force of gravity operating on the solution in the tube exactly balances the tendency of water to move into the tube, where its concentration is lower. (c) Osmotic pressure (Π) is measured as the force that must be applied to return the solution in the tube to the level of the water in the beaker. This force is proportional to the height, *h*, of the column in (b).

or more ionic species. The term c is the solute's molar concentration, and ic is the **osmolarity** of the solution, the product of the van't Hoff factor i and c. In dilute NaCl solutions, the solute completely dissociates into Na^+ and Cl^-, doubling the number of solute particles, and thus $i = 2$. For all nonionizing solutes, $i = 1$. For solutions of several (n) solutes, Π is the sum of the contributions of each species:

$$\Pi = RT(i_1c_1 + i_2c_2 + i_3c_3 + \cdots + i_nc_n)$$

Osmosis, water movement across a semipermeable membrane driven by differences in osmotic pressure, is an important factor in the life of most cells. Plasma membranes are more permeable to water than to most other small molecules, ions, and macromolecules because protein channels (aquaporins; see Table 11-3) in the membrane selectively permit the passage of water. Solutions of osmolarity equal to that of a cell's cytosol are said to be **isotonic** relative to that cell. Surrounded by an isotonic solution, a cell neither gains nor loses water (**Fig. 2-12**). In a **hypertonic** solution, one with higher osmolarity than that of the cytosol, the cell shrinks as water moves out. In a **hypotonic** solution, one with a lower osmolarity than the cytosol, the cell swells as water enters. In their natural environments, cells generally contain higher concentrations of biomolecules and ions than their surroundings, so osmotic pressure tends to drive water into cells. If not somehow counterbalanced, this inward movement of water would distend the plasma membrane and eventually cause bursting of the cell (osmotic lysis).



(a) Cell in **isotonic** solution; no net water movement.

(b) Cell in **hypertonic** solution; water moves out and cell shrinks.

(c) Cell in **hypotonic** solution; water moves in, creating outward pressure; cell swells, may burst.

FIGURE 2-12 Effect of extracellular osmolarity on water movement across a plasma membrane. When a cell in osmotic balance with its surrounding medium—that is, a cell in (a) an isotonic medium—is transferred into (b) a hypertonic solution or (c) a hypotonic solution, water moves across the plasma membrane in the direction that tends to equalize osmolarity outside and inside the cell.

Several mechanisms have evolved to prevent this catastrophe. In bacteria and plants, the plasma membrane is surrounded by a nonexpandable cell wall of sufficient rigidity and strength to resist osmotic pressure and prevent osmotic lysis. Certain freshwater protists that live in a highly hypotonic medium have an organelle (contractile vacuole) that pumps water out of the cell. In multicellular animals, blood plasma and interstitial fluid (the extracellular fluid of tissues) are maintained at an osmolarity close to that of the cytosol. The high concentration of albumin and other proteins in blood plasma contributes to its osmolarity. Cells also actively pump out Na^+ and other ions into the interstitial fluid to stay in osmotic balance with their surroundings.

Because the effect of solutes on osmolarity depends on the *number* of dissolved particles, not their *mass*, macromolecules (proteins, nucleic acids, polysaccharides) have far less effect on the osmolarity of a solution than an equal mass of their monomeric components would have. For example, a *gram* of a polysaccharide composed of 1,000 glucose units has about the same effect on osmolarity as a *milligram* of glucose. Storing fuel as polysaccharides (starch or glycogen) rather than as glucose or other simple sugars avoids an enormous increase in osmotic pressure in the storage cell.

Plants use osmotic pressure to achieve mechanical rigidity. The very high solute concentration in the plant cell vacuole draws water into the cell, but the nonexpandable cell wall prevents swelling; instead, the pressure exerted against the cell wall (turgor pressure) increases, stiffening the cell, the tissue, and the plant body. When the lettuce in your salad wilts, it is because loss of water has reduced turgor pressure. Osmosis also has consequences for laboratory protocols. Mitochondria, chloroplasts, and lysosomes, for example, are enclosed by semipermeable membranes. In isolating these organelles from broken cells, biochemists must perform the fractionations in isotonic solutions (see Fig. 1-7) to prevent excessive entry of water into the organelles and the swelling and bursting that would follow. Buffers used in cellular fractionations commonly contain sufficient concentrations of sucrose or some other inert solute to protect the organelles from osmotic lysis.

WORKED EXAMPLE 2-1 *Osmotic Strength of an Organelle*

Suppose the major solutes in intact lysosomes are KCl (~0.1 M) and NaCl (~0.03 M). When isolating lysosomes, what concentration of sucrose is required in the extracting solution at room temperature (25 °C) to prevent swelling and lysis?

SOLUTION: We want to find a concentration of sucrose that gives an osmotic strength equal to that produced by the KCl and NaCl in the lysosomes. The equation for calculating osmotic strength (the van't Hoff equation) is

$$\Pi = RT(i_1c_1 + i_2c_2 + i_3c_3 + \cdots + i_nc_n)$$

where R is the gas constant 8.315 J/mol • K; T is the absolute temperature (Kelvin); c_1, c_2, and c_3 are the molar concentrations of each solute; and i_1, i_2, and i_3 are the numbers of particles each solute yields in solution ($i = 2$ for KCl and NaCl).

The osmotic strength of the lysosomal contents is

$$\begin{aligned}\Pi_{\text{lysosome}} &= RT(i_{\text{KCl}}c_{\text{KCl}} + i_{\text{NaCl}}c_{\text{NaCl}})\\ &= RT[(2)(0.1\ \text{mol/L}) + (2)(0.03\ \text{mol/L})]\\ &= RT(0.26\ \text{mol/L})\end{aligned}$$

The osmotic strength of a sucrose solution is given by

$$\Pi_{\text{sucrose}} = RT(i_{\text{sucrose}}c_{\text{sucrose}})$$

In this case, $i_{\text{sucrose}} = 1$, because sucrose does not ionize. Thus,

$$\Pi_{\text{sucrose}} = RT(c_{\text{sucrose}})$$

The osmotic strength of the lysosomal contents equals that of the sucrose solution when

$$\begin{aligned}\Pi_{\text{sucrose}} &= \Pi_{\text{lysosome}}\\ RT(c_{\text{sucrose}}) &= RT(0.26\ \text{mol/L})\\ c_{\text{sucrose}} &= 0.26\ \text{mol/L}\end{aligned}$$

Sucrose has a formula weight (FW) of 342, so the required sucrose concentration is (0.26 mol/L)(342 g/mol) = 88.92 g/L. Because the solute concentrations are accurate to only one significant figure, $c_{\text{sucrose}} = 0.09$ kg/L.

As we'll see later (p. 242), cells of liver and muscle store carbohydrate not as low molecular weight sugars, such as glucose or sucrose, but as the high molecular weight polymer glycogen. This allows the cell to contain a large mass of glycogen with a minimal effect on the osmolarity of the cytosol.

SUMMARY 2.1 *Weak Interactions in Aqueous Systems*

- The very different electronegativities of H and O make water a highly polar molecule, capable of forming hydrogen bonds with itself and with solutes. Hydrogen bonds are fleeting, primarily electrostatic, and weaker than covalent bonds.
- Alcohols, aldehydes, ketones, and compounds containing N—H bonds all form hydrogen bonds with water and are therefore water soluble.
- By screening the electrical charges of ions and by increasing the entropy of the system, water dissolves crystals of ionizable solutes.
- N_2, O_2, and CO_2 are nonpolar and poorly soluble in water. NH_3 and H_2S are ionizable and therefore very water soluble.
- Nonpolar (hydrophobic) compounds dissolve poorly in water; they cannot hydrogen-bond with the solvent, and their presence forces an energetically unfavorable ordering of water molecules at their hydrophobic surfaces. To minimize the surface exposed to water, nonpolar and amphipathic compounds such as lipids form aggregates (micelles and bilayer vesicles) in which the hydrophobic moieties are sequestered in the interior, an association driven by the hydrophobic effect, and only the more polar moieties interact with water.
- van der Waals interactions exist when two nearby nuclei induce dipoles in each other. The nearest approach of two atoms defines the van der Waals radius of each.
- Weak, noncovalent interactions, in large numbers, decisively influence the folding of macromolecules such as proteins and nucleic acids. The most stable macromolecular conformations are those in which hydrogen bonding is maximized within the molecule and between the molecule and the solvent, and in which hydrophobic moieties cluster in the interior of the molecule away from the aqueous solvent.
- When two aqueous compartments are separated by a semipermeable membrane (such as the plasma membrane separating a cell from its surroundings), water moves across that membrane to equalize the osmolarity in the two compartments. This tendency for water to move across a semipermeable membrane produces the osmotic pressure.

2.2 Ionization of Water, Weak Acids, and Weak Bases

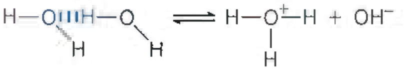
Although many of the solvent properties of water can be explained in terms of the uncharged H_2O molecule, the small degree of ionization of water to hydrogen ions (H^+) and hydroxide ions (OH^-) must also be taken into account. Like all reversible reactions, the ionization of water can be described by an equilibrium constant. When weak acids are dissolved in water, they contribute H^+ by ionizing; weak bases consume H^+ by becoming protonated. These processes are also governed by equilibrium constants. The total hydrogen ion concentration from all sources is experimentally measurable and is expressed as the pH of the solution. To predict the state of ionization of solutes in water, we must take into account the relevant equilibrium constants for each ionization reaction. Therefore, we now turn to a brief discussion of the ionization of water and of weak acids and bases dissolved in water.

Pure Water Is Slightly Ionized

Water molecules have a slight tendency to undergo reversible ionization to yield a hydrogen ion (a proton) and a hydroxide ion, giving the equilibrium

$$H_2O \rightleftharpoons H^+ + OH^- \qquad (2\text{-}1)$$

Although we commonly show the dissociation product of water as H^+, free protons do not exist in solution; hydrogen ions formed in water are immediately hydrated to form **hydronium ions** (H_3O^+). Hydrogen bonding between water molecules makes the hydration of dissociating protons virtually instantaneous:



$$H{-}O{\cdots}H{-}O(H)(H) \rightleftharpoons H{-}\overset{+}{O}(H){-}H + OH^-$$

The ionization of water can be measured by its electrical conductivity; pure water carries electrical current as H_3O^+ migrates toward the cathode and OH^- migrates toward the anode. The movement of hydronium and hydroxide ions in the electric field is extremely fast compared with that of other ions such as Na^+, K^+, and Cl^-. This high ionic mobility results from the kind of "proton hopping" shown in **Figure 2-13**. No individual proton moves very far through the bulk solution, but a series of proton hops between hydrogen-bonded water molecules causes the *net* movement of a proton over a long distance in a remarkably short time. (OH^- also moves rapidly by proton hopping, but in the opposite direction.) As a result of the high ionic mobility of H^+, acid-base reactions in aqueous solutions are exceptionally fast. As noted earlier, proton hopping very likely also plays a role in biological proton-transfer reactions (Fig. 2-10).

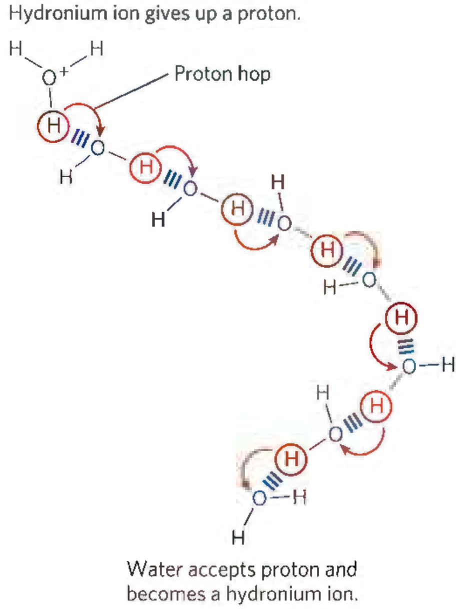

Hydronium ion gives up a proton.
Proton hop
Water accepts proton and becomes a hydronium ion.

FIGURE 2-13 Proton hopping. Short "hops" of protons between a series of hydrogen-bonded water molecules result in an extremely rapid net movement of a proton over a long distance. As a hydronium ion (upper left) gives up a proton, a water molecule some distance away (bottom) acquires one, becoming a hydronium ion. Proton hopping is much faster than true diffusion and explains the remarkably high ionic mobility of H^+ ions compared with other monovalent cations such as Na^+ and K^+.

Because reversible ionization is crucial to the role of water in cellular function, we must have a means of expressing the extent of ionization of water in quantitative terms. A brief review of some properties of reversible chemical reactions shows how this can be done.

The position of equilibrium of any chemical reaction is given by its **equilibrium constant, K_{eq}** (sometimes expressed simply as K). For the generalized reaction

$$A + B \rightleftharpoons C + D \qquad (2\text{-}2)$$

the equilibrium constant K_{eq} can be defined in terms of the concentrations of reactants (A and B) and products (C and D) at equilibrium:

$$K_{eq} = \frac{[C]_{eq}[D]_{eq}}{[A]_{eq}[B]_{eq}}$$

Strictly speaking, the concentration terms should be the *activities*, or effective concentrations in nonideal solutions, of each species. Except in very accurate work, however, the equilibrium constant may be approximated by measuring the *concentrations* at equilibrium. For reasons beyond the scope of this discussion, equilibrium constants are dimensionless. Nonetheless, we have generally retained the concentration units (M) in the equilibrium expressions used in this book to remind you that molarity is the unit of concentration used in calculating K_{eq}.

The equilibrium constant is fixed and characteristic for any given chemical reaction at a specified temperature. It defines the composition of the final equilibrium mixture, regardless of the starting amounts of reactants and products. Conversely, we can calculate the equilibrium constant for a given reaction at a given temperature if the equilibrium concentrations of all its reactants and products are known. As we showed in Chapter 1 (p. 24), the standard free-energy change ($\Delta G°$) is directly related to ln K_{eq}.

The Ionization of Water Is Expressed by an Equilibrium Constant

The degree of ionization of water at equilibrium (Eqn 2-1) is small; at 25 °C only about two of every 10^9 molecules in pure water are ionized at any instant. The equilibrium constant for the reversible ionization of water is

$$K_{eq} = \frac{[H^+][OH^-]}{[H_2O]} \qquad (2\text{-}3)$$

In pure water at 25 °C, the concentration of water is 55.5 M—grams of H_2O in 1 L divided by its gram molecular weight: (1,000 g/L)/(18.015 g/mol)—and is essentially constant in relation to the very low concentrations of H^+ and OH^-, namely 1×10^{-7} M. Accordingly, we can substitute 55.5 M in the equilibrium constant expression (Eqn 2-3) to yield

$$K_{eq} = \frac{[H^+][OH^-]}{[55.5\ \text{M}]}$$

On rearranging, this becomes

$$(55.5\ \text{M})(K_{eq}) = [H^+][OH^-] = K_w \qquad (2\text{-}4)$$

where K_w designates the product $(55.5\ \text{M})(K_{eq})$, the **ion product of water** at 25 °C.

The value for K_{eq}, determined by electrical-conductivity measurements of pure water, is 1.8×10^{-16} M at 25 °C. Substituting this value for K_{eq} in Equation 2-4 gives the value of the ion product of water:

$$\begin{aligned} K_w &= [H^+][OH^-] = (55.5\ \text{M})(1.8 \times 10^{-16}\ \text{M}) \\ &= 1.0 \times 10^{-14}\ \text{M}^2 \end{aligned}$$

Thus the product $[H^+][OH^-]$ in aqueous solutions at 25 °C always equals 1×10^{-14} M^2. When there are exactly equal concentrations of H^+ and OH^-, as in pure water, the solution is said to be at **neutral pH**. At this pH, the concentrations of H^+ and OH^- can be calculated from the ion product of water as follows:

$$K_w = [H^+][OH^-] = [H^+]^2 = [OH^-]^2$$

Solving for $[H^+]$ gives

$$\begin{aligned} [H^+] &= \sqrt{K_w} = \sqrt{1 \times 10^{-14}\ \text{M}^2} \\ [H^+] &= [OH^-] = 10^{-7}\ \text{M} \end{aligned}$$

P3 As the ion product of water is constant, whenever $[H^+]$ is greater than 1×10^{-7} M, $[OH^-]$ must be less than 1×10^{-7} M, and vice versa. When $[H^+]$ is very high, as in a solution of hydrochloric acid, $[OH^-]$ must be very low. From the ion product of water, we can calculate $[H^+]$ if we know $[OH^-]$, and vice versa.

WORKED EXAMPLE 2-2 *Calculation of [H⁺]*

What is the concentration of H^+ in a solution of 0.1 M NaOH? Because NaOH is a strong base, it dissociates completely into Na^+ and OH^-.

SOLUTION: We begin with the equation for the ion product of water:

$$K_w = [H^+][OH^-]$$

With $[OH^-] = 0.1$ M, solving for $[H^+]$ gives

$$\begin{aligned} [H^+] &= \frac{K_w}{[OH^-]} = \frac{1 \times 10^{-14}\ \text{M}^2}{0.1\ \text{M}} = \frac{10^{-14}\ \text{M}^2}{10^{-1}\ \text{M}} \\ &= 10^{-13}\ \text{M} \end{aligned}$$

WORKED EXAMPLE 2-3 *Calculation of [OH⁻]*

What is the concentration of OH^- in a solution with an H^+ concentration of 1.3×10^{-4} M?

SOLUTION: We begin with the equation for the ion product of water:

$$K_w = [H^+][OH^-]$$

With $[H^+] = 1.3 \times 10^{-4}$ M, solving for $[OH^-]$ gives

$$[OH^-] = \frac{K_w}{[H^+]} = \frac{1 \times 10^{-14}\ \text{M}^2}{1.3 \times 10^{-4}\ \text{M}} = 7.7 \times 10^{-11}\ \text{M}$$

In all calculations, be sure to round your answer to the correct number of significant figures, as here.

The pH Scale Designates the H⁺ and OH⁻ Concentrations

The ion product of water, K_w, is the basis for the **pH scale** (Table 2-5). It is a convenient means of designating the concentration of H^+ (and thus of OH^-) in any aqueous solution in the range between 1.0 M H^+ and 1.0 M OH^-. The symbol p denotes "negative logarithm of." The term **pH** is defined by the expression

$$\text{pH} = \log \frac{1}{[H^+]} = -\log [H^+]$$

For a precisely neutral solution at 25 °C, in which the concentration of hydrogen ions is 1.0×10^{-7} M, the pH can be calculated as follows:

$$\text{pH} = \log \frac{1}{1.0 \times 10^{-7}} = 7.0$$

Note that the concentration of H^+ must be expressed in molar (M) terms.

The value of 7 for the pH of a precisely neutral solution is not an arbitrarily chosen figure; it is derived from the absolute value of the ion product of water at 25 °C, which by convenient coincidence is a round number. Solutions having a pH greater than 7 are alkaline or basic;

TABLE 2-5 The pH Scale

$[H^+]$ (M)	pH	$[OH^-]$ (M)	pOH[a]
10^0 (1)	0	10^{-14}	14
10^{-1}	1	10^{-13}	13
10^{-2}	2	10^{-12}	12
10^{-3}	3	10^{-11}	11
10^{-4}	4	10^{-10}	10
10^{-5}	5	10^{-9}	9
10^{-6}	6	10^{-8}	8
10^{-7}	7	10^{-7}	7
10^{-8}	8	10^{-6}	6
10^{-9}	9	10^{-5}	5
10^{-10}	10	10^{-4}	4
10^{-11}	11	10^{-3}	3
10^{-12}	12	10^{-2}	2
10^{-13}	13	10^{-1}	1
10^{-14}	14	10^0 (1)	0

[a] The expression pOH is sometimes used to describe the basicity, or OH^- concentration, of a solution; pOH is defined by the expression $\text{pOH} = -\log [OH^-]$, which is analogous to the expression for pH. Note that in all cases, $\text{pH} + \text{pOH} = 14$.

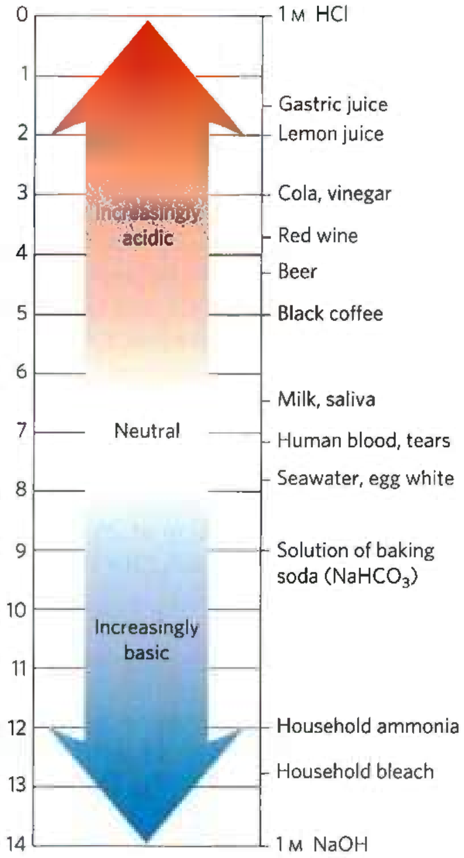


FIGURE 2-14 **The pH of some aqueous fluids.**

the concentration of OH^- is greater than that of H^+. Conversely, solutions having a pH less than 7 are acidic.

Keep in mind that the pH scale is logarithmic, not arithmetic. To say that two solutions differ in pH by 1 pH unit means that one solution has ten times the H^+ concentration of the other, but it does not tell us the absolute magnitude of the difference. **Figure 2-14** gives the pH values of some common aqueous fluids. A cola drink (pH 3.0) or red wine (pH 3.7) has an H^+ concentration approximately 10,000 times that of blood (pH 7.4).

Accurate determinations of pH in the chemical or clinical laboratory are made with a glass electrode that is selectively sensitive to H^+ concentration but insensitive to Na^+, K^+, and other cations. In a pH meter, the signal from the glass electrode placed in a test solution is amplified and compared with the signal generated by a solution of accurately known pH.

Measurement of pH is one of the most important and frequently used procedures in biochemistry. The pH affects the structure and activity of biological macromolecules, so a small change in pH can cause a large change in the structure and function of a protein. Measurements of the pH of blood and urine are commonly used in medical diagnoses. The pH of the blood plasma of people with severe, uncontrolled diabetes, for example, is often below the normal value of 7.4; this condition is called **acidosis** (described in more detail below). In certain other diseases the pH of the blood is higher than normal, a condition known as **alkalosis**. Extreme acidosis or alkalosis can be life-threatening (see **Box 2-1**).

BOX 2-1 **MEDICINE**

On Being One's Own Rabbit (Don't Try This at Home!)

> I wanted to find out what happened to a man when one made him more acid or more alkaline . . . One might, of course, have tried experiments on a rabbit first, and some work had been done along these lines; but it is difficult to be sure how a rabbit feels at any time. Indeed, some rabbits make no serious attempt to cooperate with one.
>
> —J. B. S. Haldane, *Possible Worlds*, 1928

A century ago, physiologist and geneticist J. B. S. Haldane and his colleague H. W. Davies decided to experiment on themselves, to study how the body controls blood pH. They made themselves alkaline by hyperventilating and ingesting sodium bicarbonate, which left them panting and with violent headaches. They tried to acidify themselves by drinking hydrochloric acid, but calculated that it would take a gallon and a half of dilute HCl to get the desired effect, and a pint was enough to dissolve their teeth and burn their throats. Finally, it occurred to Haldane that if he ate ammonium chloride, it would break down in the body to release hydrochloric acid and ammonia. The ammonia would be converted to harmless urea in the liver (this process is described in detail in Fig. 18-10). The hydrochloric acid would combine with the sodium bicarbonate present in all tissues, producing sodium chloride and carbon dioxide. In this experiment, the resulting shortness of breath mimicked that in diabetic acidosis or end-stage kidney disease.

Meanwhile, Ernst Freudenberg and Paul György, pediatricians in Heidelberg, were studying tetany—muscle contractions occurring in the hands, arms, feet, and larynx—in infants. They knew that tetany was sometimes seen in patients who had lost large amounts of hydrochloric acid by constant vomiting, and they reasoned that if tissue alkalinity produced tetany, acidity might be expected to cure it. The moment they read Haldane's paper on the effects of ammonium chloride, they tried giving ammonium chloride to babies with tetany, and they were delighted to find that the tetany cleared up in a few hours. This treatment didn't remove the primary cause of the tetany, but it did give the infant and the physician time to deal with that cause.

Weak Acids and Bases Have Characteristic Acid Dissociation Constants

Hydrochloric, sulfuric, and nitric acids, commonly called strong acids, are completely ionized in dilute aqueous solutions; the strong bases NaOH and KOH are also completely ionized. Of more interest to biochemists is the behavior of weak acids and bases—those not completely ionized when dissolved in water. These are ubiquitous in biological systems and play important roles in metabolism and its regulation. The behavior of aqueous solutions of weak acids and bases is best understood if we first define some terms.

Acids (in the Brønsted-Lowry definition) are proton donors, and bases are proton acceptors. When a proton donor such as acetic acid (CH_3COOH) loses a proton, it becomes the corresponding proton acceptor, in this case the acetate anion (CH_3COO^-). A proton donor and its corresponding proton acceptor make up a **conjugate acid-base pair** (Fig. 2-15), related by the reversible reaction

$$CH_3COOH \rightleftharpoons CH_3COO^- + H^+$$

Each acid has a characteristic tendency to lose its proton in an aqueous solution. The stronger the acid, the greater its tendency to lose its proton. **P2** The tendency of any acid (HA) to lose a proton and form its conjugate base (A^-) is defined by the equilibrium constant (K_{eq}) for the reversible reaction

$$HA \rightleftharpoons H^+ + A^-$$

for which

$$K_{eq} = \frac{[H^+]\,[A^-]}{[HA]} = K_a$$

Equilibrium constants for ionization reactions are usually called **ionization constants** or **acid dissociation constants**, often designated K_a. The dissociation constants of some acids are given in Figure 2-15. Stronger acids, such as phosphoric and carbonic acids, have larger ionization constants; weaker acids, such as monohydrogen phosphate (HPO_4^{2-}), have smaller ionization constants.

Also included in Figure 2-15 are values of **pK_a**, which is analogous to pH and is defined by the equation

$$pK_a = \log \frac{1}{K_a} = -\log K_a$$

The stronger the tendency to dissociate a proton, the stronger the acid and the lower its pK_a. As we shall now see, the pK_a of any weak acid can be determined experimentally.

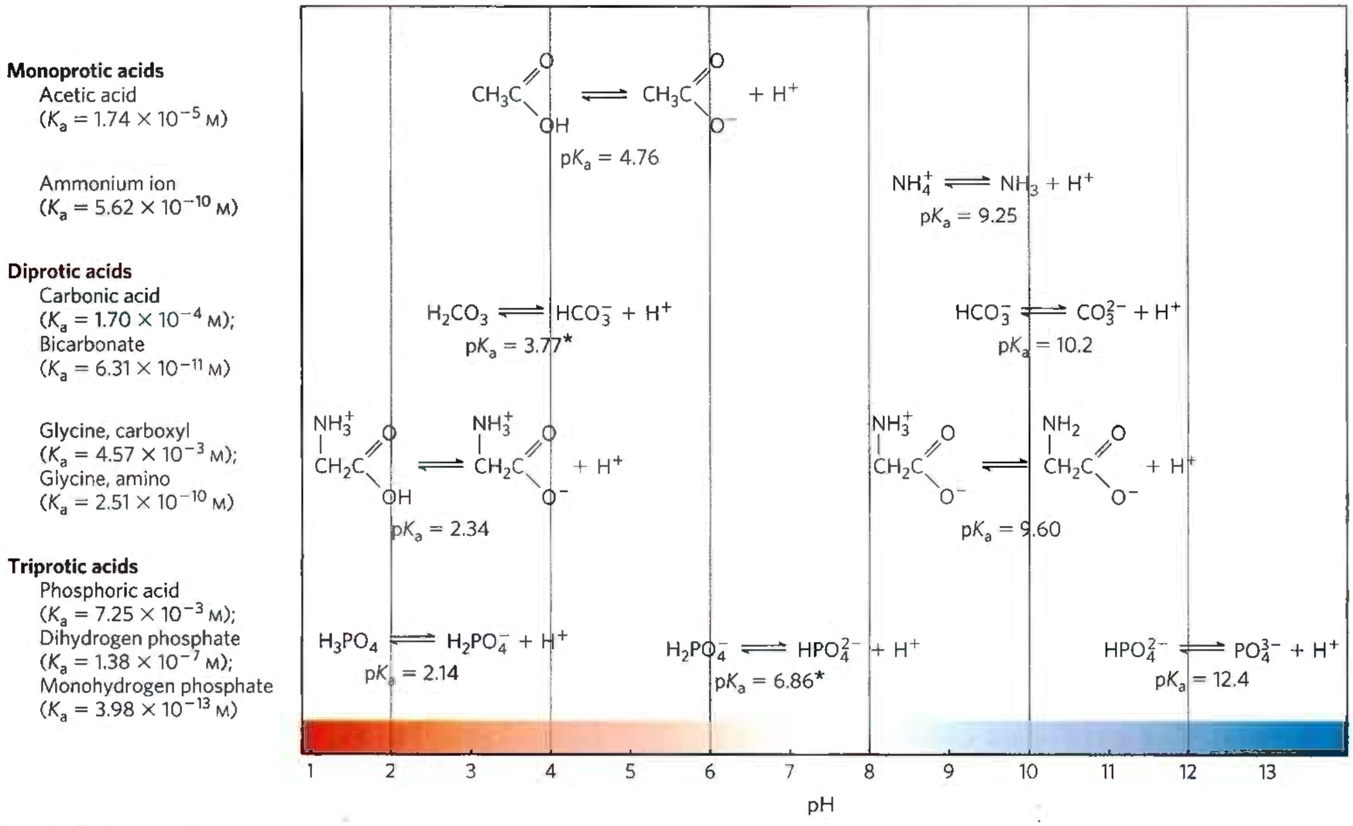

Monoprotic acids
Acetic acid
($K_a = 1.74 \times 10^{-5}$ M)
Ammonium ion
($K_a = 5.62 \times 10^{-10}$ M)
Diprotic acids
Carbonic acid
($K_a = 1.70 \times 10^{-4}$ M);
Bicarbonate
($K_a = 6.31 \times 10^{-11}$ M)
Glycine, carboxyl
($K_a = 4.57 \times 10^{-3}$ M);
Glycine, amino
($K_a = 2.51 \times 10^{-10}$ M)
Triprotic acids
Phosphoric acid
($K_a = 7.25 \times 10^{-3}$ M);
Dihydrogen phosphate
($K_a = 1.38 \times 10^{-7}$ M);
Monohydrogen phosphate
($K_a = 3.98 \times 10^{-13}$ M)
$CH_3COOH \rightleftharpoons CH_3COO^- + H^+$
pK_a = 4.76
$NH_4^+ \rightleftharpoons NH_3 + H^+$
pK_a = 9.25
$H_2CO_3 \rightleftharpoons HCO_3^- + H^+$
pK_a = 3.77*
$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+$
pK_a = 10.2
NH_3^+, CH_2C, O, OH ⇌ NH_3^+, CH_2C, O, O^- + H^+
pK_a = 2.34
NH_3^+, CH_2C, O, O^- ⇌ NH_2, CH_2C, O, O^- + H^+
pK_a = 9.60
$H_3PO_4 \rightleftharpoons H_2PO_4^- + H^+$
pK_a = 2.14
$H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$
pK_a = 6.86*
$HPO_4^{2-} \rightleftharpoons PO_4^{3-} + H^+$
pK_a = 12.4
1 2 3 4 5 6 7 8 9 10 11 12 13
pH

FIGURE 2-15 Conjugate acid-base pairs consist of a proton donor and a proton acceptor. Some compounds, such as acetic acid and ammonium ion, are monoprotic: they can give up only one proton. Others are diprotic (carbonic acid and glycine) or triprotic (phosphoric acid). The dissociation reactions for each pair are shown where they occur along a pH gradient. The equilibrium or dissociation constant (K) and its negative logarithm, the pK_a, are shown for each reaction. *For an explanation of apparent discrepancies in pK_a values for carbonic acid (H_2CO_3), see p. 63; and for dihydrogen phosphate ($H_2PO_4^-$), see p. 58.

Titration Curves Reveal the pK_a of Weak Acids

Titration can be used to determine the amount of an acid in a given solution. A measured volume of the acid is titrated with a solution of a strong base, usually sodium hydroxide (NaOH), of known concentration. The NaOH is added in small increments until the acid is consumed (neutralized), as determined with an indicator dye or a pH meter. The concentration of the acid in the original solution can be calculated from the volume and concentration of NaOH added. The amounts of acid and base in titrations are often expressed in terms of equivalents, where one equivalent is the amount of a substance that will react with, or supply, one mole of hydrogen ions in an acid-base reaction. Recall that for monoprotic acids such as HCl, 1 mol = 1 equivalent; for diprotic acids such as H_2SO_4, 1 mol = 2 equivalents.

A plot of pH against the amount of NaOH added (a **titration curve**) reveals the pK_a of the weak acid. Consider the titration of a 0.1 M solution of acetic acid with 0.1 M NaOH at 25 °C (**Fig. 2-16**). Two reversible equilibria are involved in the process (here, for simplicity, acetic acid is denoted HAc):

$$H_2O \rightleftharpoons H^+ + OH^- \qquad (2\text{-}5)$$

$$HAc \rightleftharpoons H^+ + Ac^- \qquad (2\text{-}6)$$

The equilibria must simultaneously conform to their characteristic equilibrium constants, which are, respectively,

$$K_w = [H^+][OH^-] = 1 \times 10^{-14}\ \text{M}^2 \qquad (2\text{-}7)$$

$$K_a = \frac{[H^+][Ac^-]}{[HAc]} = 1.74 \times 10^{-5}\ \text{M} \qquad (2\text{-}8)$$

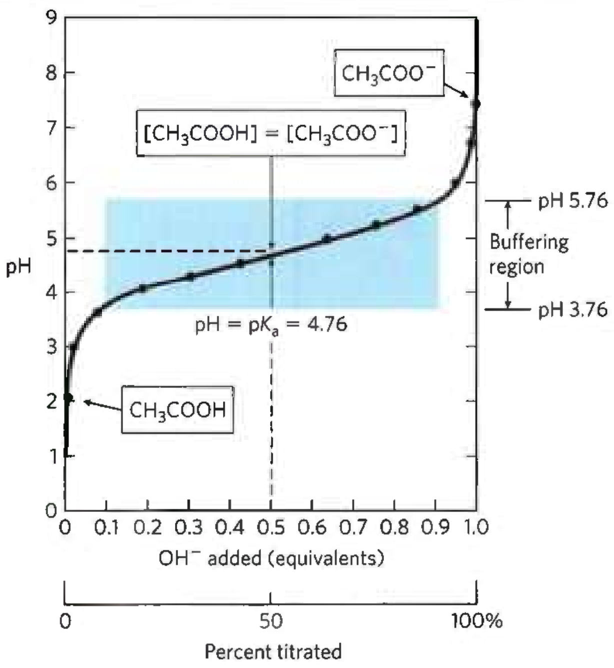


FIGURE 2-16 The titration curve of acetic acid. After addition of each increment of NaOH to the acetic acid solution, the pH of the mixture is measured. This value is plotted against the amount of NaOH added, expressed as a fraction of the total NaOH required to convert all the acetic acid (CH_3COOH) to its deprotonated form, acetate (CH_3COO^-). The points so obtained yield the titration curve. Shown in the boxes are the predominant ionic forms at the points designated. At the midpoint of the titration, the concentrations of the proton donor and the proton acceptor are equal, and the pH is numerically equal to the pK_a. The shaded zone is the useful region of buffering power, generally between 10% and 90% titration of the weak acid.

At the beginning of the titration, before any NaOH is added, the acetic acid is already slightly ionized, to an extent that can be calculated from its ionization constant (Eqn 2-8).

As NaOH is gradually introduced, the added OH^- combines with the free H^+ in the solution to form H_2O, to an extent that satisfies the equilibrium relationship in Equation 2-7. As free H^+ is removed, HAc dissociates further to satisfy its own equilibrium constant (Eqn 2-8). The net result as the titration proceeds is that more and more HAc ionizes, forming Ac^-, as the NaOH is added. At the midpoint of the titration, at which exactly 0.5 equivalent of NaOH has been added per equivalent of the acid, one-half of the original acetic acid has undergone dissociation, so that the concentration of the proton donor, [HAc], now equals that of the proton acceptor, [Ac^-]. **P2** At this midpoint a very important relationship holds: the pH of the equimolar solution of acetic acid and acetate is exactly equal to the pK_a of acetic acid (pK_a = 4.76; Figs 2-15, 2-16). The basis for this relationship, which holds for all weak acids, will soon become clear.

As the titration is continued by adding further increments of NaOH, the remaining nondissociated acetic acid is gradually converted into acetate. The end point of the titration occurs at about pH 7.0: all the acetic acid has lost its protons to OH^-, to form H_2O and acetate. Throughout the titration the two equilibria (Eqns 2-5, 2-6) coexist, each always conforming to its equilibrium constant.

Figure 2-17 compares the titration curves of three weak acids with very different ionization constants: acetic acid (pK_a = 4.76); dihydrogen phosphate, $H_2PO_4^-$ (pK_a = 6.86); and ammonium ion, NH_4^+ (pK_a = 9.25). Although the titration curves of these acids have the same shape, they are displaced along the pH axis because the three acids have different strengths. Acetic acid, with the highest K_a (lowest pK_a) of the three, is the strongest of the three weak acids (loses its proton most readily); it is already half dissociated at pH 4.76. Dihydrogen phosphate loses a proton less readily, being half dissociated at pH 6.86. Ammonium ion is the weakest acid of the three and does not become half dissociated until pH 9.25. The titration curve of these weak acids shows graphically that a weak acid and its anion—a conjugate acid-base pair—can act as a buffer, as we describe in the next section.

Like all equilibrium constants, K_a and pK_a are defined for specific conditions of concentration (components at 1 M) and temperature (25 °C). Concentrated buffer solutions do not show ideal behavior. For example, the pK_a of dihydrogen phosphate is sometimes given as 7.2, sometimes as 6.86. The higher value (the apparent pK_a) is not corrected for the effects of buffer

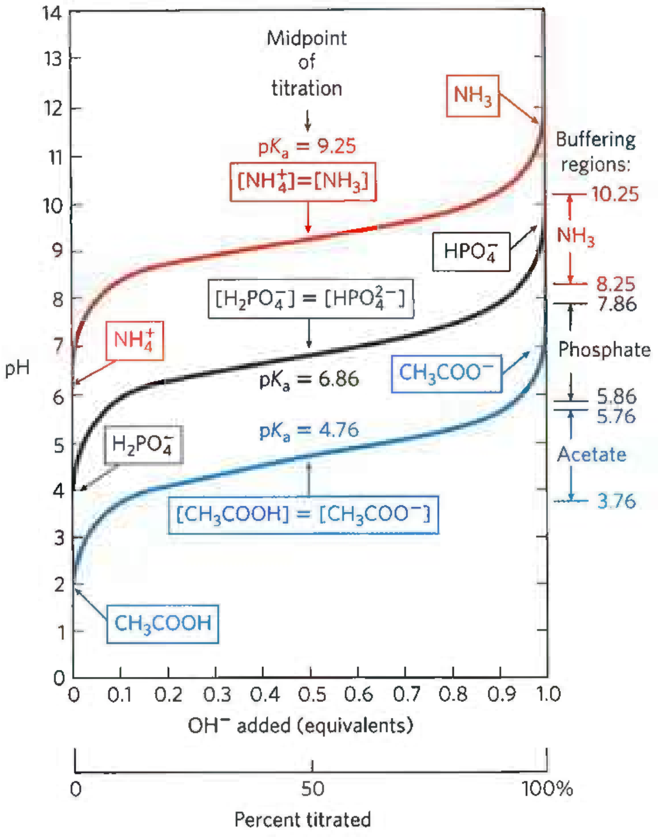


FIGURE 2-17 Comparison of the titration curves of three weak acids. Shown here are the titration curves for CH_3COOH, $H_2PO_4^-$, and NH_4^+. The predominant ionic forms at designated points in the titration are given in boxes. The regions of buffering capacity are indicated at the right. Conjugate acid-base pairs are effective buffers between approximately 10% and 90% neutralization of the proton-donor species.

concentration, and is defined at a temperature of 25 °C. The value of 6.86 is corrected for buffer concentration and measured at physiological temperature (37 °C), and is probably a closer approximation to the relevant value of pK_a in warm-blooded animals. We therefore use the value pK_a = 6.86 for dihydrogen phosphate throughout this book.

SUMMARY 2.2 *Ionization of Water, Weak Acids, and Weak Bases*

- Pure water ionizes slightly, forming equal numbers of hydrogen ions (hydronium ions, H_3O^+) and hydroxide ions.
- The extent of ionization is described by an equilibrium constant, $K_{eq} = \frac{[H^+][OH^-]}{[H_2O]}$, from which the ion product of water, K_w, is derived. At 25 °C,

$$K_w = [H^+][OH^-] = (55.5\ \text{M})(K_{eq}) = 10^{-14}\ \text{M}^2$$

- The pH of an aqueous solution reflects, on a logarithmic scale, the concentration of hydrogen ions:

$$\text{pH} = \log \frac{1}{[H^+]} = -\log [H^+]$$

- Weak acids partially ionize to release a hydrogen ion, thus lowering the pH of the aqueous solution. Weak bases accept a hydrogen ion, increasing the pH. The extent of these processes is characteristic of each particular weak acid or base and is expressed as an acid dissociation constant:

$$K_{eq} = \frac{[H^+][A^-]}{[HA]} = K_a$$

- The pK_a expresses, on a logarithmic scale, the relative strength of a weak acid or base:

$$\text{p}K_a = \log \frac{1}{[K_a]} = -\log K_a$$

- The stronger the acid, the smaller its pK_a; the stronger the base, the larger the pK_a of its conjugate acid. The pK_a can be determined experimentally; it is the pH at the midpoint of the titration curve.

2.3 Buffering against pH Changes in Biological Systems

Almost every biological process is pH-dependent; a small change in pH produces a large change in the rate of the process. This is true not only for the many reactions in which the H^+ ion is a direct participant, but also for those reactions in which there is no apparent role for H^+ ions. P4 The enzymes that catalyze cellular reactions, and many of the molecules on which they act, contain ionizable groups with characteristic pK_a values. The protonated amino and carboxyl groups of amino acids and the phosphate groups of nucleotides, for example, function as weak acids; their ionic state is determined by the pH of the surrounding medium. (When an ionizable group is sequestered in the middle of a protein, away from the aqueous solvent, its pK_a, or apparent pK_a, can be significantly different from its pK_a in water.) As we noted above, ionic interactions are among the forces that stabilize a protein molecule and allow an enzyme to recognize and bind to its substrate.

Cells and organisms maintain a specific and constant cytosolic pH, usually near pH 7, keeping biomolecules in their optimal ionic state. In multicellular organisms, the pH of extracellular fluids is also tightly regulated. Constancy of pH is achieved primarily by biological buffers: mixtures of weak acids and their conjugate bases.

Buffers Are Mixtures of Weak Acids and Their Conjugate Bases

Buffers are aqueous systems that tend to resist changes in pH when small amounts of acid (H^+) or base (OH^-) are added. A buffer system consists of a weak acid (the proton donor) and its conjugate base (the proton acceptor). As an example, a mixture of equal concentrations of acetic acid and acetate ion, found at the midpoint of the titration curve in Figure 2-16, is a buffer system. Notice that the titration curve of acetic acid has a relatively flat zone extending about 1 pH unit on either side of its midpoint pH of 4.76. In this zone, a given amount of H^+ or OH^- added to the system has much less effect on pH than the same amount added outside the zone. This relatively flat zone is the **buffering region** of the acetic acid–acetate buffer pair. At the midpoint of the buffering region, where the concentration of the proton donor (acetic acid) exactly equals that of the proton acceptor (acetate), the buffering power of the system is maximal; that is, its pH changes least on addition of H^+ or OH^-. The pH at this point in the titration curve of acetic acid is equal to its apparent pK_a. The pH of the acetate buffer system does change slightly when a small amount of H^+ or OH^- is added, but this change is very small compared with the pH change that would result if the same amount of H^+ or OH^- were added to pure water or to a solution of the salt of a strong acid and strong base, such as NaCl, which has no buffering power.

Buffering results from two reversible reaction equilibria occurring in a solution of nearly equal concentrations of a proton donor and its conjugate proton acceptor. **Figure 2-18** explains how a buffer system works. Whenever H^+ or OH^- is added to a buffer, the result is a small change in the ratio of the relative concentrations of the weak acid and its anion and thus a small change in pH. The decrease in concentration of one component of the system is balanced exactly by an increase in the other. The sum of the buffer components does not change; only their ratio changes.

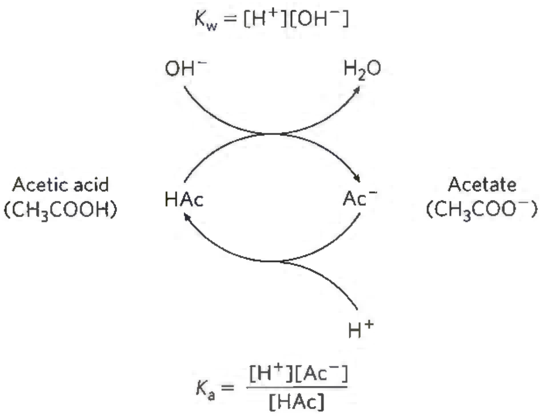


FIGURE 2-18 The acetic acid–acetate pair as a buffer system. The system is capable of absorbing either H^+ or OH^- through the reversibility of the dissociation of acetic acid. The proton donor, acetic acid (HAc), contains a reserve of bound H^+, which can be released to neutralize an addition of OH^- to the system, forming H_2O. This happens because the product $[H^+][OH^-]$ transiently exceeds K_w (1×10^{-14} M²). The equilibrium quickly adjusts to restore the product to 1×10^{-14} M² (at 25 °C), thus transiently reducing the concentration of H^+. But now the quotient $[H^+][Ac^-]/[HAc]$ is less than K_a, so HAc dissociates further to restore equilibrium. Similarly, the conjugate base, Ac^-, can react with H^+ ions added to the system; again, the two ionization reactions simultaneously come to equilibrium. Thus, a conjugate acid-base pair, such as acetic acid and acetate ion, tends to resist a change in pH when small amounts of acid or base are added. Buffering action is simply the consequence of two reversible reactions taking place simultaneously and reaching their points of equilibrium as governed by their equilibrium constants, K_w and K_a.

Each conjugate acid-base pair has a characteristic pH zone in which it is an effective buffer (Fig. 2-17). The $H_2PO_4^-/HPO_4^{2-}$ pair has a pK_a of 6.86 and thus can serve as an effective buffer system between approximately pH 5.9 and pH 7.9; the NH_4^+/NH_3 pair, with a pK_a of 9.25, can act as a buffer between approximately pH 8.3 and pH 10.3.

The Henderson-Hasselbalch Equation Relates pH, pK_a, and Buffer Concentration

The titration curves of acetic acid, $H_2PO_4^{2-}$, and NH_4^+ (Fig. 2-17) have nearly identical shapes, suggesting that these curves reflect a fundamental law or relationship. This is indeed the case. P3 The shape of the titration curve of any weak acid is described by the Henderson-Hasselbalch equation, which is important for understanding buffer action and acid-base balance in the blood and tissues of vertebrates. This equation is simply a useful way of restating the expression for the ionization constant of an acid. For the ionization of a weak acid HA, the Henderson-Hasselbalch equation can be derived as follows:

$$K_a = \frac{[H^+]\,[A^-]}{[HA]}$$

First solve for $[H^+]$:

$$[H^+] = K_a \frac{[HA]}{[A^-]}$$

Then take the negative logarithm of both sides:

$$-\log\,[H^+] = -\log\,K_a - \log\frac{[HA]}{[A^-]}$$

Substitute pH for $-\log\,[H^+]$ and pK_a for $-\log K_a$:

$$\text{pH} = pK_a - \log\frac{[HA]}{[A^-]}$$

Now invert $-\log\,[HA]/[A^-]$, which requires changing its sign, to obtain the **Henderson-Hasselbalch equation**:

$$\text{pH} = pK_a + \log\frac{[A^-]}{[HA]} \tag{2-9}$$

This equation fits the titration curve of all weak acids and enables us to deduce some important quantitative relationships. For example, it shows why the pK_a of a weak acid is equal to the pH of the solution at the midpoint of its titration. At that point, $[HA] = [A^-]$, and

$$\text{pH} = pK_a + \log 1 = pK_a + 0 = pK_a$$

The Henderson-Hasselbalch equation also allows us (1) to calculate pK_a, given pH and the molar ratio of proton donor and acceptor; (2) to calculate pH, given pK_a and the molar ratio of proton donor and acceptor; and (3) to calculate the molar ratio of proton donor and acceptor, given pH and pK_a.

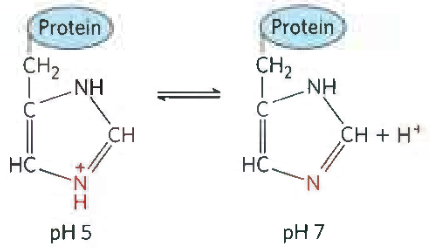


FIGURE 2-19 Ionization of histidine. The amino acid histidine, a component of proteins, is a weak acid. The pK_a of the protonated nitrogen of the side chain is 6.0.

Weak Acids or Bases Buffer Cells and Tissues against pH Changes

P3 The intracellular and extracellular fluids of multicellular organisms have a characteristic and nearly constant pH. The organism's first line of defense against changes in internal pH is provided by buffer systems. The cytoplasm of most cells contains high concentrations of proteins, and these proteins contain many amino acids with functional groups that are weak acids or weak bases. For example, the side chain of histidine (**Fig. 2-19**) has a pK_a of 6.0 and thus can exist in either the protonated form or the unprotonated form near neutral pH. Proteins containing histidine residues therefore buffer effectively near neutral pH.

WORKED EXAMPLE 2-4 *Ionization of Histidine*

Calculate the fraction of histidine that has its imidazole side chain protonated at pH 7.3. The pK_a values for histidine are $pK_1 = 1.8$, $pK_2(\text{imidazole}) = 6.0$, and $pK_3 = 9.2$ (see Fig. 3-12b).

SOLUTION: The three ionizable groups in histidine have sufficiently different pK_a values (different by at least 2 pH units) that the first acid (—COOH) is almost completely ionized before the second acid (protonated imidazole) begins to dissociate a proton, and the second ionizes almost completely before the third (—NH_3^+) begins to dissociate its proton. (With the Henderson-Hasselbalch equation, we can easily show that a weak acid goes from 1% ionized at 2 pH units below its pK_a to 99% ionized at 2 pH units above its pK_a; see also Fig. 3-12b.) At pH 7.3, the carboxyl group of histidine is entirely deprotonated (—COO^-) and the α-amino group is fully protonated (—NH_3^+). We can therefore assume that at pH 7.3, the only group that is partially dissociated is the imidazole group, which can be protonated (we'll abbreviate as $HisH^+$) or not (His).

We use the Henderson-Hasselbalch equation:

$$\text{pH} = pK_a + \log\frac{[A^-]}{[HA]}$$

Substituting $pK_2 = 6.0$ and pH = 7.3:

$$7.3 = 6.0 + \log\frac{[\text{His}]}{[\text{HisH}^+]}$$

$$1.3 = +\log\frac{[\text{His}]}{[\text{HisH}^+]}$$

$$\text{antilog } 1.3 = \frac{[\text{His}]}{[\text{HisH}^+]} = 2.0 \times 10^1$$

This gives us the *ratio* of [His] to [$HisH^+$] (20 to 1 in this case). We want to convert this ratio to the *fraction* of total histidine that is in the unprotonated form (His) at pH 7.3. That fraction is 20/21 (20 parts His per 1 part $HisH^+$, *in a total of 21 parts histidine* in either form), or about 95.2%; the remainder (100% minus 95.2%) is protonated—about 5%.

Nucleotides such as ATP, as well as many metabolites of low molecular weight, contain ionizable groups that can contribute buffering power to the cytoplasm. Some highly specialized organelles and extracellular compartments have high concentrations of compounds that contribute buffering capacity: organic acids buffer the vacuoles of plant cells; ammonia buffers urine.

Two especially important biological buffers are the phosphate and bicarbonate systems. The phosphate buffer system, which acts in the cytoplasm of all cells, consists of $H_2PO_4^-$ as proton donor and HPO_4^{2-} as proton acceptor:

$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-}$$

The phosphate buffer system is maximally effective at a pH close to its pK_a of 6.86 (Figs 2-15, 2-17) and thus tends to resist pH changes in the range between about 5.9 and 7.9. It is therefore an effective buffer in biological fluids; in mammals, for example, extracellular fluids and most cytoplasmic compartments have a pH in the range of 6.9 to 7.4.

WORKED EXAMPLE 2-5 *Phosphate Buffers*

(a) What is the pH of a mixture of 0.042 M NaH_2PO_4 and 0.058 M Na_2HPO_4?

SOLUTION: We use the Henderson-Hasselbalch equation, which we'll express here as

$$\text{pH} = pK_a + \log\frac{[\text{conjugate base}]}{[\text{acid}]}$$

In this case, the acid (the species that gives up a proton) is $H_2PO_4^-$, and the conjugate base (the species that gains a proton) is HPO_4^{2-}. Substituting the given concentrations of acid and conjugate base and the pK_a (6.86) results in

$$\text{pH} = 6.86 + \log\frac{[0.058]}{[0.042]} = 6.86 + 0.14 = 7.0$$

We can roughly check this answer. When more conjugate base than acid is present, the acid is more than 50% titrated and thus the pH is above the pK_a (6.86), where the acid is exactly 50% titrated.

(b) If 1.0 mL of 10.0 M NaOH is added to a liter of the buffer prepared in (a), how much will the pH change?

SOLUTION: A liter of the buffer contains 0.042 mol of NaH_2PO_4. Adding 1.0 mL of 10.0 M NaOH (0.010 mol) would titrate an equivalent amount (0.010 mol) of NaH_2PO_4 to Na_2HPO_4, resulting in 0.032 mol of NaH_2PO_4 and 0.068 mol of Na_2HPO_4. The new pH is

$$\begin{aligned} pH &= pK_a + \log \frac{[HPO_4^{2-}]}{[H_2PO_4^-]} \\ &= 6.86 + \log \frac{0.068}{0.032} = 6.86 + 0.33 = 7.2 \end{aligned}$$

(c) If 1.0 mL of 10.0 M NaOH is added to a liter of pure water at pH 7.0, what is the final pH? Compare this with the answer in (b).

SOLUTION: The NaOH dissociates completely into Na^+ and OH^-, giving $[OH^-] = 0.010\ mol/L = 1.0 \times 10^{-2}$ M. We can define a term pOH analogous with pH to express $[OH^-]$ of a solution. The pOH is the negative logarithm of $[OH^-]$, so in our example pOH = 2.0. Given that in all solutions, pH + pOH = 14 (see Table 2-5), the pH of the solution is 12. So, an amount of NaOH that increases the pH of water from 7 to 12 increases the pH of a buffered solution, as in (b), from 7.0 to just 7.2. Such is the power of buffering!

Why is pH + pOH = 14?

$$K_w = 10^{-14} = [H^+][OH^-]$$

Taking the negative log of both sides of the equation gives

$$\begin{aligned} -\log(10^{-14}) &= -\log[H^+] + -\log[OH^-] \\ 14 &= -\log[H^+] + -\log[OH^-] \\ 14 &= pH + pOH \end{aligned}$$

P3 Blood plasma is buffered in part by the bicarbonate system, consisting of carbonic acid (H_2CO_3) as proton donor and bicarbonate (HCO_3^-) as proton acceptor (K_1 is the first of several equilibrium constants in the bicarbonate buffering system):

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

$$K_1 = \frac{[H^+][HCO_3^-]}{[H_2CO_3]}$$

This buffer system is more complex than other conjugate acid-base pairs because one of its components, carbonic acid (H_2CO_3), is formed from dissolved (aq) carbon dioxide and water, in a reversible reaction:

$$CO_2(aq) + H_2O \rightleftharpoons H_2CO_3$$

$$K_2 = \frac{[H_2CO_3]}{[CO_2(aq)][H_2O]}$$

Carbon dioxide is a gas under normal conditions, and CO_2 dissolved in an aqueous solution is in equilibrium with CO_2 in the gas (g) phase:

$$CO_2(g) \rightleftharpoons CO_2(aq)$$

$$K_a = \frac{[CO_2(aq)]}{[CO_2(g)]}$$

The pH of a bicarbonate buffer system depends on the concentrations of H_2CO_3 and HCO_3^-, the proton donor and acceptor components. The concentration of H_2CO_3 in turn depends on the concentration of dissolved CO_2, which in turn depends on the concentration of CO_2 in the gas phase, or the **partial pressure** of CO_2, denoted pCO_2. Thus, the pH of a bicarbonate buffer exposed to a gas phase is ultimately determined by the concentration of HCO_3^- in the aqueous phase and by pCO_2 in the gas phase.

The bicarbonate buffer system is an effective physiological buffer near pH 7.4, because the H_2CO_3 of blood plasma is in equilibrium with a large reserve capacity of $CO_2(g)$ in the air space of the lungs. As noted above, this buffer system involves three reversible equilibria, in this case between gaseous CO_2 in the lungs and bicarbonate (HCO_3^-) in the blood plasma (**Fig. 2-20**).

Blood can pick up H^+, such as from the lactic acid produced in muscle tissue during vigorous exercise. Alternatively, it can lose H^+, such as by protonation of the NH_3 produced during protein catabolism. When H^+ is added to blood as it passes through the tissues, reaction 1 in Figure 2-20 proceeds toward a new equilibrium, in which $[H_2CO_3]$ is increased. This in turn increases $[CO_2(aq)]$ in the blood (reaction 2) and thus increases the partial pressure of $CO_2(g)$ in the air space of the lungs (reaction 3); the extra CO_2 is exhaled. Conversely, when H^+ is lost from the blood, the opposite events occur: more H_2CO_3 dissociates into H^+ and HCO_3^-, and thus more $CO_2(g)$ from the lungs dissolves in blood plasma. The rate of respiration—that is, the rate of inhaling and exhaling—can quickly adjust these equilibria to keep the blood pH nearly constant. The rate of respiration is controlled by the brain stem, where detection of an increased blood pCO_2 or decreased blood pH triggers deeper and more frequent breathing.

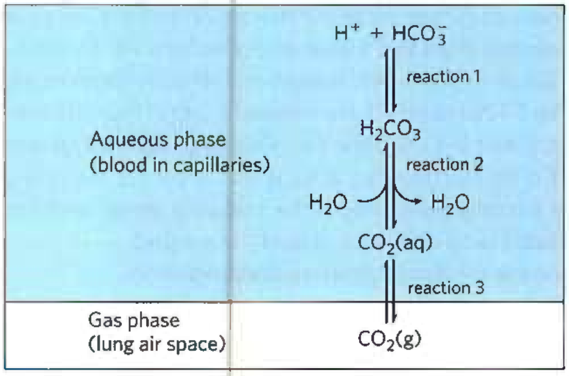


FIGURE 2-20 The bicarbonate buffer system. CO_2 in the air space of the lungs is in equilibrium with the bicarbonate buffer in the blood plasma passing through the lung capillaries. Because the concentration of dissolved CO_2 can be adjusted rapidly through changes in the rate of breathing, the bicarbonate buffer system of the blood is in near-equilibrium with a large potential reservoir of CO_2.

Hyperventilation, the rapid breathing sometimes elicited by stress or anxiety, tips the normal balance of O_2 breathed in and CO_2 breathed out in favor of too much CO_2 breathed out, raising the blood pH to 7.45 or higher and causing alkalosis. This alkalosis can lead to dizziness, headache, weakness, and fainting. One home remedy for mild alkalosis is to breathe briefly into a paper bag. The air in the bag becomes enriched in CO_2, and inhaling this air increases the CO_2 concentration in the body and blood and decreases blood pH.

At the normal pH of blood plasma (7.4), very little H_2CO_3 is present relative to HCO_3^-, and the addition of just a small amount of base (NH_3 or OH^-) would titrate this H_2CO_3, exhausting the buffering capacity. The important role of H_2CO_3 ($pK_a = 3.57$ at 37 °C) in buffering blood plasma (pH ~7.4) seems inconsistent with our earlier statement that a buffer is most effective in the range of 1 pH unit above and below its pK_a. The explanation for this apparent paradox is the large reservoir of CO_2 dissolved in blood, which we refer to as CO_2(aq). Its rapid equilibration with H_2CO_3 results in the formation of additional H_2CO_3:

$$CO_2(aq) + H_2O \rightleftharpoons H_2CO_3$$

It is useful in clinical medicine to have a simple expression for blood pH in terms of CO_2(aq), which is commonly monitored along with other blood gases. We can define a constant, K_h, which is the equilibrium constant for the hydration of CO_2 to form H_2CO_3:

$$K_h = \frac{[H_2CO_3]}{[CO_2(aq)]}$$

(The concentration of water is so high (55.5 M) that dissolving CO_2 doesn't change $[H_2O]$ appreciably, so $[H_2O]$ is made part of the constant K_h.) Then, to take the CO_2(aq) reservoir into account, we can express $[H_2CO_3]$ as $K_h[CO_2(aq)]$ and substitute this expression for $[H_2CO_3]$ in the equation for the acid dissociation of H_2CO_3:

$$K_a = \frac{[H^+]\,[HCO_3^-]}{[H_2CO_3]} = \frac{[H^+]\,[HCO_3^-]}{K_h[CO_2(aq)]}$$

Now, the overall equilibrium for dissociation of H_2CO_3 can be expressed in these terms:

$$K_hK_a = K_{\text{combined}} = \frac{[H^+]\,[HCO_3^-]}{[CO_2(aq)]}$$

We can calculate the value of the new constant, K_{combined}, and the corresponding apparent pK, or pK_{combined}, from the experimentally determined values of K_h(3.0×10^{-3} M) and K_a(2.7×10^{-4} M) at 37° C:

$$\begin{aligned} K_{\text{combined}} &= (3.0 \times 10^{-3}\ \text{M})(2.7 \times 10^{-4}\ \text{M}) \\ &= 8.1 \times 10^{-7}\ \text{M}^2 \\ pK_{\text{combined}} &= 6.1 \end{aligned}$$

In clinical medicine, it is common to refer to CO_2(aq) as the conjugate acid and to use the apparent, or combined, pK_a of 6.1 to simplify calculation of pH from $[CO_2(aq)]$. The concentration of dissolved CO_2 is a function of pCO_2, which in the lung is about 4.8 kilopascals (kPa), corresponding to $[H_2CO_3] \approx 1.2$ mM. Plasma $[HCO_3^-]$ is normally about 24 mM, so $[HCO_3^-]/[H_2CO_3]$ is about 20, and the blood pH is $6.1 + \log 20 \approx 7.4$.

Untreated Diabetes Produces Life-Threatening Acidosis

P4 Many of the enzymes that function in the blood have evolved to have maximal activity between pH 7.35 and 7.45, the normal pH range of human blood plasma. Enzymes typically show maximal catalytic activity at a characteristic pH, called the **optimum pH** (Fig. 2-21). On either side of this optimum pH, catalytic activity often declines sharply. Thus, a small change in pH can make a large difference in the rate of some crucial enzyme-catalyzed reactions. Biological control of the pH of cells and body fluids is therefore of central importance in all aspects of metabolism and cellular activities, and changes in blood pH have marked physiological consequences, as we know from the alarming experiments described in Box 2-1.

In individuals with untreated diabetes mellitus, the lack of insulin, or insensitivity to insulin, disrupts the uptake of glucose from blood into the tissues and forces the tissues to use stored fatty acids as their primary fuel. For reasons we describe in detail later in the book (see Chapter 23), this dependence on fatty acids results

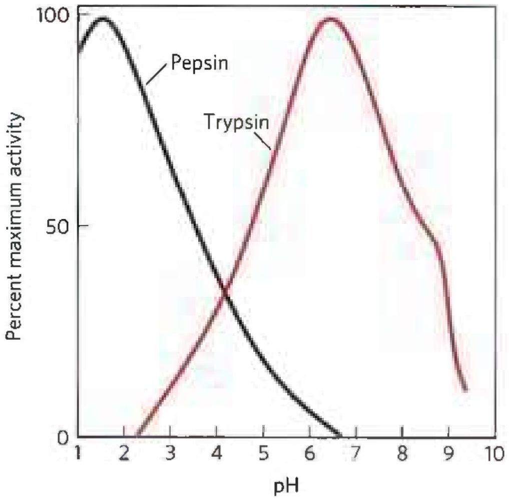


FIGURE 2-21 The pH optima of two enzymes. Pepsin is a digestive enzyme secreted into gastric juice, which has a pH of ~1.5, allowing pepsin to act optimally. Trypsin, a digestive enzyme that acts in the small intestine, has a pH optimum that matches the neutral pH in the lumen of the small intestine.

in acidosis, the accumulation of high concentrations of two carboxylic acids, β-hydroxybutyric acid and acetoacetic acid (a combined blood plasma level of 90 mg/100 mL, compared with <3 mg/100 mL in control (healthy) individuals; urinary excretion of 5 g/24 h, compared with <125 mg/24 h in controls). Dissociation of these acids lowers the pH of blood plasma to less than 7.35. **P3** Severe acidosis (characterized by low blood pH) produces headache, drowsiness, nausea, vomiting, and diarrhea, followed by stupor, coma, and convulsions, presumably because, at the lower pH, some enzyme(s) do not function optimally. When a patient is found to have high blood glucose, low plasma pH, and high levels of β-hydroxybutyric acid and acetoacetic acid in blood and urine, diabetes mellitus is the likely diagnosis.

Other conditions can also produce acidosis. For example, fasting and starvation force the use of stored fatty acids as fuel, with the same consequences as for diabetes. Very heavy exertion, such as a sprint by runners or cyclists, leads to temporary accumulation of lactic acid in the blood. Kidney failure results in a diminished capacity to regulate bicarbonate levels. Lung diseases (such as emphysema, pneumonia, and asthma) reduce the capacity to dispose of the CO_2 produced by fuel oxidation in the tissues, with the resulting accumulation of H_2CO_3.

Acidosis is treated by dealing with the underlying condition—administering insulin to people with diabetes, and steroids or antibiotics to people with lung disease. Severe acidosis can be reversed by administering bicarbonate solution intravenously.

WORKED EXAMPLE 2-6 *Treatment of Acidosis with Bicarbonate*

Why does intravenous administration of a bicarbonate solution raise the plasma pH?

SOLUTION: The ratio of $[HCO_3^-]$ to $[CO_2(aq)]$ determines the pH of the bicarbonate buffer, according to the equation

$$\text{pH} = 6.1 + \log \frac{[HCO_3^-]}{H_2CO_3}$$

where $[H_2CO_3]$ is directly related to pCO_2, the partial pressure of CO_2. So, if $[HCO_3^-]$ is increased with no change in pCO_2, the blood pH will rise.

SUMMARY 2.3 *Buffering against pH Changes in Biological Systems*

- A mixture of a weak acid (or base) and its salt resists changes in pH caused by the addition of H^+ or OH^-. The mixture thus functions as a buffer.
- The pH of a solution of a weak acid (or base) and its salt is given by the Henderson-Hasselbalch equation:

$$\text{pH} = \text{p}K_a + \log \frac{[A^-]}{[HA]}$$

- In cells and tissues, phosphate and bicarbonate buffer systems maintain intracellular and extracellular fluids near pH 7.4. Enzymes generally work optimally near this physiological pH.
- Medical conditions such as untreated diabetes that lower the pH of blood, causing acidosis, or raise it, causing alkalosis, can be life-threatening.

KEY TERMS

Terms in bold are defined in the glossary.

hydrogen bond	44
bond energy	44
hydrophilic	46
hydrophobic	46
amphipathic	46
hydrophobic effect	48
micelle	48
van der Waals interactions	49
osmolarity	52
osmosis	52
isotonic	52
hypertonic	52
hypotonic	52
equilibrium constant (K_{eq})	54
ion product of water (K_w)	55
pH	55
acidosis	56
alkalosis	56
conjugate acid-base pair	57
acid dissociation constant (K_a)	57
pK_a	57
titration curve	58
buffer	59
buffering region	60
Henderson-Hasselbalch equation	60
optimum pH	63

PROBLEMS

1. Effect of Local Environment on Ionic Bond Strength The ATP-binding site of an enzyme is buried in the interior of the enzyme, in a hydrophobic environment. Suppose that the ionic interaction between enzyme and ATP took place at the surface of the enzyme, exposed to water. Would this enzyme-substrate interaction be stronger or would it be weaker? Why?

2. Biological Advantage of Weak Interactions The associations between biomolecules are often stabilized by hydrogen bonds, electrostatic interactions, the hydrophobic effect, and van der Waals interactions. How are weak interactions such as these advantageous to an organism?

3. Solubility of Ethanol in Water Ethane (CH_3CH_3) and ethanol (CH_3CH_2OH) differ in their molecular makeup by only one atom, yet ethanol is much more soluble in water than ethane. Describe the features of ethanol that make it more water soluble than ethane.

4. Calculation of pH from Hydrogen Ion Concentration What is the pH of a solution that has an H^+ concentration of **(a)** 1.75×10^{-5} mol/L; **(b)** 6.50×10^{-10} mol/L; **(c)** 1.0×10^{-4} mol/L; **(d)** 1.50×10^{-5} mol/L?

5. Calculation of Hydrogen Ion Concentration from pH What is the H^+ concentration of a solution with pH of **(a)** 3.82; **(b)** 6.52; **(c)** 11.11?

6. Acidity of Gastric HCl A technician in a hospital laboratory obtained a 10.0 mL sample of gastric juice from a patient several hours after a meal and titrated the sample with 0.1 M NaOH to neutrality. The neutralization of gastric HCl required 7.2 mL of NaOH. The patient's stomach contained no ingested food or drink at the time of sample harvest. Therefore, assume that no buffers were present. What was the pH of the gastric juice?

7. Calculation of the pH of a Strong Acid or Base

(a) Write out the acid dissociation reaction for hydrochloric acid.

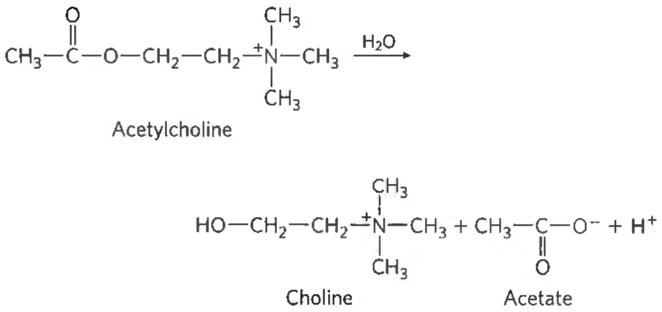
(b) Calculate the pH of a solution of 5×10^{-4} M hydrochloric acid at 25 °C.

(c) Write out the acid dissociation reaction for sodium hydroxide.

(d) Calculate the pH of a solution of 7×10^{-5} M sodium hydroxide at 25 °C.

8. Calculation of pH from Concentration of Strong Acid Calculate the pH of a solution prepared by diluting 3.0 mL of 2.5 M HCl to a final volume of 100 mL with H_2O.

9. Measurement of Acetylcholine Levels by pH Changes You have a 15 mL sample of acetylcholine (a neurotransmitter) with an unknown concentration and a pH of 7.65. You incubate this sample with the enzyme acetylcholinesterase to convert all of the acetylcholine to choline and acetic acid. The acetic acid dissociates to yield acetate and hydrogen ions.



At the end of the incubation period, you measure the pH again and find that it has decreased to 6.87. Assuming there was no buffer in the assay mixture, determine the number of nanomoles of acetylcholine in the original 15 mL sample.

10. Relationship Between pK_a and pH Which aqueous solution has the lowest pH: 0.1 M hydrofluoric acid ($pK_a = 3.20$); 0.1 M acetic acid ($pK_a = 4.86$); 0.1 M formic acid ($pK_a = 3.75$); or 0.1 M lactic acid ($pK_a = 7.86$)?

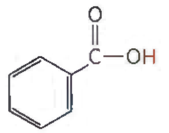
11. Properties of Strong and Weak Acids Classify each acid or property as representing a strong acid or a weak acid: **(a)** hydrochloric acid; **(b)** acetic acid; **(c)** strong tendency to dissociate protons; **(d)** larger K_a; **(e)** partially dissociates into ions; **(f)** larger pK_a.

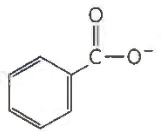
12. Simulated Vinegar One way to make vinegar is to prepare a solution of acetic acid, the sole acid component of vinegar, at the proper pH (see Fig. 2-14) and add appropriate flavoring agents. Acetic acid is a liquid at 25 °C, with a relative molecular mass (M_r) of 60, density of 1.049 g/mL, and acid dissociation constant (K_a) of 1.7×10^{-5} M. Calculate the volume of acetic acid needed to produce 1 L of simulated vinegar from distilled water (see Fig. 2-15).

13. Identifying Conjugate Bases Write the conjugate base for each acid: **(a)** H_3PO_4; **(b)** H_2CO_3; **(c)** CH_3COOH; **(d)** $CH_3NH_3^+$

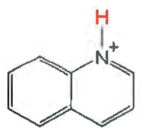
14. Calculation of the pH of a Mixture of a Weak Acid and Its Conjugate Base Calculate the pH of a dilute solution that contains a molar ratio of potassium acetate to acetic acid ($pK_a = 4.76$) of **(a)** 2:1; **(b)** 1:3; **(c)** 5:1; **(d)** 1:1; **(e)** 1:10.

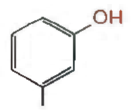
15. Effect of pH on Solubility The strongly polar, hydrogen-bonding properties of water make it an excellent solvent for ionic (charged) species. By contrast, nonionized, nonpolar organic molecules, such as benzene, are relatively insoluble in water. In principle, the aqueous solubility of any organic acid or base can be increased by converting the molecules to charged species. For example, the solubility of benzoic acid in water is low. Adding sodium bicarbonate to a mixture of water and benzoic acid raises the pH and deprotonates the benzoic acid to form benzoate ion, which is quite soluble in water.

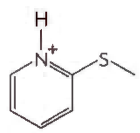

Benzoic acid
$pK_a \approx 5$


Benzoate ion

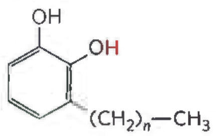
Categorize the given compounds based on whether they are more soluble in an aqueous solution of 0.1 M NaOH or 0.1 M HCl. (The dissociable protons are shown in red.)


Quinoline ion
$pK_a = 4.9$
(a)


m-Cresol
$pK_a = 10.1$
(b)


2-(Methylthio)pyridine ion
$pK_a = 3.6$
(c)

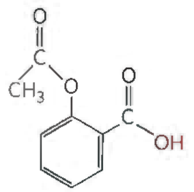
16. Treatment of Poison Ivy Rash Urushiol, the component of poison ivy that is responsible for the characteristic itchy rash, is a mixture of catechols substituted with various long-chain alkyl groups.


$pK_a \approx 8$

Which of these treatments would be most effective at removing catechols from the surface of the skin after exposure to poison ivy? Justify your choice.

(a) Wash the area with cold water.
(b) Wash the area with dilute vinegar or lemon juice.
(c) Wash the area with soap and water.
(d) Wash the area with soap, water, and baking soda (sodium bicarbonate).

17. pH and Drug Absorption Aspirin is a weak acid with a pK_a of 3.5 (the ionizable H is shown in red):



Aspirin is absorbed into the blood through the cells lining the stomach and the small intestine. Absorption requires passage through the plasma membrane. The polarity of the molecule determines the absorption rate: charged and highly polar molecules pass slowly, whereas neutral hydrophobic molecules pass rapidly. The pH of the stomach contents is about 1.5, and the pH of the contents of the small intestine is about 6. Based on this information, is more aspirin absorbed into the bloodstream from the stomach or from the small intestine? Clearly justify your choice.

18. Calculation of pH from Molar Concentrations The pK_a of NH_4^+/NH_3 is 9.25. Calculate the pH of a solution containing 0.12 M NH_4Cl and 0.03 M NaOH.

19. Calculation of pH after Titration of Weak Acid A compound has a pK_a of 7.4. You add 100 mL of a 1.0 M solution of this compound at pH 8.0 to 30 mL of 1.0 M hydrochloric acid. What is the pH of the resulting solution?

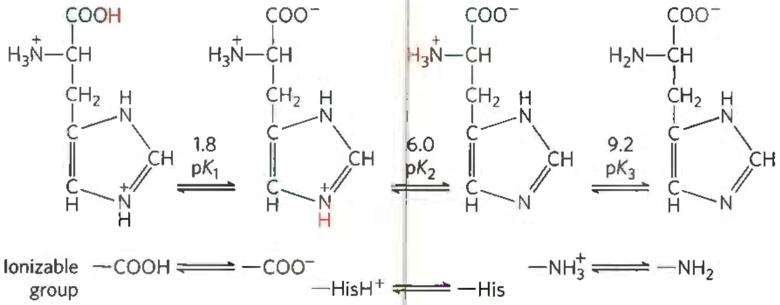
20. Properties of a Buffer The amino acid glycine is often used as the main ingredient of a buffer in biochemical experiments. The amino group of glycine, which has a pK_a of 9.6, can exist either in the protonated form ($—NH_3^+$) or as the free base ($—NH_2$), because of the reversible equilibrium

$$R—NH_3^+ \rightleftharpoons R—NH_2 + H^+$$

(a) In what pH range can glycine be used as an effective buffer due to its amino group?
(b) In a 0.1 M solution of glycine at pH 9.0, what fraction of glycine has its amino group in the $—NH_3^+$ form?
(c) How much 5 M KOH must be added to 1.0 L of 0.1 M glycine at pH 9.0 to bring its pH to exactly 10.0?
(d) When 99% of the glycine is in its $—NH_3^+$ form, what is the numerical relation between the pH of the solution and the pK_a of the amino group?

21. Calculation of the pK_a of an Ionizable Group by Titration Suppose a biochemist has 10 mL of a 1.0 M solution of a compound with two ionizable groups at a pH of 8.00. She adds 10.0 mL of 1.00 M HCl, which changes the pH to 3.20. The pK_a value of one of the groups (pK_1) is 3.8 and it is known that pK_2 is between 7 and 10. What is the exact value of pK_2?

22. Calculation of the pH of a Solution of a Polyprotic Acid The amino acid histidine has ionizable groups with pK_a values of 1.8, 6.0, and 9.2, as shown (His = imidazole group). A biochemist makes up 100 mL of a 0.10 M solution of histidine at a pH of 5.40. She then adds 40 mL of 0.10 M HCl. What is the pH of the resulting solution?



23. Calculation of Original pH from Final pH after Titration A biochemist has 100 mL of a 0.100 M solution of a weak acid with a pK_a of 6.3. He adds 6.0 mL of 1.0 M HCl, which changes the pH to 5.7. What was the pH of the original solution?

24. Preparation of a Phosphate Buffer Phosphoric acid (H_3PO_4), a triprotic acid, has three pK_a values: 2.14, 6.86, and 12.4. What molar ratio of HPO_4^{2-} to $H_2PO_4^-$ in solution would produce a pH of 7.0? Hint: Only one of the pK_a values is relevant here.

25. Preparation of Standard Buffer for Calibration of a pH Meter The glass electrode used in commercial pH meters gives an electrical response proportional to the concentration of hydrogen ion. Converting these responses to a pH reading requires calibration of the electrode against standard solutions of known H^+ concentration. Preparation of the pH 7.00 standard buffer uses dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$; FW 138) and disodium hydrogen phosphate (Na_2HPO_4; FW 142). Phosphoric acid (H_3PO_4), a triprotic acid, has three pK_a values: 2.14, 6.86, and 12.4. Calculate the weight in grams of sodium dihydrogen phosphate and disodium hydrogen phosphate needed to prepare 1.0 L of a standard buffer with a total phosphate concentration of 0.10 M (see Fig. 2-15).

26. Calculation of Molar Ratios of Conjugate Base to Weak Acid from pH For a weak acid with a pK_a of 6.00, calculate the ratio of conjugate base to acid at a pH of 5.00.

27. Preparation of Buffer of Known pH and Strength You have 0.10 M solutions of acetic acid (pK_a = 4.76) and sodium acetate. If you wanted to prepare 1.0 L of 0.10 M acetate buffer of pH 4.00, how many milliliters of acetic acid and sodium acetate would you mix together?

28. Choice of Weak Acid for a Buffer Determine whether each weak acid would best buffer at pH 3.0, at pH 5.0, or at pH 9.0: **(a)** formic acid (pK_a = 3.8); **(b)** acetic acid (pK_a = 4.76); **(c)** ammonium (pK_a = 9.25); **(d)** boric acid (pK_a = 9.24); **(e)** chloroacetic acid (pK_a = 2.87); **(f)** hydrazoic acid (pK_a = 4.6). Briefly justify your answer.

29. Working with Buffers A buffer contains 0.010 mol of lactic acid (pK_a = 3.86) and 0.050 mol of sodium lactate per liter.
(a) Calculate the pH of the buffer.

(b) Calculate the change in pH after adding 5.0 mL of 0.5 M HCl to 1 L of the buffer.
(c) What pH change would you expect if you added the same quantity of HCl to 1 L of pure water?

30. Use of Molar Concentrations to Calculate pH What is the pH of a solution that contains 0.20 M sodium acetate and 0.60 M acetic acid ($pK_a = 4.76$)?

31. Preparation of an Acetate Buffer Calculate the concentrations of acetic acid ($pK_a = 4.76$) and sodium acetate necessary to prepare a 0.2 M buffer solution at pH 5.0.

32. pH of Insect Defensive Secretion You have been observing an insect that defends itself from enemies by secreting a caustic liquid. Analysis of the liquid shows it to have a total concentration of formate plus formic acid ($K_a = 1.8 \times 10^{-4}$) of 1.45 M. Further analysis reveals that the concentration of formate ion is 0.015 M. What is the pH of the secretion?

33. Calculation of pK_a An unknown compound, X, is thought to have a carboxyl group with a pK_a of 2.0 and another ionizable group with a pK_a between 5 and 8. When 75 mL of 0.1 M NaOH is added to 100 mL of a 0.1 M solution of X at pH 2.0, the pH increases to 6.72. Calculate the pK_a of the second ionizable group of X.

34. Ionized Forms of Amino Acids at Different pH Levels Glycine is a diprotic acid that can undergo two dissociation reactions, one for the α-amino group ($—NH_3^+$) and the other for the carboxyl (—COOH) group. Therefore, it has two pK_a values. The carboxyl group has a pK_1 of 2.34 and the α-amino group has a pK_2 of 9.60. Glycine can exist in fully deprotonated ($NH_2—CH_2—COO^-$), fully protonated ($^+NH_3—CH_2—COOH$), or zwitterionic form ($^+NH_3—CH_2—COO^-$). Determine which form of glycine would be present in the highest concentration in a solution of **(a)** pH 1.0; **(b)** pH 6.0; **(c)** pH 7.0; **(d)** pH 8.0; **(e)** pH 11.9. Explain your answers in terms of pH relative to the two pK_a values.

35. Control of Blood pH by Respiratory Rate
(a) The partial pressure of CO_2 (pCO_2) in the lungs can be varied rapidly by the rate and depth of breathing. For example, a common remedy to alleviate hiccups is to increase the concentration of CO_2 in the lungs. This can be achieved by holding one's breath, by very slow and shallow breathing (hypoventilation), or by breathing in and out of a paper bag. Under such conditions, pCO_2 in the air space of the lungs rises above normal. How would increasing pCO_2 in the air space of the lungs affect blood pH?
(b) It is common practice among competitive short-distance runners to breathe rapidly and deeply (hyperventilate) for about half a minute to remove CO_2 from their lungs just before a race begins. Under these conditions, blood pH may rise to 7.6. Explain how hyperventilation elicits an increase in blood pH.
(c) During a short-distance run, the muscles produce a large amount of lactic acid ($CH_3CH(OH)COOH$; $K_a = 1.38 \times 10^{-4}$ M) from their glucose stores. Why might hyperventilation before a short-distance run be useful?

36. Calculation of Blood pH from CO_2 and Bicarbonate Levels Calculate the pH of a blood plasma sample with a total CO_2 concentration of 26.9 mM and bicarbonate concentration of 25.6 mM. Recall from page 63 that the relevant pK_a of carbonic acid is 6.1.

37. Effect of Holding One's Breath on Blood pH The pH of the extracellular fluid is buffered by the bicarbonate/carbonic acid system. Holding your breath can increase the concentration of $CO_2(aq)$ in the blood. What effect might this have on the pH of the extracellular fluid? Explain the effect on pH by writing the relevant equilibrium equation(s) for this buffer system.

38. Boiling Point of Alcohols and Diols
(a) Arrange these compounds in order of expected boiling point.

$CH_3—CH_2—OH$
$HO—CH_2CH_2CH_2—OH$
$CH_3—OH$
$HO—CH_2CH_2—OH$

(b) What factors are important in predicting the boiling points of these compounds?

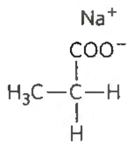
39. Duration of Hydrogen Bonds PCR is a laboratory process in which specific DNA sequences are copied and amplified manyfold. The two DNA strands, which are held together in part by hydrogen bonds between them, are heated in a buffered solution to separate the two strands, then cooled to allow them to reassociate. What do you predict about the average duration of H bonds at the high temperature in comparison to the low temperature?

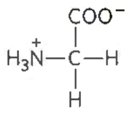
40. Electronegativity and Hydrogen Bonding The Pauling electronegativity is a measure of the affinity of an atom for the electron in a covalent bond. The larger the electronegativity value, the greater the affinity of the atom for an electron shared with another atom.

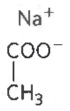
Atom	Electronegativity
H	2.1
C	2.55
S	2.58
N	3.04
O	3.44

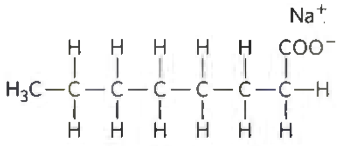
Note that S is directly beneath O in the periodic table.
(a) Do you expect H_2S to form hydrogen bonds with itself? With H_2O?
(b) Water boils at 100 °C. Is the boiling point for H_2S higher or lower than for H_2O?
(c) Is H_2S a more polar solvent than H_2O?

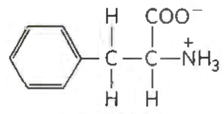
41. Solubility of Low Molecular Weight Compounds Several low molecular weight compounds found in cells are shown in the ionic form in which they exist in water at pH 7.


$$Na^+ \quad H_3C—\overset{COO^-}{\underset{H}{C}}—H$$
Sodium propionate


$$H_3\overset{+}{N}—\overset{COO^-}{\underset{H}{C}}—H$$
Glycine


$$Na^+ \quad \overset{COO^-}{CH_3}$$
Sodium acetate


Sodium octanoate


L-Phenylalanine

List the five compounds in order from most soluble to least soluble in water.

42. Relative Solubility of Alcohols List the alcohols in order from most soluble to least soluble in water.

$$CH_3{-}(CH_2)_5{-}OH \quad CH_3{-}(CH_2)_{10}{-}OH$$

$$CH_3{-}(CHOH){-}CH_2{-}CHOH{-}CH_2{-}OH$$

43. Determining Charge and Solubility of Organic Acids Suppose that, for a typical carboxyl-containing compound, the pK_a is approximately 3. Suppose $HOOC{-}(CH_2)_4{-}COOH$, $CH_3{-}(CH_2)_4{-}COOH$, and $HOOC{-}(CH_2)_2{-}COOH$ are added to water at pH 7.

(a) What is the net charge of each compound in the solution?

(b) List the compounds in order from most soluble to least soluble.

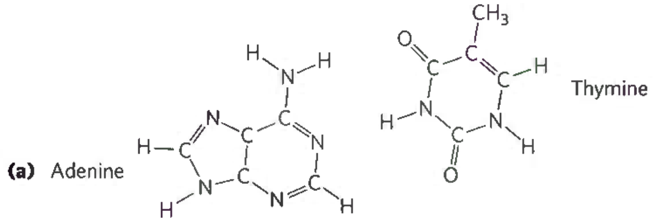
44. Ecological Effects of pH The defendant in a lawsuit over industrial pollution is accused of releasing effluent of pH 10 into a trout stream. The plaintiff has asked that the defendant reduce the effluent's pH to no higher than 7. The defendant's attorney, aiming to please the court, promises that his client will do even better than that: the defendant will bring the pH of the effluent down to 1!

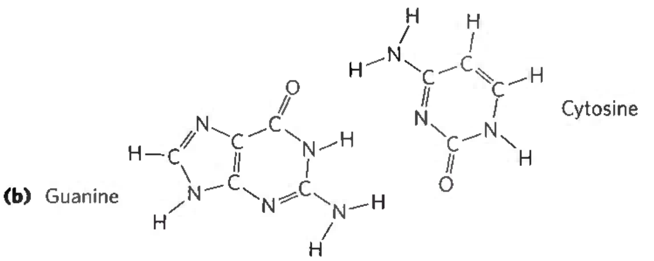
(a) Will the defense attorney's suggested remedy be acceptable to the plaintiff? Why or why not?

(b) What facts about pH does the defense attorney need to understand?

45. Phosphate-Buffered Saline pH and Osmolarity Phosphate-buffered saline (PBS) is a solution commonly used in studies of animal tissues and cells. Its composition is 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 ($pK_a = 2.14$), 1.8 mM KH_2PO_4 ($pK_a = 6.86$). Calculate the pH and osmolarity of PBS. Give the osmolarity in units of osmoles per liter (osm/L).

46. Hydrogen Bonding in Watson-Crick Base Pairs In 1953, James Watson and Francis Crick discovered that the purine base adenine forms a base pair with the pyrimidine base thymine (or uracil). Likewise, the purine base guanine forms a base pair with the pyrimidine base cytosine. These base pairs form due to hydrogen bonding between purines and pyrimidines. Show the hydrogen bonds that form **(a)** when adenine base-pairs with thymine and **(b)** when guanine base-pairs with cytosine.





DATA ANALYSIS PROBLEM

47. "Switchable" Surfactants Hydrophobic molecules do not dissolve well in water. This makes certain processes very difficult: washing oily food residue off dishes, cleaning up spilled oil, keeping the oil and water phases of salad dressings well mixed, and carrying out chemical reactions that involve both hydrophobic and hydrophilic components.

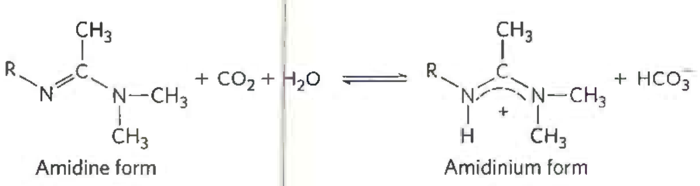
Surfactants are a class of amphipathic compounds that includes soaps, detergents, and emulsifiers. With the use of surfactants, hydrophobic compounds can be suspended in aqueous solution by forming micelles (see Fig. 2-7). A micelle has a hydrophobic core consisting of the hydrophobic compound and the hydrophobic "tails" of the surfactant; the hydrophilic "heads" of the surfactant cover the surface of the micelle. A suspension of micelles is called an emulsion. The more hydrophilic the head group of the surfactant, the more powerful it is—that is, the greater its capacity to emulsify hydrophobic material.

When you use soap to remove grease from dirty dishes, the soap forms an emulsion with the grease that is easily removed by water through interaction with the hydrophilic head of the soap molecules. Likewise, a detergent can be used to emulsify spilled oil for removal by water. And emulsifiers in commercial salad dressings keep the oil suspended evenly throughout the water-based mixture.

There are some situations, such as oil spill cleanups, in which it would be very useful to have a "switchable" surfactant: a molecule that could be reversibly converted between a surfactant and a nonsurfactant.

(a) Imagine that such a "switchable" surfactant existed. How would you use it to clean up and then recover the oil from an oil spill?

Liu and colleagues describe a prototypical switchable surfactant in their 2006 article "Switchable Surfactants." The switching is based on the following reaction:



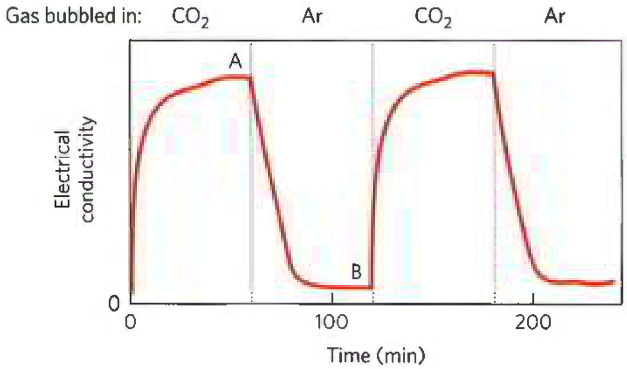
(b) Given that the pK_a of a typical amidinium ion is 12.4, in which direction (left or right) would you expect the equilibrium of the above reaction to lie? (See Fig. 2-15 for relevant pK_a values.) Justify your answer. Hint: Remember the reaction $H_2O + C_2O \rightleftharpoons H_2CO_3$.

Liu and colleagues produced a switchable surfactant for which $R = C_{16}H_{33}$. We will call the molecule s-surf.

(c) The amidinium form of s-surf is a powerful surfactant; the amidine form is not. Explain this observation.

Liu and colleagues found that they could switch between the two forms of s-surf by changing the gas that they bubbled through a solution of the surfactant. They demonstrated this switch by measuring the electrical conductivity of the s-surf solution; aqueous solutions of ionic compounds have higher conductivity than solutions of nonionic compounds. They started with a solution of the amidine form of s-surf in water.

Their results are shown below; dotted lines indicate the switch from one gas to another.



(d) In which form is the majority of s-surf at point A? At point B?

(e) Why does the electrical conductivity rise from time 0 to point A?

(f) Why does the electrical conductivity fall from point A to point B?

(g) Explain how you would use s-surf to clean up and recover the oil from an oil spill.

Reference

Liu, Y., P.G. Jessop, M. Cunningham, C.A. Eckert, and C.L. Liotta. 2006. Switchable surfactants. *Science* 313(5789):958–960. https://doi.org/10.1126/science.1128142.

Chapter 3

AMINO ACIDS, PEPTIDES, AND PROTEINS

3.1 Amino Acids *70*
3.2 Peptides and Proteins *80*
3.3 Working with Proteins *83*
3.4 The Structure of Proteins: Primary Structure *90*

Proteins mediate virtually every process that takes place in a cell, exhibiting an almost endless diversity of functions. To explore the molecular mechanism of a biological process, a biochemist almost inevitably studies one or more proteins. Proteins are the most abundant biological macromolecules, occurring in all cells and all parts of cells. Proteins also occur in great variety; thousands of different kinds may be found in a single cell. Proteins are the molecular instruments through which genetic information is expressed—the important final products of the information pathways discussed in Part III of this book.

Cells produce proteins with strikingly different properties and activities by joining a common set of 20 amino acids in many different combinations and sequences. From these building blocks, different organisms can make such widely diverse products as enzymes, hormones, antibodies, transporters, light-harvesting complexes in plants, the flagella of bacteria, muscle fibers, feathers, spider webs, rhinoceros horn, antibiotics, and myriad other substances that have distinct biological functions (**Fig. 3-1**). Among these protein products, the enzymes are the most varied and specialized. As the catalysts of almost all cellular reactions, enzymes are one of the keys to understanding the chemistry of life, and thus they provide a focal point for any course in biochemistry.

Protein structure and function are the topics of this and the next three chapters. Here, we begin with a description of the fundamental chemical properties of amino acids, peptides, and proteins. We also consider how a biochemist works with proteins. The material is organized around four principles:

P1 **In every living organism, proteins are constructed from a common set of 20 amino acids.** Each amino acid has a side chain with distinctive chemical properties. Amino acids may be regarded as the alphabet in which the language of protein structure is written.

P2 **In proteins, amino acids are joined in characteristic linear sequences through a common amide linkage, the peptide bond.** The amino acid sequence of a protein constitutes its primary structure, a first level we will introduce within the broader complexities of protein structure.

P3 **For study, individual proteins can be separated from the thousands of other proteins present in a cell, based on differences in their chemical and functional properties arising from their distinct amino acid sequences.** As proteins are central to biochemistry, the purification of individual proteins for study is a quintessential biochemical endeavor.

P4 **Shaped by evolution, amino acid sequences are a key resource for understanding the function of individual proteins and for tracing broader functional and evolutionary relationships.**

3.1 Amino Acids

Proteins are polymers of amino acids, with each **amino acid residue** joined to its neighbor by a specific type of covalent bond. (The term "residue" reflects the loss of the elements of water when one amino acid is joined to another.) Proteins can be broken down (hydrolyzed) to their constituent amino acids by a variety of methods, and the earliest studies of proteins naturally focused on the free amino acids derived from them. Twenty different amino acids are commonly found in proteins. The first to be discovered was asparagine, in 1806. The last of the 20 to be found, threonine, was not identified until 1938. All the amino acids have trivial or common names, in some cases derived from the source from which they were first isolated. Asparagine was first found in asparagus, and glutamate in wheat gluten; tyrosine was first isolated from cheese (its name is derived from the Greek *tyros*, "cheese"); glycine (Greek *glykos*, "sweet") was so named because of its sweet taste.

FIGURE 3-1 **Some functions of proteins.** (a) The light produced by fireflies is the result of a reaction involving the protein luciferin and ATP, catalyzed by the enzyme luciferase (see Box 13-1). (b) Erythrocytes contain large amounts of the oxygen-transporting protein hemoglobin. (c) The protein keratin, formed by all vertebrates, is the chief structural component of hair, scales, horn, wool, nails, and feathers. The black rhinoceros is extinct in the wild because of the belief prevalent in some parts of the world that a powder derived from its horn has aphrodisiac properties. In reality, the chemical properties of powdered rhinoceros horn are no different from those of powdered bovine hooves or human fingernails. [(a) Jeff J. Daly/Alamy. (b) Bill Longcore/Science Source. (c) Mary Cooke/Animals Animals.]

Learning the names, structures, and chemical properties of the 20 common amino acids found in proteins is one of the key memorization trials of every beginning biochemistry student. The necessity rapidly becomes apparent in succeeding chapters. It is impossible to discuss protein structure, protein function, ligand-binding sites, enzyme active sites, and most other biochemical topics without this foundation. The amino acids are part of the biochemistry vocabulary.

Amino Acids Share Common Structural Features

P1 All 20 of the common amino acids are α-amino acids. They have a carboxyl group and an amino group bonded to the same carbon atom (the α carbon) (**Fig. 3-2**). They differ from each other in their side chains, or **R groups**, which vary in structure, size, and electric charge, and which influence the solubility of the amino acids in water. In addition to these 20 amino acids, there are many less common ones. Some are residues modified after a protein has been synthesized, others are amino acids present in living organisms but not as constituents of proteins, and two are special cases found in just a few proteins. The common amino acids of proteins have been assigned three-letter abbreviations and one-letter symbols (see Table 3-1), which are used as shorthand to indicate the composition and sequence of amino acids polymerized in proteins.

KEY CONVENTION The three-letter code is easily understood, the abbreviations generally consisting of the first three letters of the amino acid name. The one-letter code was devised by Margaret Oakley Dayhoff, considered by many to be the founder of the field of bioinformatics. The one-letter code reflects an attempt to reduce the size of the data files (in an era of limited computer memory) used to describe amino acid sequences. It was designed to be easily memorized, and understanding its origin can help students do just that. For six amino acids (CHIMSV), the first letter of the amino acid name is unique and thus is used as the symbol. For five others (AGLPT), the first letter of the name is not unique but is assigned to the amino acid that is most common in proteins (for example, leucine is more common than lysine). For another four, the letter used is phonetically suggestive (RFYW: aRginine, Fenylalanine, tYrosine, tWiptophan). The rest were harder to assign. Four (DNEQ) were assigned letters found within or suggested by their names (asparDic, asparagiNe, glutamEke, Q-tamine). That left lysine. Only a few letters were left, and K was chosen because it was the closest to L. ■

Margaret Oakley Dayhoff, 1925–1983 [Photo by Dr. Ruth Dayhoff, courtesy Vincent Brannigan.]

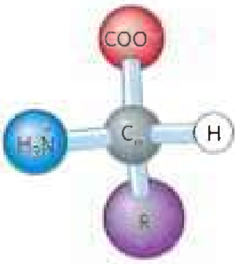


FIGURE 3-2 **General structure of an amino acid.** This structure is common to all but one of the α-amino acids. (Proline, a cyclic amino acid, is the exception.) The R group, or side chain (purple), attached to the α carbon (gray) is different in each amino acid.

For all the common amino acids except glycine, the α carbon is bonded to four different groups: a carboxyl group, an amino group, an R group, and a hydrogen atom (Fig. 3-2; in glycine, the R group is another hydrogen atom). The α-carbon atom is thus a **chiral center** (p. 61). Because of the tetrahedral arrangement of the bonding orbitals around the α-carbon atom, the four different groups can occupy two unique spatial arrangements, and thus amino acids have two possible stereoisomers. Since they are nonsuperposable mirror images of each other (**Fig. 3-3**), the two forms represent a class of stereoisomers called **enantiomers** (see Fig. 1-21). All molecules with a chiral center are also **optically active**—that is, they rotate the plane of plane-polarized light (see Box 1-2).

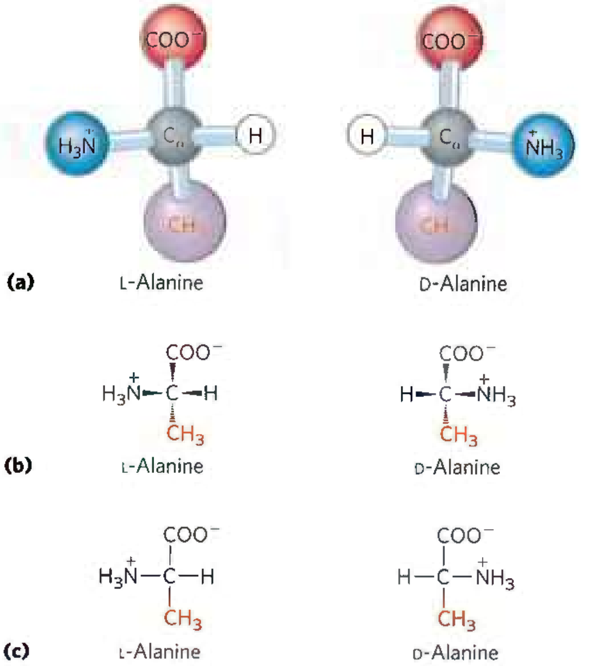
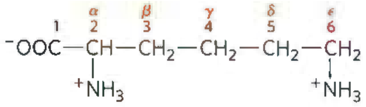


FIGURE 3-3 Stereoisomerism in α-amino acids. (a) The two stereoisomers of alanine, L- and D-alanine, are nonsuperposable mirror images of each other (enantiomers). (b, c) Two different conventions for showing the configurations in space of stereoisomers. In perspective formulas (b), the solid wedge-shaped bonds project out of the plane of the paper, the dashed bonds behind it. In projection formulas (c), the horizontal bonds are assumed to project out of the plane of the paper, the vertical bonds behind. However, projection formulas are often used casually and are not always intended to portray a specific stereochemical configuration. See Figure 3-4 for an explanation of the D, L-system for specifying absolute configuration.

KEY CONVENTION Two conventions are used to identify the carbons in an amino acid — a practice that can be confusing. The additional carbons in an R group are commonly designated $\beta, \gamma, \delta, \varepsilon$, and so forth, proceeding out from the α carbon. For most other organic molecules, carbon atoms are simply numbered from one end, giving highest priority (C-1) to the carbon with the substituent containing the atom of highest atomic number. Within this latter convention, the carboxyl carbon of an amino acid would be C-1 and the α carbon would be C-2.



$$\begin{array}{cccccc} & \alpha & \beta & \gamma & \delta & \varepsilon \\ 1 & 2 & 3 & 4 & 5 & 6 \\ {}^-\mathrm{OOC}- & \mathrm{CH}- & \mathrm{CH_2}- & \mathrm{CH_2}- & \mathrm{CH_2}- & \mathrm{CH_2} \\ & {}^+\mathrm{NH_3} & & & & {}^+\mathrm{NH_3} \end{array}$$

Lysine

In cases such as amino acids with heterocyclic R groups (e.g., histidine), where the Greek lettering system is ambiguous, the numbering system is used. For branched amino acid side chains, equivalent carbons are given numbers after the Greek letters. Leucine thus has $\delta 1$ and $\delta 2$ carbons (see the structure in Fig. 3-5). ■

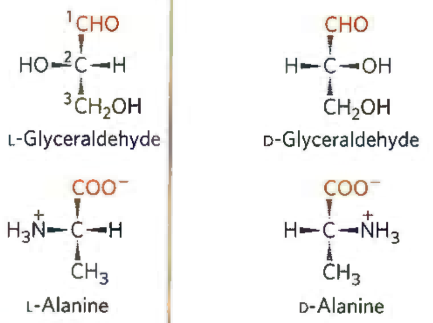


FIGURE 3-4 Steric relationship of the stereoisomers of alanine to the absolute configuration of L- and D-glyceraldehyde. In these perspective formulas, the carbons are lined up vertically, with the chiral atom in the center. The carbons in these molecules are numbered beginning with the terminal aldehyde or carboxyl carbon (red), 1 to 3 from top to bottom as shown. When presented in this way, the R group of the amino acid (in this case the methyl group of alanine) is always below the α carbon. L-Amino acids are those with the α-amino group on the left, and D-amino acids have the α-amino group on the right.

Special nomenclature has been developed to specify the **absolute configuration** of the four substituents of asymmetric carbon atoms. The absolute configurations of simple sugars and amino acids are specified by the **D, L system** (Fig. 3-4), based on the absolute configuration of the three-carbon sugar glyceraldehyde, a convention proposed by Emil Fischer in 1891. (Fischer knew what groups surrounded the asymmetric carbon of glyceraldehyde but had to guess at their absolute configuration; he guessed right, as was later confirmed by x-ray diffraction analysis.) For all chiral compounds, stereoisomers having a configuration related to that of L-glyceraldehyde are designated L, and stereoisomers related to D-glyceraldehyde are designated D. The functional groups of L-alanine are matched with those of L-glyceraldehyde by aligning those that can be interconverted by simple, one-step chemical reactions. Thus the carboxyl group of L-alanine occupies the same position about the chiral carbon as does the aldehyde group of L-glyceraldehyde, because an aldehyde is readily converted to a carboxyl group via a one-step oxidation. Historically, the similar *l* and *d* designations were used for levorotatory (rotating plane-polarized light to the left) and dextrorotatory (rotating light to the right). However, not all L-amino acids are levorotatory, and the convention shown in Figure 3-4 was needed to avoid potential ambiguities about absolute configuration. By Fischer's convention, L and D refer *only* to the absolute configuration of the four substituents around the chiral carbon, not to optical properties of the molecule.

Another system of specifying configuration around a chiral center is the **RS system**, which is used in the systematic nomenclature of organic chemistry and describes more precisely the configuration of molecules with more than one chiral center (p. 17).

The Amino Acid Residues in Proteins Are L Stereoisomers

Nearly all biological compounds with a chiral center occur naturally in only one stereoisomeric form, either D or L. The amino acid residues in protein molecules are almost all L stereoisomers, with less than 1% being found in the D-configuration. The rare D-amino acid residues generally have a precise structural purpose, and they are introduced to a protein by enzyme-catalyzed reactions that occur after the proteins are synthesized on a ribosome.

It is remarkable that virtually all amino acid residues in proteins are L stereoisomers. When chiral compounds are formed by ordinary chemical reactions, the result is a racemic mixture of D and L isomers, which are difficult for a chemist to distinguish and separate. But to a living system, D and L isomers are as different as the right hand and the left. The formation of stable, repeating substructures in proteins (Chapter 4) requires that their constituent amino acids be of one stereochemical series. Cells are able to specifically synthesize the L isomers of amino acids because the active sites of enzymes are asymmetric, causing the reactions they catalyze to be stereospecific.

Amino Acids Can Be Classified by R Group

Knowledge of the chemical properties of the common amino acids is central to an understanding of biochemistry. The topic can be simplified by grouping the amino acids into five main classes based on the properties of their R groups (**Table 3-1**), particularly their **polarity**, or tendency

TABLE 3-1 Properties and Conventions Associated with the Common Amino Acids Found in Proteins

			pK_a values							
Amino acid	Abbreviation/ symbol	M_r[a]	pK_1 (—COOH)	pK_2 (—NH_3^+)	pK_R (R group)	pI	Hydropathy index[b]	Occurrence in proteins (%)[c]		
Nonpolar, aliphatic R groups										
Glycine	Gly G	75	2.34	9.60		5.97	–0.4	7.2	7.3	7.3
Alanine	Ala A	89	2.34	9.69		6.01	1.8	7.8	9.4	7.2
Proline	Pro P	115	1.99	10.96		6.48	–1.6[d]	5.2	4.4	4.2
Valine	Val V	117	2.32	9.62		5.97	4.2	6.6	7.1	8.2
Leucine	Leu L	131	2.36	9.60		5.98	3.8	9.1	10.6	9.9
Isoleucine	Ile I	131	2.36	9.68		6.02	4.5	5.3	6.0	7.6
Methionine	Met M	149	2.28	9.21		5.74	1.9	2.3	2.2	2.2
Aromatic R groups										
Phenylalanine	Phe F	165	1.83	9.13		5.48	2.8	3.9	4.0	4.5
Tyrosine	Tyr Y	181	2.20	9.11	10.07	5.66	–1.3	3.2	3.0	3.9
Tryptophan	Trp W	204	2.38	9.39		5.89	–0.9	1.4	1.3	1.1
Polar, uncharged R groups										
Serine	Ser S	105	2.21	9.15		5.68	–0.8	6.8	6.1	5.7
Threonine	Thr T	119	2.11	9.62		5.87	–0.7	5.9	5.4	4.5
Cysteine[e]	Cys C	121	1.96	10.28	8.18	5.07	2.5	1.9	1.2	0.8
Asparagine	Asn N	132	2.02	8.80		5.41	–3.5	4.3	3.7	3.4
Glutamine	Gln Q	146	2.17	9.13		5.65	–3.5	4.2	4.5	2.0
Positively charged R groups										
Lysine	Lys K	146	2.18	8.95	10.53	9.74	–3.9	5.9	4.7	6.8
Histidine	His H	155	1.82	9.17	6.00	7.59	–3.2	2.3	2.4	1.6
Arginine	Arg R	174	2.17	9.04	12.48	10.76	–4.5	5.1	5.6	5.9
Negatively charged R groups										
Aspartate	Asp D	133	1.88	9.60	3.65	2.77	–3.5	5.3	5.1	5.0
Glutamate	Glu E	147	2.19	9.67	4.25	3.22	–3.5	6.3	6.0	8.2

[a] M_r values reflect the structures as shown in Figure 3-5. The elements of water (M_r 18) are deleted when the amino acid is incorporated into a polypeptide.

[b] A scale combining hydrophobicity and hydrophilicity of R groups. The values reflect the free energy (ΔG) of transfer of the amino acid side chain from a hydrophobic environment to water. This transfer is favorable ($\Delta G < 0$; negative value in the index) for charged or polar amino acid side chains, and it is unfavorable ($\Delta G > 0$; positive value in the index) for amino acids with nonpolar or more hydrophobic side chains. See Chapter 11. Source: Data from J. Kyte and R. F. Doolittle, *J. Mol. Biol.* 157:105, 1982.

[c] The first value in each row is the average occurrence in more than 1,150 proteins. Source: Data from R. F. Doolittle, in *Prediction of Protein Structure and the Principles of Protein Conformation* (G. D. Fasman, ed.), p. 599, Plenum Press, 1989. The second and third values are, respectively, from the complete proteomes of nine mesophilic bacterial species and seven thermophilic bacterial species. Mesophiles grow at commonly encountered temperatures, whereas thermophiles grow at elevated temperatures up to and beyond the boiling point of water. The decline in glutamine occurrence in thermophiles may reflect a tendency of this amino acid to deaminate at high temperatures. Source: Data from A. C. Singer and D. A. Hickey, *Gene* 317:39, 2003.

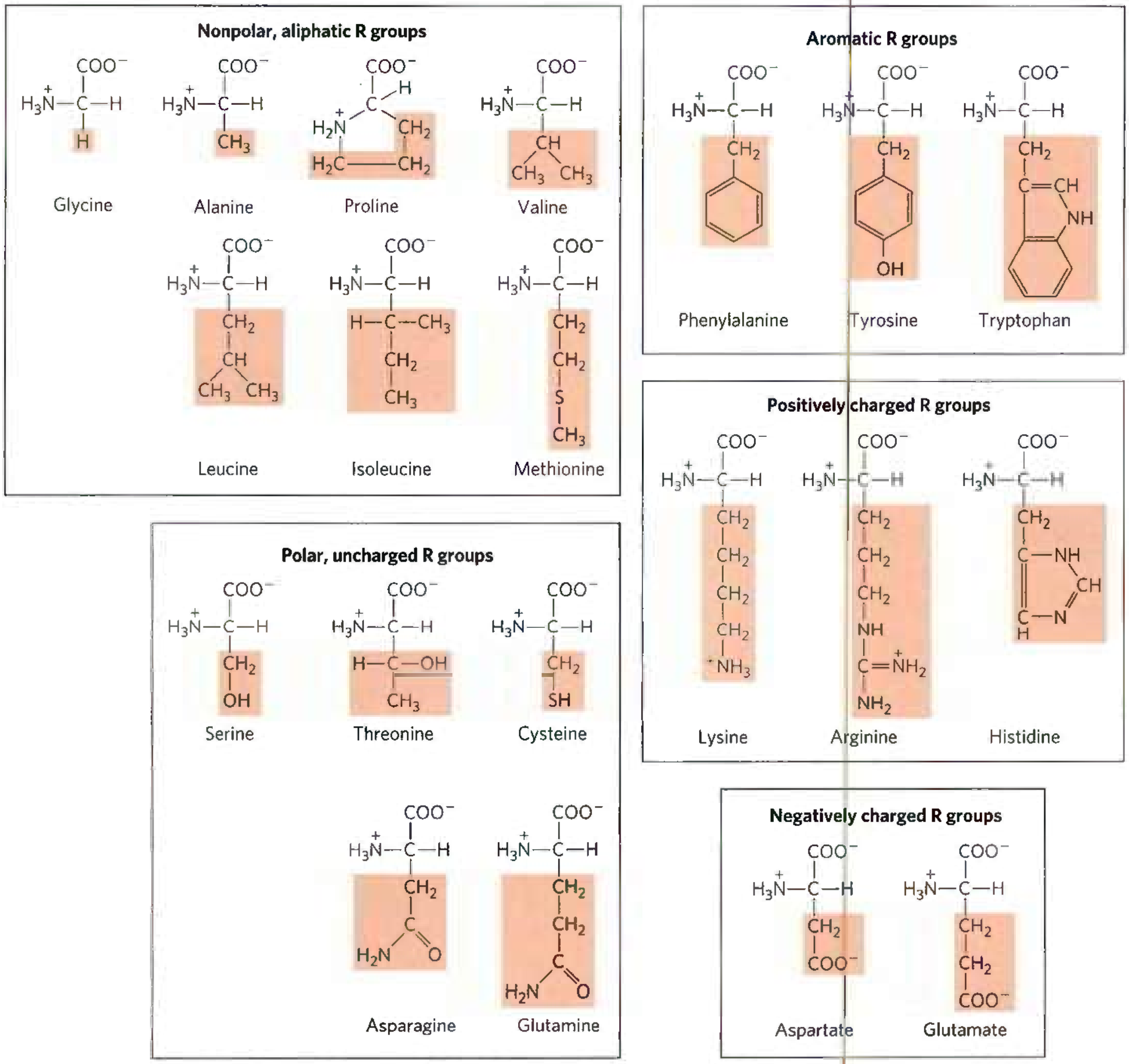
[d] As originally composed, the hydropathy index takes into account the frequency with which an amino acid residue appears on the surface of a protein. As proline often appears on the surface in β turns, it has a lower score than its chain of methylene groups would suggest.

[e] Cysteine is generally classified as polar, despite having a positive hydropathy index. This reflects the ability of the sulfhydryl group to act as a weak acid and to form a weak hydrogen bond with oxygen or nitrogen.

to interact with water at biological pH (near pH 7.0). The polarity of the R groups varies widely, from nonpolar and hydrophobic (water-insoluble) to highly polar and hydrophilic (water-soluble). A few amino acids—especially glycine, histidine, and cysteine—are somewhat difficult to characterize or do not fit perfectly in any one group. They are assigned to particular groupings based on considered judgments rather than absolutes.

P1 The structures of the 20 common amino acids are shown in **Figure 3-5**, and some of their properties are listed in Table 3-1. Within each class there are gradations of polarity, size, and shape of the R groups.

Nonpolar, Aliphatic R Groups The R groups in this class of amino acids are nonpolar and hydrophobic. The side chains of **alanine**, **valine**, **leucine**, and **isoleucine** tend to cluster together within proteins, stabilizing protein structure through the hydrophobic effect. **Glycine** has the simplest structure. Although it is most easily grouped with the nonpolar amino acids, its very small side chain makes no real contribution to interactions driven by the hydrophobic effect. **Methionine**, one of the two sulfur-containing amino acids, has a slightly nonpolar thioether group in its side chain. **Proline** has an aliphatic side chain with a distinctive cyclic structure.


Nonpolar, aliphatic R groups

Aromatic R groups

Positively charged R groups

Polar, uncharged R groups

Negatively charged R groups

FIGURE 3-5 The 20 common amino acids of proteins. The structural formulas show the state of ionization that would predominate at pH 7.0. The unshaded portions are those that are common to all the amino acids; the shaded portions are the R groups. Although the R group of histidine is shown uncharged, its pK_a (see Table 3-1) is such that a small but significant fraction of these groups are positively charged at pH 7.0. The protonated form of histidine is shown above the graph in Figure 3-12b.

BOX 3-1 METHODS

Absorption of Light by Molecules: The Lambert-Beer Law

A wide range of biomolecules absorb light at characteristic wavelengths, just as tryptophan absorbs light at 280 nm (see Fig. 3-6). Measurement of light absorption by a spectrophotometer is used to detect and identify molecules and to measure their concentration in solution. The fraction of the incident light absorbed by a solution at a given wavelength is related to the thickness of the absorbing layer (path length) and the concentration of the absorbing species (Fig. 1). These two relationships are combined into the Lambert-Beer law,

$$\log \frac{I_0}{I} = \varepsilon c l$$

where I_0 is the intensity of the incident light, I is the intensity of the transmitted light, the ratio I/I_0 (the inverse of the ratio in the equation) is the transmittance, ε is the molar extinction coefficient (in units of liters per mole-centimeter), c is the concentration of the absorbing species (in moles per liter), and l is the path length of the light-absorbing sample (in centimeters). The Lambert-Beer law assumes that the incident light is parallel and monochromatic (of a single wavelength) and that the solvent and solute molecules are randomly oriented. The expression $\log (I_0/I)$ is called the **absorbance**, designated A.

It is important to note that each successive millimeter of path length of absorbing solution in a 1.0 cm cell absorbs not a constant amount but a constant fraction of the light that is incident upon it. However, with an absorbing layer of fixed path length, *the absorbance,* A, *is directly proportional to the concentration of the absorbing solute.*

The molar extinction coefficient varies with the nature of the absorbing compound, the solvent, and the wavelength, and also with pH if the light-absorbing species is in equilibrium with an ionization state that has different absorbance properties.

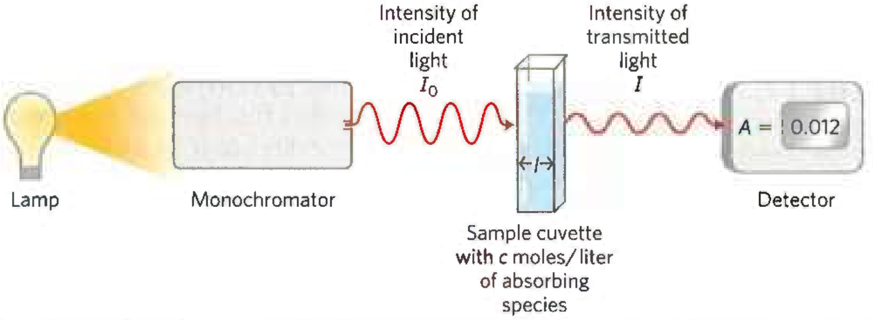

Intensity of incident light I_0
Intensity of transmitted light I
A = 0.012
Lamp
Monochromator
l
Detector
Sample cuvette with c moles/liter of absorbing species

FIGURE 1 The principal components of a spectrophotometer. A light source emits light along a broad spectrum, then the monochromator selects and transmits light of a particular wavelength. The monochromatic light passes through the sample in a cuvette of path length l. The absorbance of the sample, $\log (I_0/I)$, is proportional to the concentration of the absorbing species. The transmitted light is measured by a detector.

The secondary amino (imino) group of proline residues is held in a rigid conformation that reduces the structural flexibility of polypeptide regions containing proline.

Aromatic R Groups **Phenylalanine**, **tyrosine**, and **tryptophan**, with their aromatic side chains, are relatively nonpolar (hydrophobic). All can contribute to the hydrophobic effect. The hydroxyl group of tyrosine can form hydrogen bonds, and it is an important functional group in some enzymes. Tyrosine and tryptophan are significantly more polar than phenylalanine because of the tyrosine hydroxyl group and the nitrogen of the tryptophan indole ring.

Tryptophan and tyrosine, and to a much lesser extent phenylalanine, absorb ultraviolet light (**Fig. 3-6**; see also **Box 3-1**). This accounts for the characteristic strong absorbance of light by most proteins at a

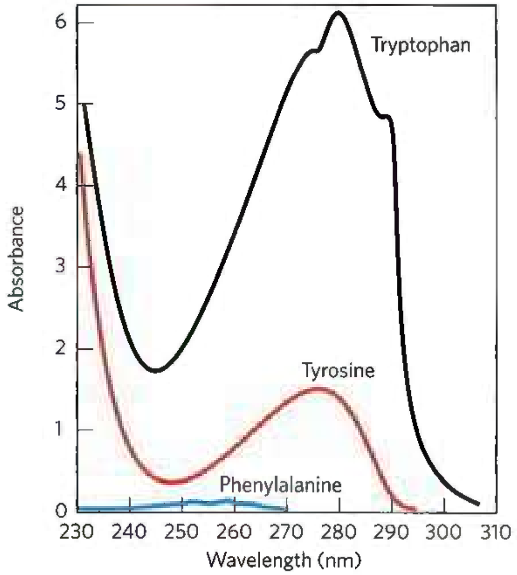


FIGURE 3-6 Absorption of ultraviolet light by aromatic amino acids. Comparison of the light absorption spectra of the aromatic amino acids tryptophan, tyrosine, and phenylalanine at pH 6.0. The amino acids are present in equimolar amounts (10^{-3} M) under identical conditions. The measured absorbance of tryptophan is more than four times that of tyrosine at a wavelength of 280 nm. Note that the maximum light absorption for both tryptophan and tyrosine occurs near 280 nm. Light absorption by phenylalanine generally contributes little to the spectroscopic properties of proteins.

wavelength of 280 nm, a property exploited by researchers in the characterization of proteins.

Polar, Uncharged R Groups The R groups of these amino acids are more soluble in water, or more hydrophilic, than those of the nonpolar amino acids because they contain functional groups that form hydrogen bonds with water. This class of amino acids includes **serine**, **threonine**, **cysteine**, **asparagine**, and **glutamine**. The polarity of serine and threonine is contributed by their hydroxyl groups, and the polarity of asparagine and glutamine is contributed by their amide groups. Cysteine is an outlier here because its polarity, contributed by its sulfhydryl group, is quite modest. Cysteine is a weak acid and can make weak hydrogen bonds with oxygen or nitrogen.

Asparagine and glutamine are the amides of two other amino acids also found in proteins—aspartate and glutamate, respectively—to which asparagine and glutamine are easily hydrolyzed by acid or base. Cysteine is readily oxidized to form a covalently linked dimeric amino acid called **cystine**, in which two cysteine molecules or residues are joined by a disulfide bond (**Fig. 3-7**). The disulfide-linked residues are strongly hydrophobic (nonpolar). Disulfide bonds play a special role in the structures of many proteins by forming covalent links between parts of a polypeptide molecule or between two different polypeptide chains.

Positively Charged (Basic) R Groups The most hydrophilic R groups are those that are either positively charged or negatively charged. The amino acids in which the R groups have significant positive charge at pH 7.0 are **lysine**, which has a second primary amino group at the ε position on its aliphatic chain; **arginine**, which has a positively charged guanidinium group; and **histidine**, which has an aromatic imidazole group. As the only common amino acid having an ionizable side chain with pK_a near neutrality, histidine may be positively charged (protonated form) or uncharged at pH 7.0. His residues facilitate many enzyme-catalyzed reactions by serving as proton donors/acceptors.

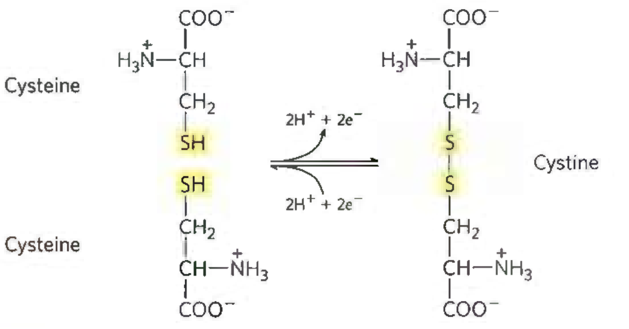


FIGURE 3-7 Reversible formation of a disulfide bond by the oxidation of two molecules of cysteine. Disulfide bonds between Cys residues stabilize the structures of many proteins.

Negatively Charged (Acidic) R Groups The two amino acids having R groups with a net negative charge at pH 7.0 are **aspartate** and **glutamate**, each of which has a second carboxyl group.

Uncommon Amino Acids Also Have Important Functions

In addition to the 20 common amino acids, proteins may contain residues created by modification of common residues already incorporated into a polypeptide—that is, through postsynthetic modification (**Fig. 3-8a**). Among these uncommon amino acids are **4-hydroxyproline**, a derivative of proline found in the fibrous protein collagen, and γ-**carboxyglutamate**, found in the blood-clotting protein prothrombin and in certain other proteins that bind Ca^{2+} as part of their biological function. More complex is **desmosine**, a derivative of four Lys residues, which is found in the fibrous protein elastin.

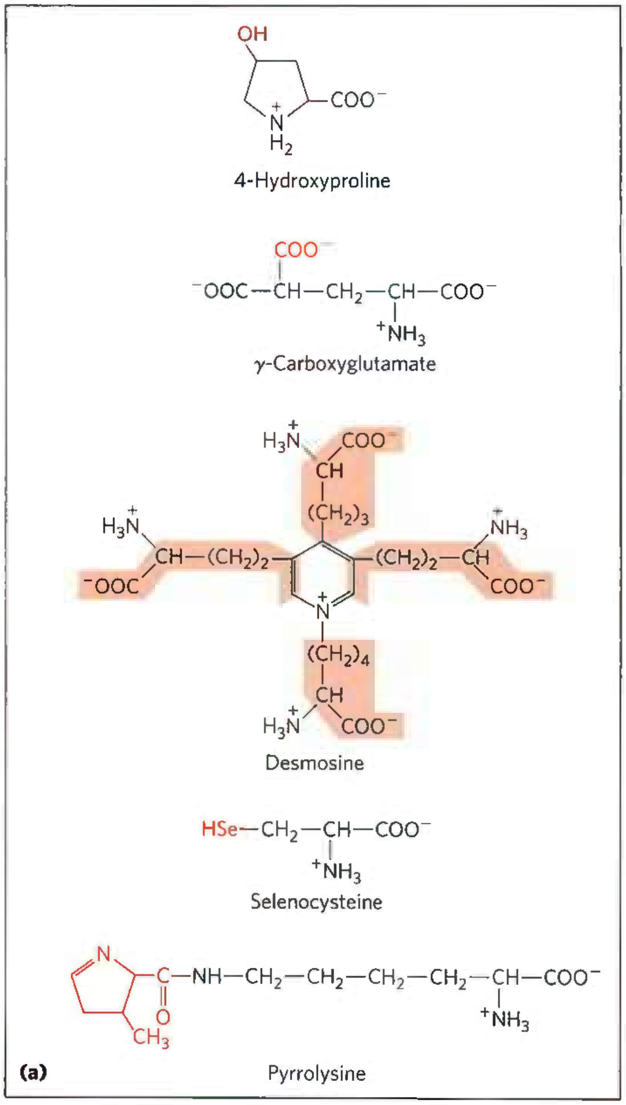
Selenocysteine and **pyrrolysine** are special cases. These rare amino acid residues are not created through a postsynthetic modification. Instead, they are introduced during protein synthesis through an unusual adaptation of the genetic code, which we describe in Chapter 27. Selenocysteine contains selenium rather than the sulfur of cysteine. Actually derived from serine, selenocysteine is a constituent of just a few known proteins. Pyrrolysine is found in a few proteins in several methanogenic (methane-producing) archaea and in one known bacterium; it plays a role in methane biosynthesis.

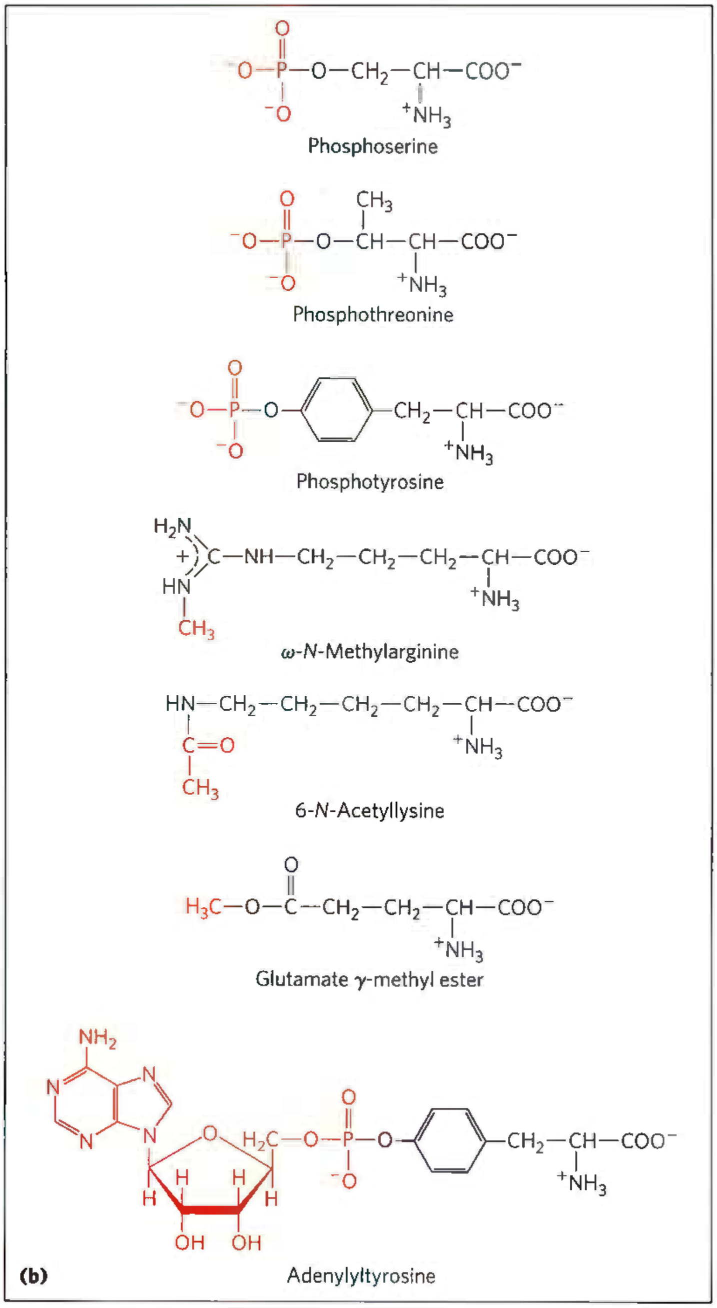
Some amino acid residues in a protein may be modified transiently to alter the protein's function. The addition of phosphoryl, methyl, acetyl, adenylyl, ADP-ribosyl, or other groups to particular amino acid residues can increase or decrease a protein's activity (Fig. 3-8b). Phosphorylation is a particularly common regulatory modification. Covalent modification as a protein regulatory strategy is discussed in more detail in Chapter 6.

Some 300 additional amino acids have been found in cells. They have a variety of functions, but not all are constituents of proteins. **Ornithine** and **citrulline** (Fig. 3-8c) deserve special note because they are key intermediates (metabolites) in the biosynthesis of arginine (Chapter 22) and in the urea cycle (Chapter 18).

Amino Acids Can Act as Acids and Bases

The amino and carboxyl groups of amino acids, along with the ionizable R groups of some amino acids, function as weak acids and bases. When an amino acid lacking an ionizable R group is dissolved in water at neutral pH, the α-amino and carboxyl groups create a


4-Hydroxyproline
γ-Carboxyglutamate
Desmosine
Selenocysteine
Pyrrolysine
(a)


Phosphoserine
Phosphothreonine
Phosphotyrosine
ω-N-Methylarginine
6-N-Acetyllysine
Glutamate γ-methyl ester
Adenylyltyrosine
(b)

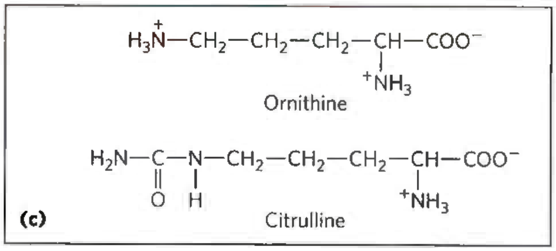
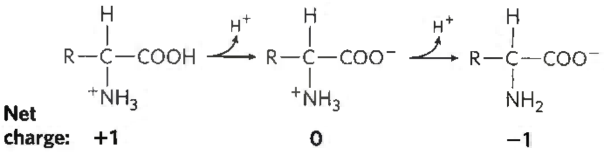

Ornithine
Citrulline
(c)

FIGURE 3-8 Uncommon amino acids. (a) Some uncommon amino acids found in proteins. Most are derived from common amino acids. (Note the use of either numbers or Greek letters in the names of these structures to identify the altered carbon atoms.) Extra functional groups added by modification reactions are shown in red. Desmosine is formed from four Lys residues (the carbon backbones are shaded in light red). Selenocysteine and pyrrolysine are exceptions: these amino acids are added during normal protein synthesis through a highly specialized expansion of the standard genetic code. Both are found in very small numbers of proteins. (b) Reversible amino acid modifications involved in regulation of protein activity. Phosphorylation is the most common type of regulatory modification. (c) Ornithine and citrulline, which are not found in proteins, are intermediates in the biosynthesis of arginine and in the urea cycle.

dipolar ion, or **zwitterion** (German for "hybrid ion"), which can act as either an acid or a base (**Fig. 3-9**). Substances having this dual (acid-base) nature are **amphoteric** and are often called **ampholytes** (from "amphoteric electrolytes"). A simple monoamino monocarboxylic α-amino acid, such as alanine, is a diprotic acid when fully protonated; it has two groups, the —COOH group and the —NH_3^+ group, that can yield protons:



$$\underset{+1}{R\text{—}\overset{H}{\underset{^+NH_3}{C}}\text{—}COOH} \xrightarrow{H^+} \underset{0}{R\text{—}\overset{H}{\underset{^+NH_3}{C}}\text{—}COO^-} \xrightarrow{H^+} \underset{-1}{R\text{—}\overset{H}{\underset{NH_2}{C}}\text{—}COO^-}$$

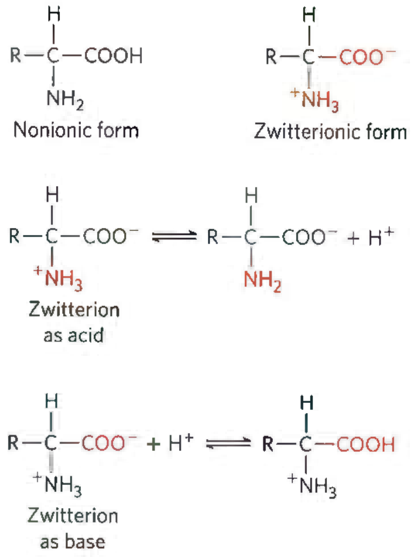


FIGURE 3-9 Nonionic and zwitterionic forms of amino acids. The nonionic form does not occur in significant amounts in aqueous solutions. The zwitterion predominates at neutral pH. A zwitterion can act as either an acid (proton donor) or a base (proton acceptor).

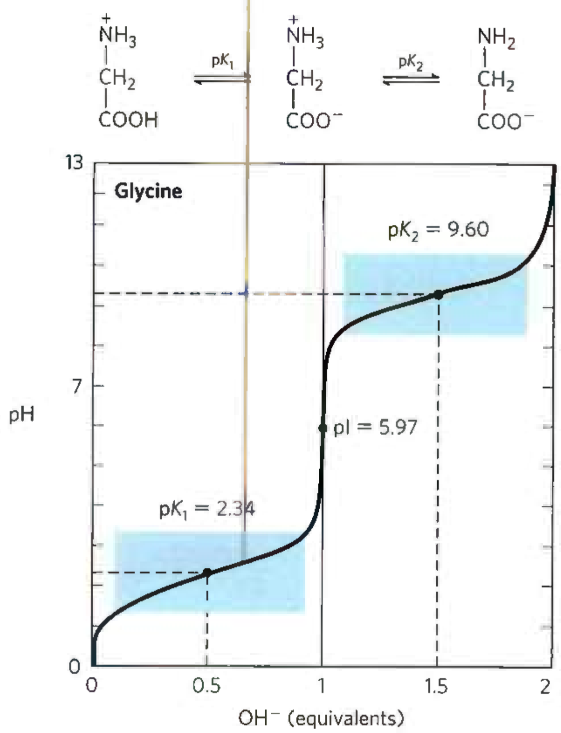


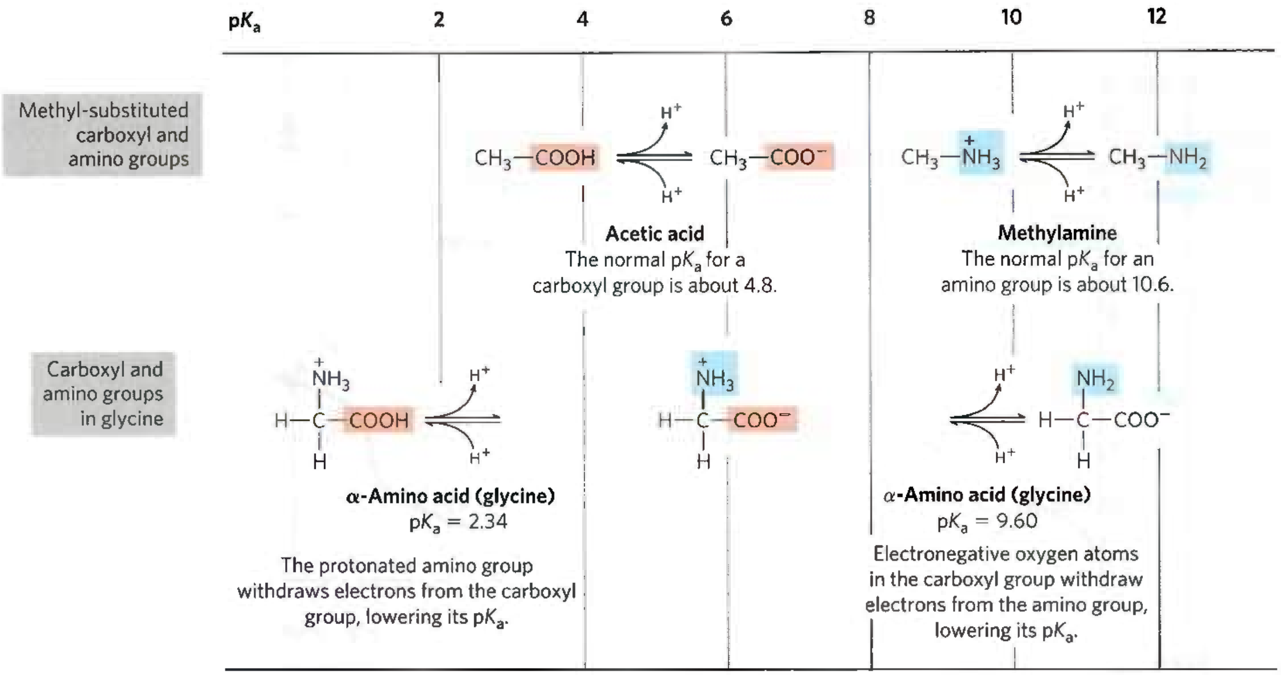
FIGURE 3-10 Titration of amino acids. The titration curve of 0.1 M glycine at 25 °C. The ionic species predominating at key points in the titration are shown above the graph. The shaded boxes, centered at about $pK_1 = 2.3$ and $pK_2 = 9.60$, indicate the regions of greatest buffering power. Note that 1 equivalent of $OH^- = 0.1$ M NaOH added. The pI occurs at the arithmetic mean between the two pK_a values, and it corresponds to the inflection point in the titration.

Acid-base titration involves the gradual addition or removal of protons (Chapter 2). **Figure 3-10** shows the titration curve of the diprotic form of glycine. The two ionizable groups of glycine, the carboxyl group and the amino group, are titrated with a strong base such as NaOH. The plot has two distinct stages, corresponding to deprotonation of two different groups on glycine. Each of the two stages resembles in shape the titration curve of a monoprotic acid, such as acetic acid (see Fig. 2-16), and can be analyzed in the same way. At very low pH, the predominant ionic species of glycine is the fully protonated form, $^+H_3N—CH_2—COOH$.

In the first stage of the titration, the —COOH group of glycine (with its lower pK_a) loses its proton. At the midpoint of this stage, equimolar concentrations of the proton-donor ($^+H_3N—CH_2—COOH$) and the proton-acceptor ($^+H_3N—CH_2—COO^-$) species are present. As in the titration of any weak acid, a point of inflection is reached at this midpoint where the pH is equal to the pK_a of the protonated group that is being titrated (see Fig. 2-17). For glycine, the pH at the midpoint is 2.34; thus its —COOH group has a pK_a (labeled pK_1 in Fig. 3-10) of 2.34. (Recall from Chapter 2 that pH and pK_a are simply convenient notations for proton concentration and the equilibrium constant for ionization, respectively. The pK_a is a measure of the tendency of a group to give up a proton, with that tendency decreasing 10-fold as the pK_a increases by one unit.) As the titration of glycine proceeds, another point of inflection is reached at pH 5.97; at this point, removal of the first proton is essentially complete and removal of the second has just begun. At this pH, glycine is present largely as the dipolar ion (zwitterion) $^+H_3N—CH_2—COO^-$. We shall return to the significance of this inflection point in the titration curve (labeled pI in Fig. 3-10) shortly.

The second stage of the titration corresponds to the removal of a proton from the $—NH_3^+$ group of glycine. The pH at the midpoint of this stage is 9.60, equal to the pK_a (labeled pK_2 in Fig. 3-10) for the $—NH_3^+$ group. The titration is essentially complete at a pH of about 12, at which point the predominant form of glycine is $H_2N—CH_2—COO^-$.

From the titration curve of glycine we can derive several important pieces of information. First, it gives a quantitative measure of the pK_a of each of the two ionizing groups: 2.34 for the —COOH group and 9.60 for the $—NH_3^+$ group. Note that the carboxyl group of glycine is over 100 times more acidic (more easily ionized) than the carboxyl group of acetic acid, which, as we saw in Chapter 2, has a pK_a of 4.76—about average for a carboxyl group attached to an otherwise unsubstituted aliphatic hydrocarbon. The perturbed pK_a of glycine is caused primarily by the nearby positively charged amino group on the α-carbon atom, an electronegative group that tends to pull electrons toward it (a process called electron withdrawal), as described in **Figure 3-11**. The opposite charges on the resulting zwitterion are also somewhat stabilizing. Similarly, the pK_a of the amino group in glycine is perturbed downward relative to the average pK_a of an amino group. This effect is due largely



pKa
2
4
6
8
10
12
Methyl-substituted carboxyl and amino groups
CH3—COOH
H+
CH3—COO−
Acetic acid
The normal pKa for a carboxyl group is about 4.8.
CH3—NH3
CH3—NH2
Methylamine
The normal pKa for an amino group is about 10.6.
Carboxyl and amino groups in glycine
α-Amino acid (glycine)
pKa = 2.34
The protonated amino group withdraws electrons from the carboxyl group, lowering its pKa.
α-Amino acid (glycine)
pKa = 9.60
Electronegative oxygen atoms in the carboxyl group withdraw electrons from the amino group, lowering its pKa.

FIGURE 3-11 Effect of the chemical environment on pK_a. The pK_a values for the ionizable groups in glycine are lower than those for simple, methyl-substituted amino and carboxyl groups. These downward perturbations of pK_a are due to intramolecular interactions. Similar effects can be caused by chemical groups that happen to be positioned nearby—for example, in the active site of an enzyme.

to electron withdrawal by the electronegative oxygen atoms in the carboxyl groups, increasing the tendency of the amino group to give up a proton. Hence, the α-amino group has a pK_a that is lower than that of an aliphatic amine such as methylamine (Fig. 3-11). In short, the pK_a of any functional group is greatly affected by its chemical environment, a phenomenon sometimes exploited in the active sites of enzymes to promote exquisitely adapted reaction mechanisms that depend on the perturbed pK_a values of proton donor/acceptor groups of specific residues.

The second piece of information provided by the titration curve of glycine is that this amino acid has two regions of buffering power. One of these is the relatively flat portion of the curve, extending for approximately 1 pH unit on either side of the first pK_a of 2.34, indicating that glycine is a good buffer near this pH. The other buffering zone is centered around pH 9.60. (Note that glycine is not a good buffer at the pH of intracellular fluid or blood, about 7.4.) Within the buffering ranges of glycine, the Henderson-Hasselbalch equation (p. 60) can be used to calculate the proportions of proton-donor and proton-acceptor species of glycine required to make a buffer at a given pH.

A final important piece of information derived from the titration curve of an amino acid is the relationship between its net charge and the pH of the solution. At pH 5.97, the point of inflection between the two stages in its titration curve, glycine is present predominantly as its dipolar form, fully ionized but with no *net* electric charge (Fig. 3-10). The characteristic pH at which the *net* electric charge is zero is called the **isoelectric point** or **isoelectric pH**, designated **pI**. For glycine, which has no ionizable group in its side chain, the isoelectric point is simply the arithmetic mean of the two pK_a values:

$$\text{pI} = \frac{1}{2}(\text{p}K_1 + \text{p}K_2) = \frac{1}{2}(2.34 + 9.60) = 5.97$$

As is evident in Figure 3-10, glycine has a net negative charge at any pH above its pI and thus will move toward the positive electrode (the anode) when placed in an electric field. At any pH below its pI, glycine has a net positive charge and will move toward the negative electrode (the cathode). The farther the pH of a glycine solution is from its isoelectric point, the greater the net electric charge of the population of glycine molecules. At pH 1.0, for example, glycine exists almost entirely as the form $^+H_3N—CH_2—COOH$ with a net positive charge of 1.0. At pH 2.34, where there is an equal mixture of $^+H_3N—CH_2—COOH$ and $^+H_3N—CH_2—COO^-$, the average or net positive charge is 0.5. The sign and the magnitude of the net charge of any amino acid at any pH can be predicted in the same way.

Amino Acids Differ in Their Acid-Base Properties

The shared properties of many amino acids permit some simplifying generalizations about their acid-base behaviors. First, all amino acids with a single α-amino group, a single α-carboxyl group, and an R group that does not ionize have titration curves resembling that of glycine (Fig. 3-10). These amino acids have very similar, although not identical, pK_a values: pK_a of the —COOH group in the range of 1.8 to 2.4, and pK_a of the $—NH_3^+$ group in the range of 8.8 to 11.0 (Table 3-1). The differences in these

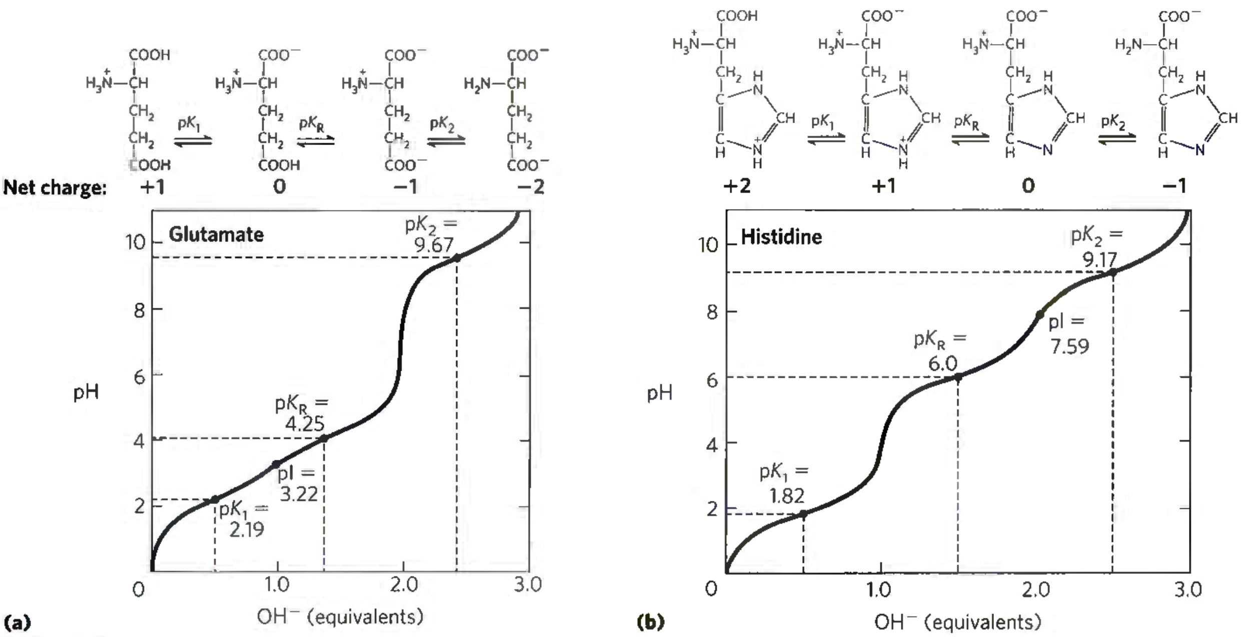


FIGURE 3-12 Titration curves for (a) glutamate and (b) histidine. The pK_a of the R group is designated here as pK_R. In both cases, the presence of three ionizable groups renders the titration curve more complex. Note that for glutamate, the pI is approximately the arithmetic mean of the pK_a of the two groups that are negatively charged. There is a net charge of 0 (the pI) when these two groups contribute a net charge of −1 (one protonated, the other not) to exactly balance the +1 charge of the protonated α-amino group. Similarly, the pI for histidine is approximately the arithmetic mean of the pK_a of the two groups that are positively charged when protonated.

pK_a values reflect the chemical environments imposed by their R groups.

Second, amino acids with an ionizable R group have more complex titration curves, with *three* stages corresponding to the three possible ionization steps; thus, they have three pK_a values. The additional stage for the titration of the ionizable R group merges to some extent with that for the titration of the α-carboxyl group, the titration of the α-amino group, or both. The titration curves for two amino acids of this type, glutamate and histidine, are shown in **Figure 3-12**. The isoelectric points reflect the nature of the ionizing R groups that are present. For example, glutamate has a pI of 3.22, considerably lower than that of glycine. This is due to the presence of two carboxyl groups, which, at the average of their pK_a values (3.22), contribute a net charge of −1 that balances the +1 contributed by the amino group. Similarly, the pI of histidine, with two groups that are positively charged when protonated, is 7.59 (the average of the pK_a values of the amino and imidazole groups), much higher than that of glycine.

Finally, in an aqueous environment, only histidine has an R group ($pK_a = 6.0$) providing significant buffering power near the neutral pH usually found in the intracellular and extracellular fluids of most animals and bacteria (Table 3-1).

SUMMARY 3.1 *Amino Acids*

- The 20 amino acids commonly found as residues in proteins contain an α-carboxyl group, an α-amino group, and a distinctive R group substituted on the α-carbon atom. The α-carbon atom of all amino acids except glycine is asymmetric, and thus amino acids can exist in at least two stereoisomeric forms.
- Only the L stereoisomers of amino acids, with a configuration related to the absolute configuration of the reference molecule L-glyceraldehyde, are found in proteins.
- Amino acids can be classified into five types on the basis of the polarity and charge (at pH 7) of their R groups.
- Other, less common amino acids also occur, either as constituents of proteins (usually through modification of common amino acid residues after protein synthesis) or as free metabolites.
- Amino acids vary in their acid-base properties and have characteristic titration curves. Monoamino monocarboxylic amino acids (with nonionizable R groups) are diprotic acids ($^+H_3NCH(R)COOH$) at low pH and exist in several different ionic forms as the pH is increased.
- Amino acids with ionizable R groups have additional ionic species, depending on the pH of the medium and the pK_a of the R group.

3.2 Peptides and Proteins

We now turn to polymers of amino acids, the **peptides** and **proteins**. Biologically occurring polypeptides range in size from small, consisting of two or three linked amino acid residues, to very large, consisting of thousands of residues.

Peptides Are Chains of Amino Acids

P2 Two amino acid molecules can be covalently joined through a substituted amide linkage, termed a **peptide bond**, to yield a dipeptide. Such a linkage is formed by removal of the elements of water (dehydration) — a hydroxyl moiety from the α-carboxyl group of one amino acid and a hydrogen atom the α-amino group of another (**Fig. 3-13**). The joined amino acids are referred to as residues, the part left over after the elements of water are removed. Peptide bond formation is an example of a **condensation** reaction, a common class of reactions in living cells. The reverse reaction, bond breakage involving water, is an example of hydrolytic cleavage or **hydrolysis**. Under standard biochemical conditions, the equilibrium for the reaction shown in Figure 3-13 favors the hydrolysis of the dipeptide into amino acids. To make the condensation reaction thermodynamically more favorable, the carboxyl group must be chemically modified or activated so that the hydroxyl group can be more readily eliminated. A chemical approach to this problem is outlined later in this chapter. The biological approach to peptide bond formation is a major topic of Chapter 27.

Three amino acids can be joined by two peptide bonds to form a tripeptide; similarly, four amino acids can be linked to form a tetrapeptide, five to form a pentapeptide, and so forth. When a few amino acids are joined in this fashion, the structure is called an **oligopeptide**. When many amino acids are joined, the product is called a **polypeptide**. Proteins may have thousands of amino acid residues. Although the terms "protein" and "polypeptide" are sometimes used interchangeably, molecules referred to as polypeptides generally have molecular weights below 10,000, and those called proteins have higher molecular weights.

Figure 3-14 shows the structure of a pentapeptide. In a peptide, the amino acid residue at the end with a free α-amino group is the **amino-terminal** (or *N*-terminal) **residue**; the residue at the other end, which has a free carboxyl group, is the **carboxyl-terminal** (*C*-terminal) **residue**.

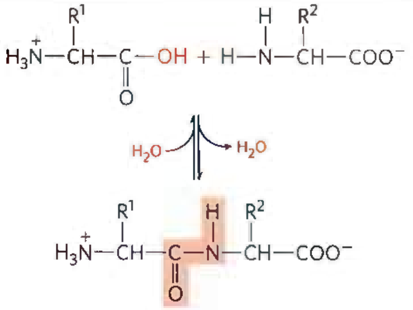


FIGURE 3-13 Formation of a peptide bond by condensation. The α-amino group of one amino acid (with the R^2 group) acts as a nucleophile to displace the hydroxyl group of another amino acid (with the R^1 group), forming a peptide bond (shaded in light red). Amino groups are good nucleophiles, but the hydroxyl group is a poor leaving group and is not readily displaced. At physiological pH, the reaction shown here does not occur to any appreciable extent.

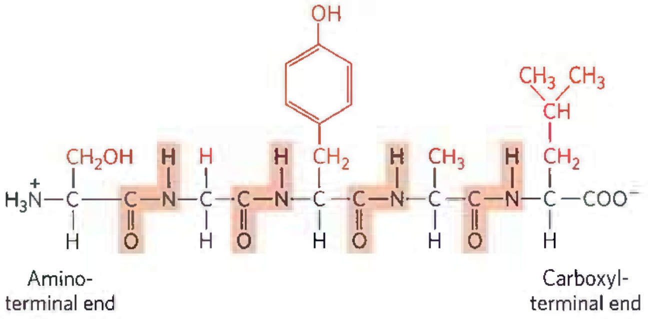


FIGURE 3-14 The pentapeptide serylglycyltyrosylalanylleucine, Ser–Gly–Tyr–Ala–Leu, or SGYAL. Peptides are named beginning with the amino-terminal residue, which by convention is placed at the left. The peptide bonds are shaded in light red; the R groups are in red.

KEY CONVENTION When an amino acid sequence of a peptide, a polypeptide, or a protein is displayed, the amino-terminal end is placed on the left and the carboxyl-terminal end is placed on the right. The sequence is read from left to right, beginning with the amino-terminal end. ■

Although hydrolysis of a peptide bond is an exergonic reaction, it occurs only slowly because it has a high activation energy (p. 25). As a result, the peptide bonds in proteins are quite stable, with an average half-life ($t_{1/2}$) of about 7 years under most intracellular conditions.

Peptides Can Be Distinguished by Their Ionization Behavior

P2 Peptides contain only one free α-amino group and one free α-carboxyl group, at opposite ends of the chain (**Fig. 3-15**). These groups ionize as they do in free

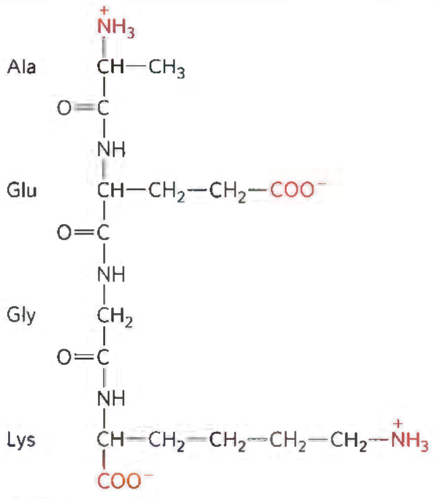


FIGURE 3-15 Alanylglutamylglycyllysine. This tetrapeptide has one free α-amino group, one free α-carboxyl group, and two ionizable R groups. The groups ionized at pH 7.0 are in red.

amino acids. The α-amino and α-carboxyl groups of all nonterminal amino acids are covalently joined in the peptide bonds. They can no longer ionize and thus do not contribute to the total acid-base behavior of peptides. Ionizable R groups in a peptide (Table 3-1) also contribute to the overall acid-base properties of the molecule (Fig. 3-15).

Like free amino acids, peptides have characteristic titration curves and a characteristic isoelectric pH (pI) at which the net charge is zero and they do not move in an electric field. These properties are exploited in some of the techniques used to separate peptides and proteins, as we describe later in the chapter. When an amino acid becomes a residue in a peptide, its chemical environment is altered, and the pK_a value for an ionizable R group can change somewhat. The pK_a values for

TABLE 3-2 Molecular Data on Some Proteins

Protein	Molecular weight	Number of residues	Number of polypeptide chains
Cytochrome *c* (human)	12,400	104	1
Myoglobin (equine heart)	16,700	153	1
Chymotrypsin (bovine pancreas)	25,200	241	3
Hemoglobin (human)	64,500	574	4
Hexokinase (yeast)	107,900	972	2
RNA polymerase (*E. coli*)	450,000	4,158	5
Glutamine synthetase (*E. coli*)	619,000	5,628	12
Titin (human)	2,993,000	26,926	1

TABLE 3-3 Amino Acid Composition of Two Proteins

	Bovine cytochrome *c*		Bovine chymotrypsinogen	
Amino acid	Number of residues per molecule	Percentage of total[a]	Number of residues per molecule	Percentage of total[a]
Ala	6	6	22	9
Arg	2	2	4	1.6
Asn	5	5	14	5.7
Asp	3	3	9	3.7
Cys	2	2	10	4
Gln	3	3	10	4
Glu	9	9	5	2
Gly	14	13	23	9.4
His	3	3	2	0.8
Ile	6	6	10	4
Leu	6	6	19	7.8
Lys	18	17	14	5.7
Met	2	2	2	0.8
Phe	4	4	6	2.4
Pro	4	4	9	3.7
Ser	1	1	28	11.4
Thr	8	8	23	9.4
Trp	1	1	8	3.3
Tyr	4	4	4	1.6
Val	3	3	23	9.4
Total	104	102[a]	245	99.7[a]

Note: In some common analyses, such as acid hydrolysis, Asp and Asn are not readily distinguished from each other and are together designated Asx (or B). Similarly, when Glu and Gln cannot be distinguished, they are together designated Glx (or Z). In addition, Trp is destroyed by acid hydrolysis. Additional procedures must be employed to obtain an accurate assessment of complete amino acid content.

[a] Percentages do not total to 100%, due to rounding.

the average molecular weight of the 20 common amino acids is about 138, the smaller amino acids predominate in most proteins. If we take into account the proportions in which the various amino acids occur in an average protein (Table 3-1; the averages are determined by surveying the amino acid compositions of more than 1,000 different proteins), the average molecular weight of protein amino acids is nearer to 128. Because a molecule of water (M_r 18) is removed to create each peptide bond, the average molecular weight of an amino acid residue in a protein is about 128 – 18 = 110.

Some Proteins Contain Chemical Groups Other Than Amino Acids

Many proteins—for example, the enzymes ribonuclease A and chymotrypsin—contain only amino acid residues and no other chemical constituents. However, some proteins contain permanently associated chemical components in addition to amino acids; these are called **conjugated proteins**. The non–amino acid part of a conjugated protein is usually called its **prosthetic group**. Conjugated proteins are classified on the basis of the chemical nature of their prosthetic groups (**Table 3-4**); for example, **lipoproteins** contain lipids, **glycoproteins** contain sugar groups, and **metalloproteins** contain a specific metal. Some proteins contain more than one prosthetic group. Usually the prosthetic group plays an important role in the protein's biological function.

TABLE 3-4 Conjugated Proteins

Class	Prosthetic group	Example
Lipoproteins	Lipids	β_1-Lipoprotein of blood (Fig. 17-2)
Glycoproteins	Carbohydrates	Immunoglobulin G (Fig. 5-20)
Phosphoproteins	Phosphate groups	Glycogen phosphorylase (Fig. 6-39)
Hemoproteins	Heme (iron porphyrin)	Hemoglobin (Figs 5-8 to 5-11)
Flavoproteins	Flavin nucleotides	Succinate dehydrogenase (Fig. 19-9)
Metalloproteins	Iron Zinc Calcium Molybdenum Copper	Ferritin (Box 16-1) Alcohol dehydrogenase (Fig. 14-12) Calmodulin (Fig. 12-17) Dinitrogenase (Fig. 22-3) Complex IV (Fig. 19-12)

SUMMARY 3.2 *Peptides and Proteins*

- Amino acids can be joined covalently through peptide bonds to form peptides and proteins. Cells generally contain thousands of different proteins, each with a different biological activity.
- The ionization behavior of peptides reflects their ionizable side chains as well as the terminal α-amino and α-carboxyl groups.
- Proteins can be very long polypeptide chains of 100 to several thousand amino acid residues. However, some naturally occurring peptides have only a few amino acid residues. Some proteins are composed of several noncovalently associated polypeptide chains, called subunits.
- Simple proteins yield only amino acids on hydrolysis; conjugated proteins contain in addition some other component, such as a metal or organic prosthetic group.

3.3 Working with Proteins

Biochemists' understanding of protein structure and function has been derived from the study of many individual proteins. To study a protein in detail, the researcher must be able to separate it from other proteins in pure form and must have the techniques to determine its properties. The necessary methods come from protein chemistry, a discipline as old as biochemistry itself and one that retains a central position in biochemical research.

Proteins Can Be Separated and Purified

A pure preparation is usually essential before a protein's properties and activities can be determined. Given that cells contain thousands of different kinds of proteins, how can one protein be purified? P3 Methods for separating proteins take advantage of properties that vary from one protein to the next, including size, charge, and binding properties. The advent of genetic engineering approaches has provided new and simpler paths for protein purification. The latter methods, described in Chapter 9, often artificially modify the protein being purified, adding a few or many amino acid residues to one or both ends. In many cases, the modifications alter protein function. Isolation of unaltered native proteins requires removal of the modification or a reliance on methods described here.

The source of a protein is generally tissue or microbial cells. The first step in any protein purification procedure is to break open these cells, releasing their proteins into a solution called a **crude extract**. If necessary, differential centrifugation can be used to prepare subcellular fractions or to isolate specific organelles (see Fig. 1-7).

Once the extract or organelle preparation is ready, various methods are available for purifying one or more of the proteins it contains. P3 Commonly, the extract is subjected to treatments that separate the proteins into different **fractions** based on a property such as size or charge; the process is referred to as **fractionation**. Early fractionation steps in a purification utilize differences in protein solubility, which is a complex function of pH, temperature, salt concentration, and other factors. The solubility of proteins is lowered in the presence of some salts, an effect called "salting out." Ammonium sulfate ($(NH_4)_2SO_4$) is particularly effective for selectively precipitating some proteins while leaving others in solution. Low-speed centrifugation is then used to remove the precipitated proteins from those remaining in solution.

A solution containing the protein of interest usually must be further altered before subsequent purification steps are possible. For example, **dialysis** is a procedure that separates proteins from small solutes by taking advantage of the proteins' larger size. The partially purified extract is placed in a bag or tube made of a semipermeable membrane, which is suspended in a much larger volume of buffered solution of appropriate ionic strength. The membrane allows the exchange of salt and buffer but not proteins. Thus dialysis retains large proteins within the membranous bag or tube while allowing the concentration of other solutes in the protein preparation to change until they come into equilibrium with the solution outside the membrane. Dialysis might be used, for example, to remove ammonium sulfate from the protein preparation.

The most efficient methods for fractionating proteins make use of **column chromatography**, which takes advantage of differences in protein charge, size, binding affinity, and other properties (**Fig. 3-16**). A porous solid material with appropriate chemical properties (the stationary phase) is held in a column, and a buffered solution (the mobile phase) migrates through it. The protein, dissolved in the same buffered solution that was used to establish the mobile phase, is layered on the top of the column. The protein then percolates through the solid matrix as an ever-expanding band within the larger mobile phase. Individual proteins migrate faster or more slowly through the column, depending on their properties.

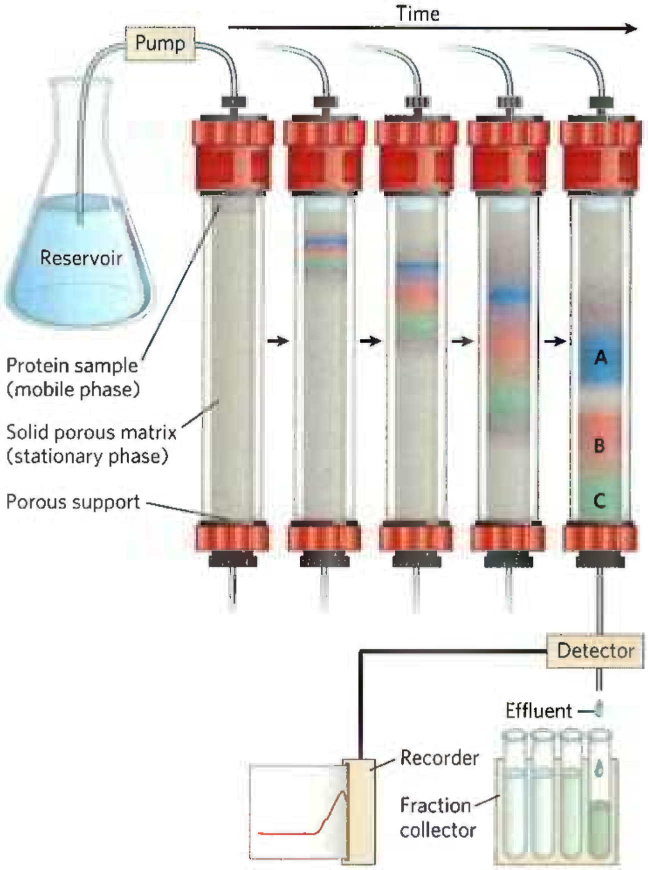
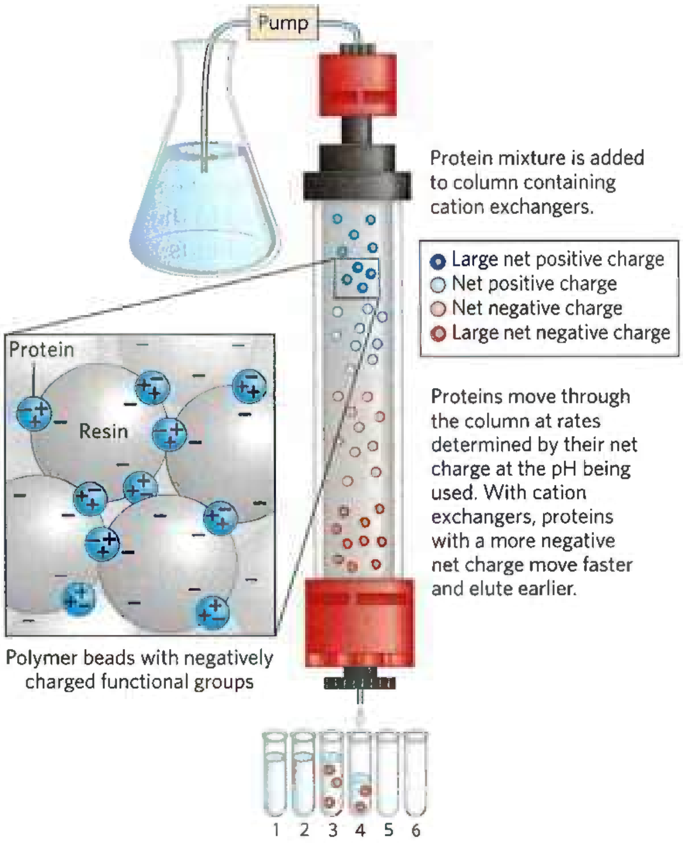
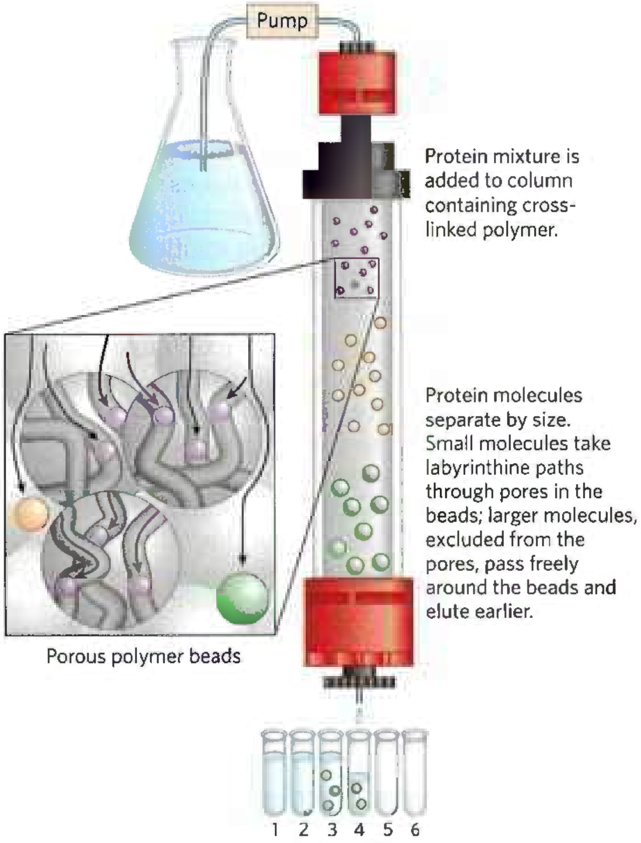


FIGURE 3-16 Column chromatography. The standard elements of a chromatographic column include a solid, porous material (matrix) supported inside a column, generally made of plastic or glass. A solution, the mobile phase, flows through the matrix, the stationary phase. The solution that passes out of the column at the bottom (the effluent) is constantly replaced by solution supplied from a reservoir at the top. The protein solution to be separated is layered on top of the column and allowed to percolate into the solid matrix. Additional solution is added on top. The protein solution forms a band within the mobile phase that is initially the depth of the protein solution applied to the column. As proteins migrate through the column (shown here at five different times), they are retarded to different degrees by their different interactions with the matrix material. The overall protein band thus widens as it moves through the column. Individual types of proteins (such as A, B, and C, shown in blue, red, and green) gradually separate from each other, forming bands within the broader protein band. Separation improves (i.e., resolution increases) as the length of the column increases. However, each individual protein band also broadens with time due to diffusional spreading, a process that decreases resolution. In this example, protein A is well separated from B and C, but diffusional spreading prevents complete separation of B and C under these conditions. Protein C is being detected and its presence recorded as it is eluted from the column.

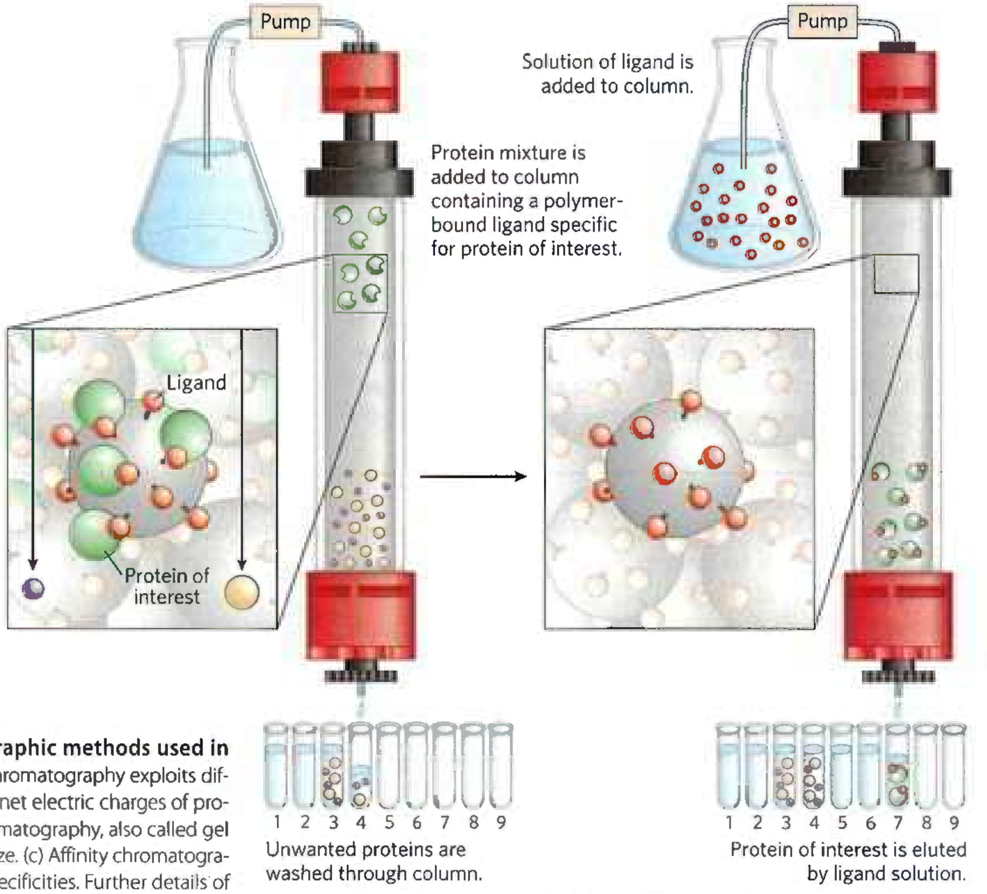
Ion-exchange chromatography exploits differences in the sign and magnitude of the net electric charge of proteins at a given pH (**Fig. 3-17a**). The column


Pump
Protein mixture is added to column containing cation exchangers.
Large net positive charge
Net positive charge
Net negative charge
Large net negative charge
Protein
Resin
Proteins move through the column at rates determined by their net charge at the pH being used. With cation exchangers, proteins with a more negative net charge move faster and elute earlier.
Polymer beads with negatively charged functional groups
1 2 3 4 5 6

(a) Ion-exchange chromatography


Pump
Protein mixture is added to column containing cross-linked polymer.
Protein molecules separate by size. Small molecules take labyrinthine paths through pores in the beads; larger molecules, excluded from the pores, pass freely around the beads and elute earlier.
Porous polymer beads
1 2 3 4 5 6

(b) Size-exclusion chromatography


Pump
Protein mixture is added to column containing a polymer-bound ligand specific for protein of interest.
Ligand
Protein of interest
1 2 3 4 5 6 7 8 9
Unwanted proteins are washed through column.
Pump
Solution of ligand is added to column.
1 2 3 4 5 6 7 8 9
Protein of interest is eluted by ligand solution.

(c) Affinity chromatography

FIGURE 3-17 Three chromatographic methods used in protein purification. (a) Ion-exchange chromatography exploits differences in the sign and magnitude of the net electric charges of proteins at a given pH. (b) Size-exclusion chromatography, also called gel filtration, separates proteins according to size. (c) Affinity chromatography separates proteins by their binding specificities. Further details of these methods are given in the text.

matrix is a synthetic polymer (resin) containing bound charged groups; those with bound anionic groups are called **cation exchangers**, and those with bound cationic groups are called **anion exchangers**. The affinity of each protein for the charged groups on the column is affected by the pH (which determines the ionization state of the molecule) and the concentration of competing free salt ions in the surrounding solution. Separation can be optimized by gradually changing the pH and/or salt concentration of the mobile phase in order to create a pH or salt gradient. In **cation-exchange chromatography**, proteins with a net positive charge migrate through the matrix more slowly than those with a net negative charge, because the migration of the former is retarded more by interaction with the stationary phase.

As the protein-containing solution exits a column, successive portions (fractions) of this effluent are collected in test tubes. Each fraction can be tested for the presence of the protein of interest as well as other properties, such as ionic strength or total protein concentration. All fractions positive for the protein of interest can be combined as the product of this chromatographic step of the protein purification.

WORKED EXAMPLE 3-1 *Ion Exchange of Peptides*

A biochemist wants to separate two peptides by ion-exchange chromatography. At the pH of the mobile phase to be used on the column, one peptide (A) has a pI of 5.1, due to the presence of more Glu and Asp residues than Arg, Lys, and His residues, and has a net negative charge at neutral pH. Peptide B has a pI of 7.8, reflecting a plurality of positively charged amino acid residues at neutral pH. At neutral pH, which peptide would elute first from a cation-exchange resin? Which would elute first from an anion-exchange resin?

SOLUTION: A cation-exchange resin has negative charges and binds positively charged molecules, retarding their progress through the column. Peptide B, with its higher pI and net positive charge, will interact more strongly than peptide A with the cation-exchange resin. Thus, peptide A will elute first. On the anion-exchange resin, peptide B will elute first. Peptide A, having a relatively low pI and a net negative charge, will be retarded by its interaction with the positively charged resin.

Figure 3-17 shows two variations of column chromatography in addition to ion exchange. **Size-exclusion chromatography**, also called gel filtration (Fig. 3-17b), separates proteins according to size. In this method, large proteins emerge from the column sooner than small ones — a somewhat counterintuitive result. The solid phase consists of cross-linked polymer beads with engineered pores or cavities of a particular size. Large proteins cannot enter the cavities and so take a shorter (and more rapid) path through the column, around the beads. Small proteins enter the cavities and are slowed by their more labyrinthine path through the column. Size-exclusion chromatography can also be used to approximate the size of a protein being purified, using methods similar to those described in Figure 3-19.

Affinity chromatography is based on binding affinity (Fig. 3-17c). The beads in the column have a covalently attached chemical group called a ligand — a group or molecule that binds to a macromolecule such as a protein. When a protein mixture is added to the column, any protein with affinity for this ligand binds to the beads, and its migration through the matrix is retarded. For example, if the biological function of a protein involves binding to ATP, then attaching a molecule that resembles ATP to the beads in the column creates an affinity matrix that can help purify the protein. Proteins that do not bind to ATP flow more rapidly through the column. Bound proteins are then eluted by a solution containing either a high concentration of salt or a free ligand — in this case, ATP or an analog of ATP. Salt weakens the binding of the protein to the immobilized ligand, interfering with ionic interactions. Free ligand competes with the ligand attached to the beads, releasing the protein from the matrix; the protein product that elutes from the column is often bound to the ligand used to elute it.

Protein purification protocols often use genetic engineering to fuse additional amino acids or peptides (tags) to the target protein. Affinity chromatography can be used to bind this tag, achieving a large increase in purity in a single step (see Fig. 9-11). In many cases, the tag can be subsequently removed, fully restoring the function of the native protein.

Chromatographic methods are typically enhanced by the use of **HPLC**, or **high-performance liquid chromatography**. HPLC makes use of high-pressure pumps that speed the movement of the protein molecules down the column; it also uses higher-quality chromatographic materials that can withstand the crushing force of the pressurized flow. By reducing the transit time on the column, HPLC can limit diffusional spreading of protein bands and thus can greatly improve resolution.

Choosing the approach to purification of a protein that has not previously been isolated is guided both by established precedents and by common sense. **P3** In most cases, several different methods must be used sequentially to purify a protein completely, each separating proteins on the basis of different properties. The choice of methods is somewhat empirical, and many strategies may be tried before the most effective one is found. Researchers can often minimize trial and error by basing the new procedure on purification techniques developed for similar proteins. Common sense dictates that inexpensive procedures such as salting out be used first, when the total volume and the number of contaminants are greatest. As each purification step is completed, the sample size generally becomes smaller (**Table 3-5**), making it feasible to use more sophisticated (and expensive) chromatographic procedures at later stages. A purification table documents the success of each step in a purification protocol. In the hypothetical purification shown

TABLE 3-5 **A Hypothetical Purification Table for an Enzyme**

Procedure or step	Fraction volume (mL)	Total protein (mg)	Activity (units)	Specific activity (units/mg)
1. Crude cellular extract	1,400	10,000	100,000	10
2. Precipitation with ammonium sulfate	280	3,000	96,000	32
3. Ion-exchange chromatography	90	400	80,000	200
4. Size-exclusion chromatography	80	100	60,000	600
5. Affinity chromatography	6	3	45,000	15,000

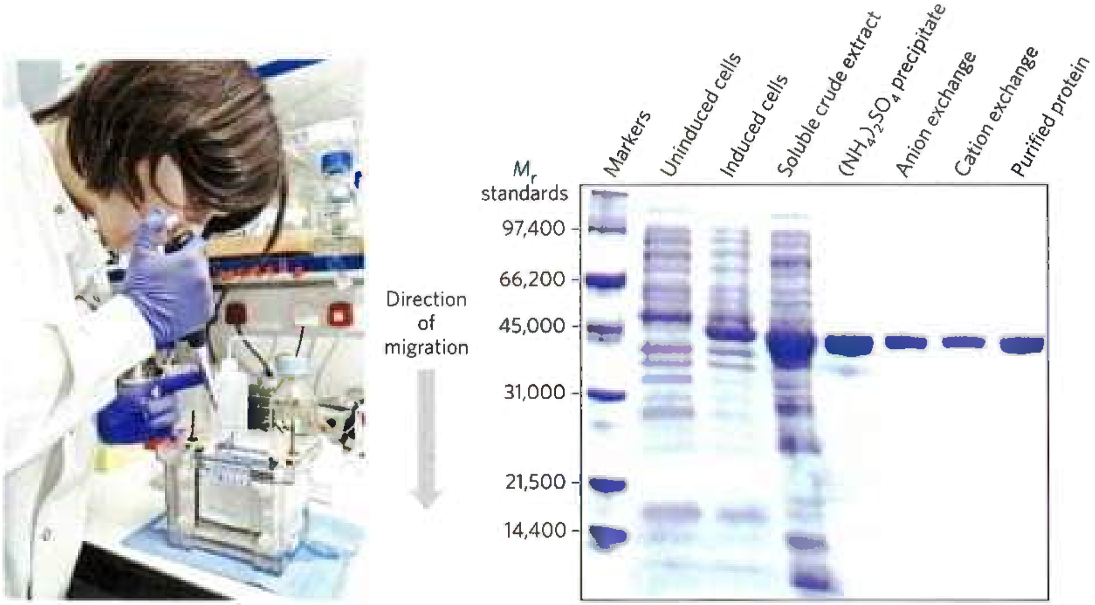
Note: All data represent the status of the sample *after* the designated procedure has been carried out. "Activity" and "specific activity" are defined on page 90.

in Table 3-5, the ratio of the final specific activity (15,000 units/mg) to the starting specific activity (10 units/mg) gives the purification factor (1,500). The percentage of the total activity at the last step (45,000 units) relative to the total activity in the starting material (100,000 units) gives the yield from the purification procedure (45%).

Proteins Can Be Separated and Characterized by Electrophoresis

Protein purification is usually complemented by **electrophoresis**, an analytical process that allows researchers to visualize and characterize proteins as they are purified. This method does not itself contribute to purification, as electrophoresis often adversely affects the structure and thus the function of proteins. However, it allows a biochemist to rapidly estimate the number of different proteins in a mixture and the degree of purity of a particular protein preparation. Also, electrophoresis can be used to determine such crucial properties of a protein as its isoelectric point and approximate molecular weight.

Electrophoresis of proteins is generally carried out in gels made up of the cross-linked polymer polyacrylamide (**Fig. 3-18**). The polyacrylamide gel acts as a molecular sieve, slowing the migration of proteins approximately in proportion to their charge-to-mass ratio. Migration may



(a) (b)

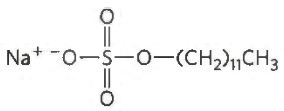
FIGURE 3-18 **Electrophoresis.** (a) Different samples are loaded in wells or depressions at the top of the SDS polyacrylamide gel. The proteins move into the gel when an electric field is applied. The gel minimizes convection currents caused by small temperature gradients, as well as protein movements other than those induced by the electric field. (b) Proteins can be visualized after electrophoresis by treating the gel with a stain such as Coomassie blue, which binds to the proteins but not to the gel itself. Each band on the gel represents a different protein (or protein subunit); smaller proteins move through the gel more rapidly than larger proteins and therefore are found nearer the bottom of the gel. This gel illustrates purification of the RecA protein of *Escherichia coli.* The gene for the RecA protein was cloned so that its expression (synthesis of the protein) could be controlled. The first lane shows a set of standard proteins (of known M_r), serving as molecular weight markers. The second and third lanes show, respectively, proteins from *E. coli* cells before and after synthesis of RecA protein was induced. The fourth lane shows the proteins in a crude cellular extract. Subsequent lanes (left to right) show the proteins that are present after successive purification steps. Although the protein looks pure in lane 6, two more steps are needed to remove minor contaminants not evident on the gel. The purified protein is a single polypeptide chain (M_r ~38,000), as seen in the rightmost lane. [(a) Gustoimages/Science Source; (b) Dr. Julia Cox.]

also be affected by protein shape. In electrophoresis, the force moving the macromolecule is the electrical potential, E. The electrophoretic mobility, μ, of a molecule is the ratio of its velocity, V, to the electrical potential. Electrophoretic mobility is also equal to the net charge, Z, of the molecule divided by the frictional coefficient, f, which reflects in part a protein's shape. Thus,

$$\mu = \frac{V}{E} = \frac{Z}{f}$$

The migration of a protein in a gel during electrophoresis is therefore a function of its size and its shape.

The electrophoretic method commonly employed for estimation of purity and molecular weight makes use of the detergent **sodium dodecyl sulfate (SDS)** ("dodecyl" denoting a 12-carbon chain).



$$Na^+\ {}^-O-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{S}}-O-(CH_2)_{11}CH_3$$

Sodium dodecyl sulfate
(SDS)

A protein will bind about 1.4 times its weight of SDS, nearly one molecule of SDS for each amino acid residue. The sulfate moieties of the bound SDS contribute a large net negative charge, rendering the intrinsic charge of the protein insignificant and conferring on each protein a similar charge-to-mass ratio. In addition, SDS binding partially unfolds proteins, such that most SDS-bound proteins assume a similar rodlike shape. Electrophoresis in the presence of SDS therefore separates proteins almost exclusively on the basis of mass (molecular weight), with smaller polypeptides migrating more rapidly. After electrophoresis, the proteins are visualized by adding a dye such as Coomassie blue, which binds to proteins but not to the gel itself (Fig. 3-18b). Thus, a researcher can monitor the progress of a protein purification procedure as the number of protein bands visible on the gel decreases after each new fractionation step. When compared with the positions to which proteins of known molecular weight migrate in the gel, the position of an unidentified protein can provide a good approximation of its molecular weight (**Fig. 3-19**). If the protein has two or more different subunits, generally the subunits are separated by the SDS treatment, and a separate band appears for each.

Isoelectric focusing is a procedure used to determine the isoelectric point (pI) of a protein (**Fig. 3-20**). A pH gradient is established by allowing a mixture of low molecular weight organic acids and bases (ampholytes; p. 77) to distribute themselves in an electric field generated across the gel. When a protein mixture is applied, each protein migrates until it reaches the pH that matches its pI. Proteins with different isoelectric points are thus distributed differently throughout the gel.

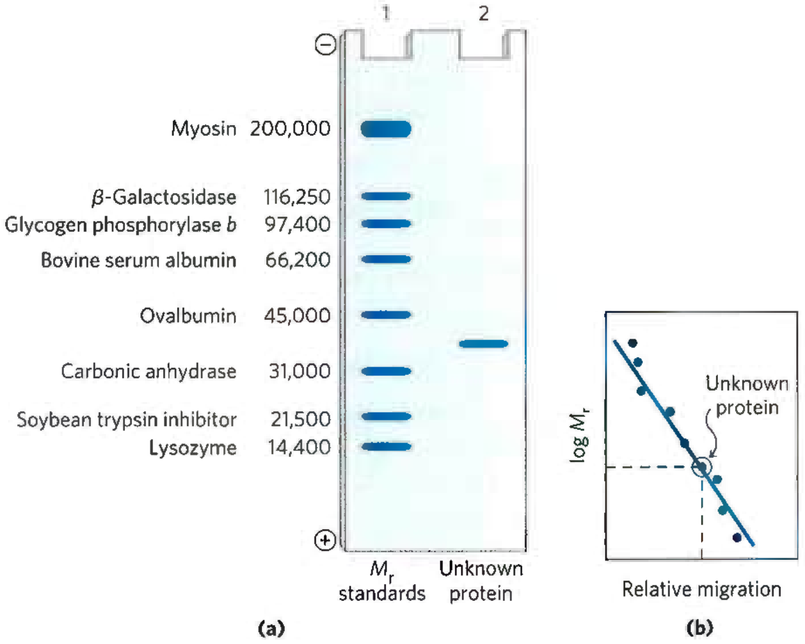


FIGURE 3-19 Estimating the molecular weight of a protein. The electrophoretic mobility of a protein on an SDS polyacrylamide gel is related to its molecular weight, M_r. (a) Standard proteins of known molecular weight are subjected to electrophoresis (lane 1). These marker proteins can be used to estimate the molecular weight of an unknown protein (lane 2). (b) A plot of log M_r of the marker proteins versus relative migration during electrophoresis is linear, which allows the molecular weight of the unknown protein to be read from the graph. (In similar fashion, a set of standard proteins with reproducible retention times on a size-exclusion column can be used to create a standard curve of retention time versus log M_r. The retention time of an unknown substance on the column can be compared with this standard curve to obtain an approximate M_r.)

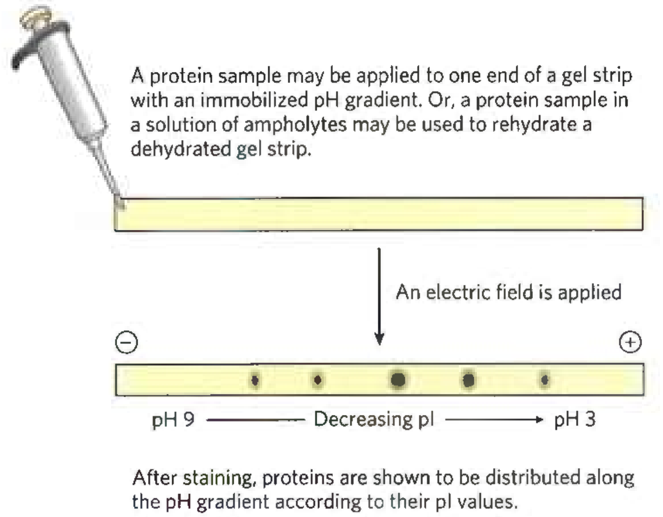

A protein sample may be applied to one end of a gel strip with an immobilized pH gradient. Or, a protein sample in a solution of ampholytes may be used to rehydrate a dehydrated gel strip.
An electric field is applied
⊖
⊕
pH 9
Decreasing pI
pH 3
After staining, proteins are shown to be distributed along the pH gradient according to their pI values.

FIGURE 3-20 Isoelectric focusing. This technique separates proteins according to their isoelectric points. A protein mixture is placed on a gel strip containing an immobilized pH gradient. With an applied electric field, proteins enter the gel and migrate until each reaches a pH that is equivalent to its pI. Remember that when pH = pI, the net charge of a protein is zero.

Combining isoelectric focusing and SDS electrophoresis sequentially in a process called **two-dimensional electrophoresis** permits the resolution of complex mixtures of proteins (**Fig. 3-21**). This is a more sensitive analytical method than either electrophoretic method alone. Two-dimensional electrophoresis separates proteins of identical molecular weight that differ in pI, or proteins with similar pI values but different molecular weights.

Unseparated Proteins Are Detected and Quantified Based on Their Functions

P3 To purify a protein, it is essential to have a way of detecting and quantifying that protein in the presence of many other proteins at each stage of the procedure. A common target of purification is one or another of the class of proteins called enzymes (Chapter 6). Each enzyme catalyzes a particular reaction that converts one biomolecule (the substrate) to another (the product). The amount of the protein in a given solution or tissue extract can be measured, or assayed, in terms of the catalytic effect the enzyme produces—that is, the *increase* in the rate at which its substrate is converted to reaction products when the enzyme is present. For this purpose the researcher must know (1) the overall equation of the reaction catalyzed, (2) an analytical procedure for determining the disappearance of the substrate or the appearance of a reaction product, (3) whether the enzyme requires cofactors such as metal ions or coenzymes, (4) the dependence of the enzyme activity on substrate concentration, (5) the optimum pH, and (6) a temperature zone in which the enzyme is stable and has high activity. Enzymes are usually assayed at their optimum pH and at some convenient temperature within the range 25 to 38 °C. Also, very high substrate concentrations are generally used so that the initial reaction rate, measured experimentally, is proportional to enzyme concentration (Chapter 6).

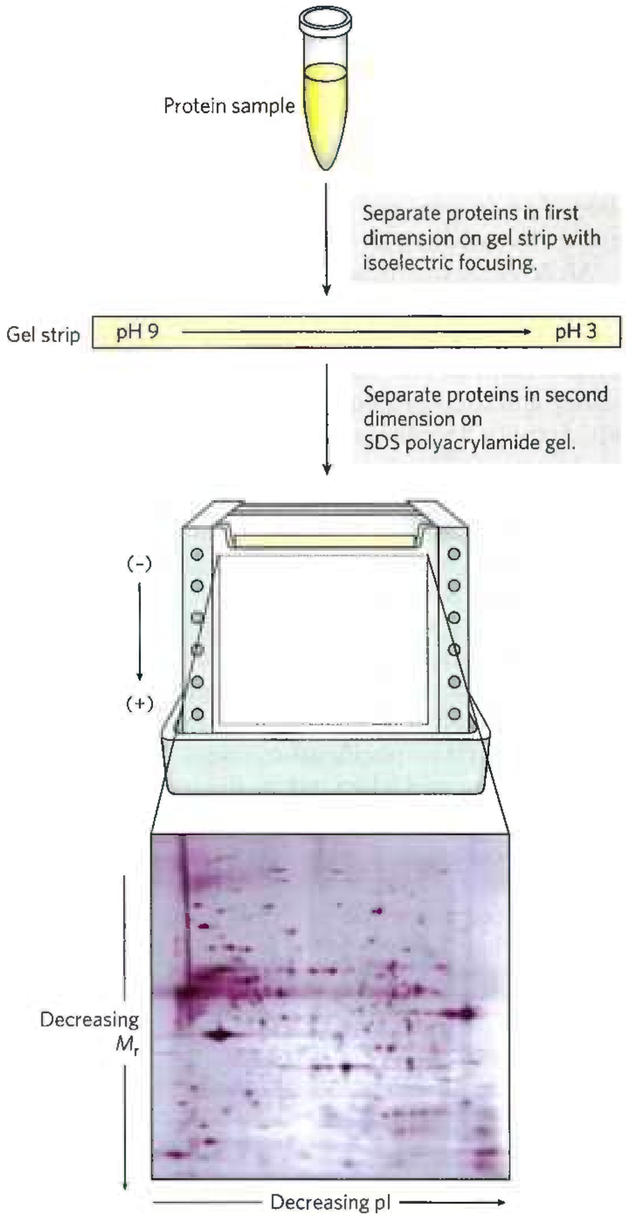

Protein sample
Separate proteins in first dimension on gel strip with isoelectric focusing.
Gel strip
pH 9
pH 3
Separate proteins in second dimension on SDS polyacrylamide gel.
(-)
(+)
Decreasing M_r
Decreasing pI

FIGURE 3-21 Two-dimensional electrophoresis. Proteins are first separated by isoelectric focusing in a thin strip gel. The gel is then laid horizontally on a second, slab-shaped gel, and the proteins are separated by SDS polyacrylamide gel electrophoresis. Horizontal separation reflects differences in pI; vertical separation reflects differences in molecular weight. The original protein complement is thus spread in two dimensions. Thousands of cellular proteins can be resolved using this technique. Individual protein spots can be cut out of the gel and identified by mass spectrometry (see Figs 3-28 and 3-29). [Wellcome Collection. CC BY.]

By international agreement, 1.0 unit of enzyme activity for most enzymes is defined as the amount of enzyme causing transformation of 1.0 μmol of substrate to product per minute at 25 °C under optimal conditions of measurement (for many enzymes, this definition is inconvenient, and a unit may be defined differently). The term **activity** refers to the total units of enzyme in a solution. The **specific activity** is the number of enzyme units per milligram of total protein (**Fig. 3-22**). The specific activity is a measure of enzyme purity: it increases during purification of an enzyme and becomes maximal and constant when the enzyme is pure (Table 3-5).

After each purification step, the activity of the preparation (in units of enzyme activity) is assayed, the total amount of protein is determined independently, and the ratio of the two gives the specific activity. Activity and total protein generally decrease with each step. Activity decreases because there is always some loss due to inactivation or nonideal interactions with chromatographic materials or other molecules in the solution. Total protein decreases because the objective is to remove as much unwanted or nonspecific protein as possible. In a successful step, the loss of nonspecific protein is much greater than the loss of activity; therefore, specific activity increases even as total activity falls. The data are assembled in a purification table similar to Table 3-5. A protein is generally considered pure when further purification steps fail to increase specific activity and when only a single protein species can be detected (for example, by electrophoresis in the presence of SDS).

For proteins that are not enzymes, other quantification methods are required. Transport proteins can be assayed by their binding to the molecule they transport, and hormones and toxins by the biological effect they produce; for example, growth hormones will stimulate the growth of certain cultured cells. Some structural proteins represent such a large fraction of a tissue mass that they can be readily extracted and purified without a functional assay. The approaches are as varied as the proteins themselves.

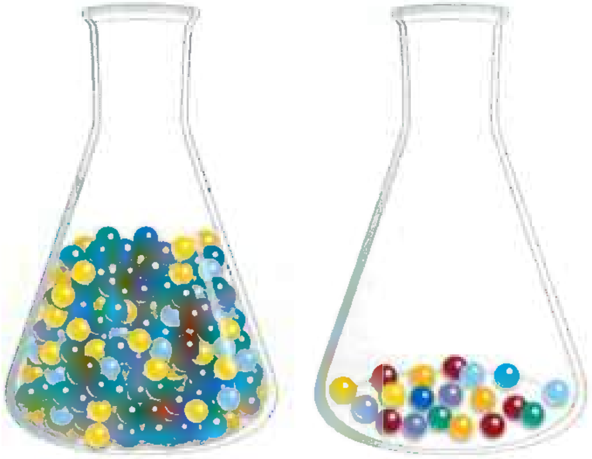


FIGURE 3-22 Activity versus specific activity. The difference between these terms can be illustrated by considering two flasks containing marbles. The flasks contain the same number of red marbles, but different numbers of marbles of other colors. If the marbles represent proteins, both flasks contain the same *activity* of the protein, represented by the red marbles. The second flask, however, has the higher *specific activity,* because red marbles represent a higher fraction of the total.

SUMMARY 3.3 *Working with Proteins*

- Proteins are separated and purified on the basis of differences in their properties. Proteins can be selectively precipitated by changes in pH or temperature, and particularly by the addition of certain salts. A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties. These include ion-exchange, size-exclusion, affinity, and high-performance liquid chromatography.
- Electrophoresis separates proteins on the basis of mass or charge for analytical purposes. SDS gel electrophoresis and isoelectric focusing can be used separately or in combination for higher resolution.
- All purification procedures require a method for quantifying or assaying the protein of interest in the presence of other proteins. Purification can be monitored by assaying specific activity.

3.4 The Structure of Proteins: Primary Structure

Purification of a protein is usually only a prelude to a detailed biochemical dissection of its structure and function. What is it that makes one protein an enzyme, another a hormone, another a structural protein, and still another an antibody? How do they differ chemically? The most obvious distinctions are structural, and to protein structure we now turn.

We can describe the structure of large molecules such as proteins at several levels of complexity, arranged in a kind of conceptual hierarchy. Four levels of protein structure are commonly defined (**Fig. 3-23**). P2 A description of all covalent bonds (mainly peptide bonds and disulfide bonds) linking amino acid residues in a polypeptide chain is its **primary structure**. The most important element of primary structure is the *sequence* of amino acid residues. **Secondary structure** refers to particularly stable arrangements of amino acid residues giving rise to recurring structural patterns. **Tertiary structure** describes all aspects of the three-dimensional folding of a polypeptide. When a protein has two or more polypeptide subunits, their arrangement in space is referred to as **quaternary structure**. Our exploration of proteins will eventually include complex protein machines consisting of dozens to thousands of subunits. Primary structure is

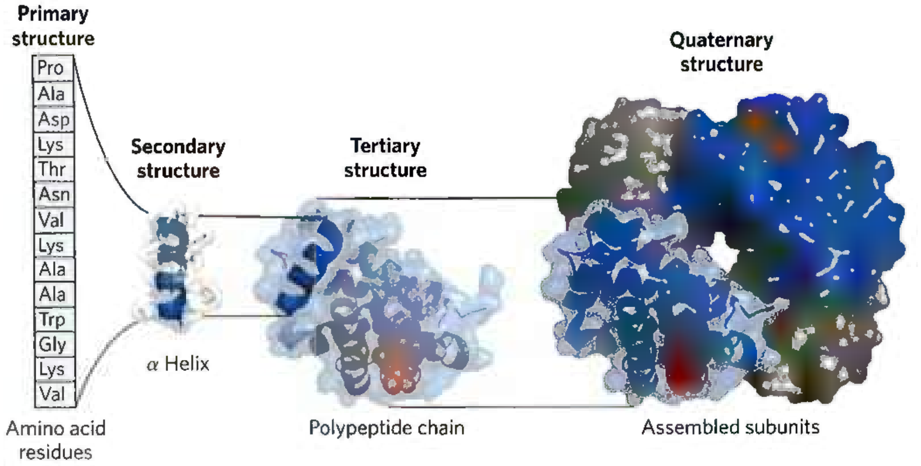


FIGURE 3-23 Levels of structure in proteins. The *primary structure* consists of a sequence of amino acids linked together by peptide bonds, and it includes any disulfide bonds. The resulting polypeptide can be arranged into units of *secondary structure*, such as an α helix. The helix is a part of the *tertiary structure* of the folded polypeptide, which is itself one of the subunits that make up the *quaternary structure* of the multisubunit protein, in this case hemoglobin. [Data from PDB ID 1HGA, R. Liddington et al., *J. Mol. Biol.* 228:551, 1992.]

the focus of the remainder of this chapter; we discuss the higher levels of structure in Chapter 4.

Primary structure now becomes our focus. We first consider empirical clues that amino acid sequence and protein function are closely linked, then describe how amino acid sequence is determined; finally, we outline the many uses to which this information can be put.

The Function of a Protein Depends on Its Amino Acid Sequence

The bacterium *Escherichia coli* produces more than 3,000 different proteins; a human has ~20,000 genes that may produce over a million different proteins (through genetic processes discussed in Part III of this text). In both species, each type of protein has a unique amino acid sequence that confers a particular three-dimensional structure. This structure in turn confers a unique function.

Amino acid sequences are important elements of the broader realm of biological information. They are a major functional expression of information stored in DNA in the form of genes. The sequences are not at all random. Each protein has a distinctive number and sequence of amino acid residues. As we shall see in Chapter 4, P4 the primary structure of a protein determines how it folds up into its unique three-dimensional structure, and this in turn determines the function of the protein.

Some simple observations illustrate the functional importance of primary structure, or the amino acid sequence of a protein. First, as we have already noted, proteins with different functions always have different amino acid sequences. Second, thousands of human genetic diseases have been traced to the production of proteins with less activity or altered activity. The alteration can range from a single change in the amino acid sequence (as in sickle cell disease, described in Chapter 5) to deletion of a larger portion of the polypeptide chain (as in most cases of Duchenne muscular dystrophy: a large deletion in the gene encoding the protein dystrophin leads to production of a shortened, inactive protein). Finally, on comparing functionally similar proteins from different species, we find that these proteins often have similar amino acid sequences. Thus, a close link between protein primary structure and function is evident.

The amino acid sequence for a particular protein is not absolutely fixed, or invariant. Virtually all of the proteins in humans are **polymorphic**, having amino acid sequence variants in the human population. Many human proteins are polymorphic even within an individual, with amino acid variations occurring due to processes that will be described in Part III of this text. Some of these variations have little or no effect on the function of the protein; others may affect function dramatically. Furthermore, proteins that carry out a broadly similar function in distantly related species can differ greatly in overall size and amino acid sequence.

Although the amino acid sequence in some regions of the primary structure might vary considerably without affecting biological function, most proteins contain crucial regions that are essential to their function and thus have sequences that are conserved. The fraction of the overall sequence that is critical varies from protein to protein, complicating the task of relating sequence to three-dimensional structure, and structure to function. Before we can consider this problem further, however, we must examine how sequence information is obtained.

In 1953, Frederick Sanger worked out the sequence of amino acid residues in the polypeptide chains of the hormone insulin (**Fig. 3-24**), surprising many researchers who had long thought that determining the amino acid sequence of a polypeptide would be a hopelessly difficult task. The elucidation of DNA structure in that same year by Watson and Crick telegraphed a likely relationship between DNA and protein sequences. Barely a decade after these discoveries, the genetic code relating the nucleotide sequence of DNA to the amino acid sequence of protein molecules was elucidated (Chapter 27).

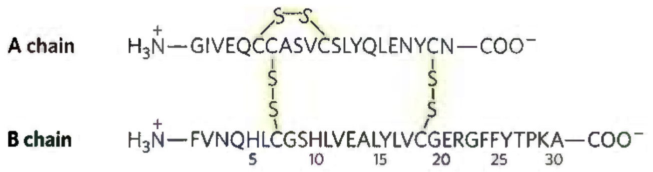

A chain
H3N—GIVEQCCASVCSLYQLENYCN—COO−
S–S
B chain
H3N—FVNQHLCGSHLVEALYLVCGERGFFYTPKA—COO−
5
10
15
20
25
30

FIGURE 3-24 Amino acid sequence of bovine insulin. The two polypeptide chains are joined by disulfide cross-linkages (yellow). The A chain of insulin is identical in human, pig, dog, rabbit, and sperm whale insulins. The B chains of the cow, pig, dog, goat, and horse are identical.

The amino acid sequences of proteins are now most often derived indirectly from the DNA sequences in genome databases. However, an array of techniques derived from traditional methods of polypeptide sequencing made important contributions to the broader field of protein chemistry. The method used by Sanger to sequence insulin is based on the classical method for direct chemical sequencing of proteins from the amino terminus, the two-step **Edman degradation** developed by Pehr Edman.

Protein Structure Is Studied Using Methods That Exploit Protein Chemistry

The sequence of a protein can be predicted from the sequence of the gene encoding it, which is usually available in genomic databases. Direct sequencing can also be provided by mass spectrometry. Many methods used in traditional protein sequencing protocols remain valuable for labeling proteins or breaking them into parts for functional and structural analysis.

For example, the amino-terminal α-amino group of a protein can be labeled with 1-fluoro-2,4-dinitrobenzene (FDNB), dansyl chloride, or dabsyl chloride (**Fig. 3-25**). These reagents also label the ε-amino group of lysine residues. Disulfide bonds within a polypeptide or between polypeptide subunits can be broken irreversibly (**Fig. 3-26**).

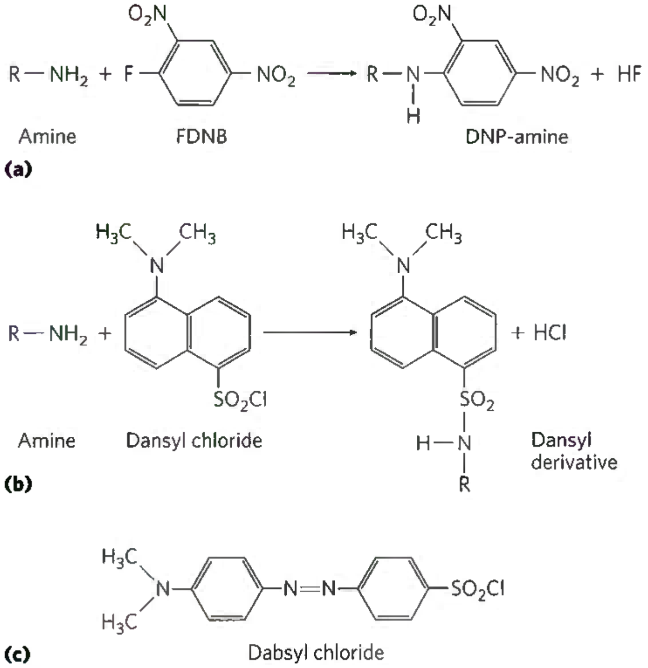
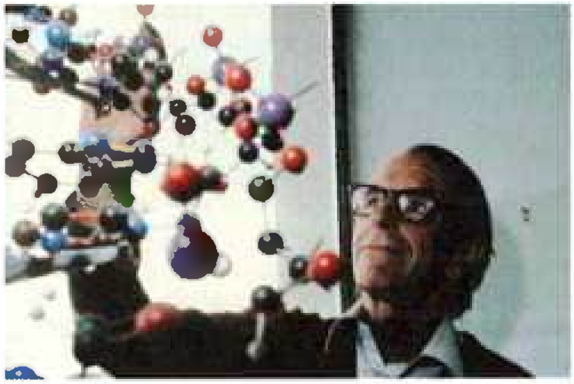


FIGURE 3-25 Modification of the α-amino group at the amino terminus. The reaction is a nucleophilic displacement of the halide ion as shown for (a) FDNB and (b) dansyl chloride. The ε-amino group of lysine will also be labeled. Dansyl chloride and (c) dabsyl chloride, another labeling reagent, have useful absorbance and/or fluorescent properties at visible wavelengths.



Frederick Sanger, 1918–2013 [Bettmann/Getty Images.]

TABLE 3-6 The Specificity of Some Common Methods for Fragmenting Polypeptide Chains

Reagent (biological source)[a]	Cleavage points[b]
Trypsin (bovine pancreas)	Lys, Arg (C)
Chymotrypsin (bovine pancreas)	Phe, Trp, Tyr (C)
Staphylococcus aureus V8 protease (bacterium *S. aureus*)	Asp, Glu (C)
Asp-*N*-protease (bacterium *Pseudomonas fragi*)	Asp, Glu (N)
Pepsin (porcine stomach)	Leu, Phe, Trp, Tyr (N)
Endoproteinase Lys C (bacterium *Lysobacter enzymogenes*)	Lys (C)
Cyanogen bromide	Met (C)

[a] All reagents except cyanogen bromide are proteases.

[b] Residues furnishing the primary recognition point for the protease or reagent; peptide bond cleavage occurs on either the carbonyl (C) side or the amino (N) side of the indicated amino acid residues.

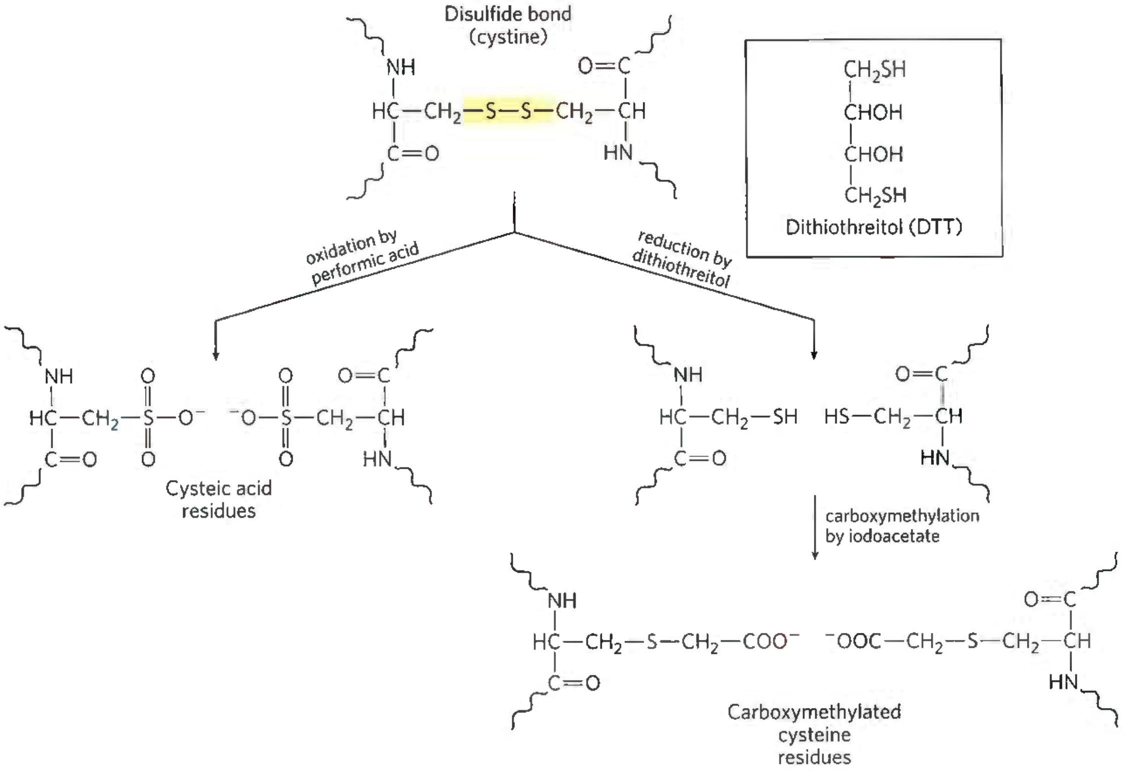

Disulfide bond
(cystine)
Dithiothreitol (DTT)
oxidation by performic acid
reduction by dithiothreitol
Cysteic acid residues
carboxymethylation by iodoacetate
Carboxymethylated cysteine residues

FIGURE 3-26 Breaking disulfide bonds in proteins. Two common methods are illustrated. Oxidation of a cystine residue with performic acid produces two cysteic acid residues. Reduction by dithiothreitol (or β-mercaptoethanol) to form Cys residues must be followed by further modification of the reactive —SH groups to prevent re-formation of the disulfide bond. Carboxymethylation by iodoacetate serves this purpose.

Enzymes called **proteases** catalyze the hydrolytic cleavage of peptide bonds and provide the most common method to break a protein into parts. Some proteases cleave only the peptide bond adjacent to particular amino acid residues (**Table 3-6**) and thus fragment a polypeptide chain in a predictable and reproducible way. A few chemical reagents also cleave the peptide bond adjacent to specific residues. Among proteases, the digestive enzyme trypsin catalyzes the hydrolysis of only those peptide bonds in which the carbonyl group is contributed by either a Lys or an Arg residue, regardless of the length or amino acid sequence of the chain. A polypeptide with three Lys and/or Arg residues will usually yield four smaller peptides upon cleavage with trypsin. Moreover, all except one of these will have a carboxyl-terminal Lys or Arg.

The capacity to modify proteins in specific ways has many applications in the lab. The methods used to break disulfide bonds can also be used to denature proteins when that is required. The development of reagents to label the amino-terminal amino acid residue led eventually to the development of an array of reagents that could react with specific groups at many locations on a protein. For example, the sulfhydryl group on Cys residues can be modified with iodoacetamides, maleimides, benzyl halides, and bromomethyl ketones (**Fig. 3-27**). Other amino acid residues can be modified by reagents linked to a dye or other molecule to aid in protein detection or functional studies. The cleavage of proteins into smaller parts with proteases has numerous applications that will be explored in subsequent chapters of this book.

Mass Spectrometry Provides Information on Molecular Mass, Amino Acid Sequence, and Entire Proteomes

Mass spectrometry can provide a highly accurate measure of the molecular mass of a protein, readily distinguishing between single proton differences. However, this technology can do much more. The sequences of multiple short polypeptide segments (20 to 30 amino acid residues each) in a protein sample can be obtained

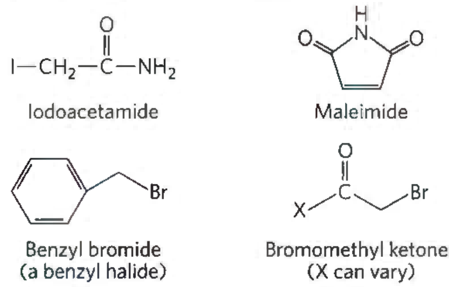

Iodoacetamide
Maleimide
Benzyl bromide
(a benzyl halide)
Bromomethyl ketones
(X can vary)

FIGURE 3-27 Reagents used to modify the sulfhydryl groups of Cys residues. (See also Fig. 3-26.)

within seconds. Unknown purified proteins can be identified, and their mass can be accurately determined. When coupled to powerful peptide separation protocols, mass spectrometry can document a complete cellular **proteome**—defined as the entire complement of proteins in a cell, including estimates of their relative abundance—in just an hour.

The mass spectrometer has been an indispensable tool in chemistry for more than a century. Molecules to be analyzed, referred to as **analytes**, are first ionized in a vacuum. When the newly charged molecules are introduced into an electric and/or magnetic field, their paths through the field are a function of their mass-to-charge ratio, m/z. This measured property of the ionized species can be used to deduce the mass (m) of the analyte with very high precision.

As the m/z measurements are made in the gas phase, the technique was long limited to relatively small molecules. In 1988, two different techniques were introduced to permit transfer of macromolecules to the gas phase while limiting decomposition; these new capabilities revolutionized protein sequencing. In one technique, proteins are placed in a light-absorbing matrix. With a short pulse of laser light, the proteins are ionized and then desorbed from the matrix into the vacuum system. This process, known as **matrix-assisted laser desorption/ionization mass spectrometry**, or **MALDI MS**, is used to measure the mass of macromolecules. In a second method, macromolecules in solution are forced directly from the liquid phase to the gas phase. A solution of analytes is passed through a charged needle that is kept at a high electrical potential, dispersing the solution into a fine mist of charged microdroplets. The solvent surrounding the macromolecules rapidly evaporates, leaving multiply charged macromolecular ions in the gas phase. This technique is called **electrospray ionization mass spectrometry**, or **ESI MS**. Protons added during passage through the needle give additional charge to the macromolecule. The m/z of the molecule can then be analyzed in the vacuum chamber. One method for analyzing m/z is called **time of flight** or **TOF**, in which ion acceleration in an electric field depends on m/z. A newer, more-efficient method is the **Orbitrap**, in which ions are trapped in orbit between an outer barrel-shaped electrode and an inner spindle electrode. The trajectory of the electrons, related to their mass and charge, is detected and converted to m/z by a Fourier transform.

The process for determining the molecular mass of a protein with ESI MS is illustrated in Figure 3-28. As a protein is injected into the gas phase, it acquires a variable number of protons, and thus positive charges, from the solvent. The variable addition of these charges creates a spectrum of species with different mass-to-charge ratios. Each successive peak corresponds to a species that differs from that of its neighboring peak by a charge of 1 and a mass of 1 (one proton). The mass of the protein can be determined from any two neighboring peaks.

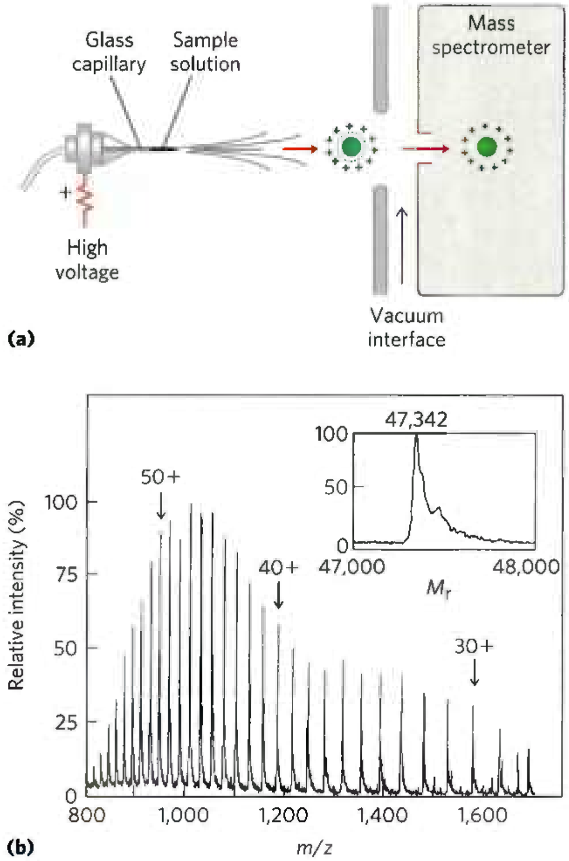


FIGURE 3-28 Electrospray ionization mass spectrometry of a protein. (a) A protein solution is dispersed into highly charged droplets by passage through a needle under the influence of a high-voltage electric field. The droplets evaporate, and the ions (with added protons in this case) enter the mass spectrometer for m/z measurement. (b) The spectrum generated is a family of peaks, with each successive peak (from right to left) corresponding to a charged species with both mass and charge increased by 1. The inset shows a computer-generated transformation of this spectrum. [Information from M. Mann and M. Wilm, *Trends Biochem. Sci.* 20:219, 1995.]

Amino acid sequence information is extracted using a technique called **tandem MS**, or **MS/MS**. A solution containing the protein or multiple proteins under investigation is first treated with a protease (often trypsin, due to its high specificity) to hydrolyze it to a mixture of shorter peptides. The mixture is then injected into a mass spectrometer that has two mass filters in tandem (**Fig. 3-29a**, top). In the first, the peptide mixture is sorted so that only one of the several types of peptides produced by cleavage emerges at the other end. The sample of the selected peptide, each molecule of which has a charge somewhere along its length, then travels through a vacuum chamber between the two mass spectrometers. In this collision cell, the peptide is further fragmented by high-energy impact with a "collision gas" such as helium or argon that is bled into the vacuum chamber. Each individual peptide is broken in only one place, on average. Although the breaks are not hydrolytic, most occur at the peptide bonds.

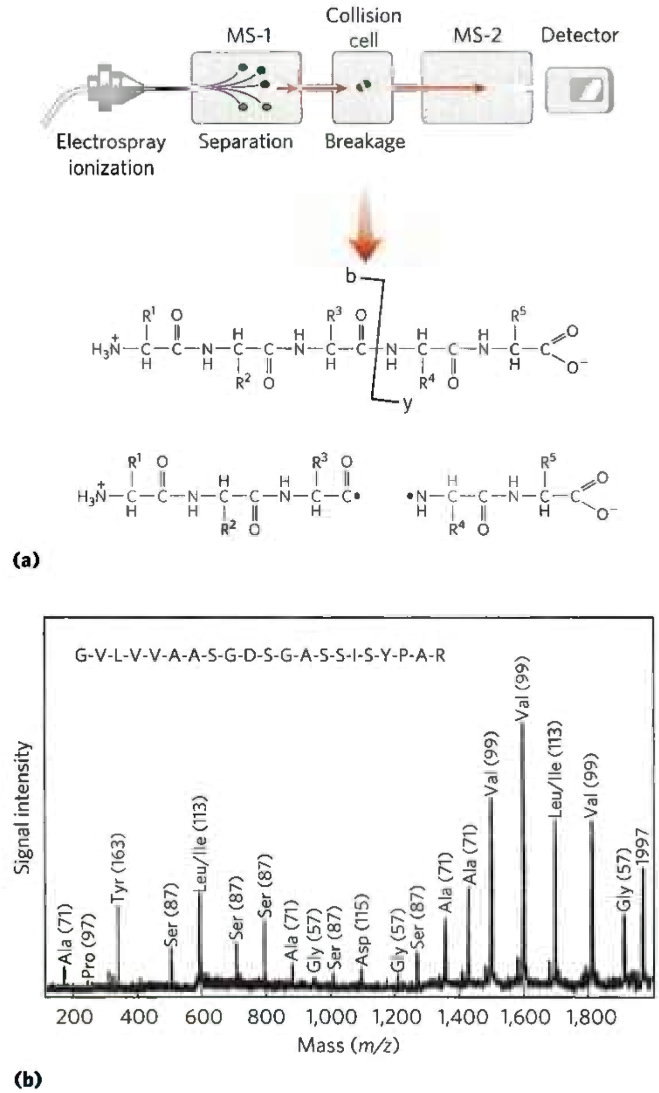


FIGURE 3-29 Obtaining protein sequence information with tandem MS. (a) After proteolytic hydrolysis, a protein solution is injected into a mass spectrometer (MS-1). The different peptides are sorted so that only one type is selected for further analysis. The selected peptide is further fragmented in a chamber between the two mass spectrometers, and *m/z* for each fragment is measured in the second mass spectrometer (MS-2). Many of the ions generated during this second fragmentation result from breakage of the peptide bond, as shown. These are called b-type or y-type ions, depending on whether the charge is retained on the amino- or carboxyl-terminal side, respectively. (b) A typical spectrum with peaks representing the peptide fragments generated from a sample of one small peptide (21 residues). The labeled peaks are y-type ions derived from amino acid residues. The number in parentheses over each peak is the molecular weight of the amino acid ion. The successive peaks differ by the mass of a particular amino acid in the original peptide. The deduced sequence is shown at the top. [Information from T. Keough et al., *Proc. Natl. Acad. Sci. USA* 96:7131, 1999, Fig. 3.]

The second mass filter then measures the *m/z* ratios of all the charged fragments. This process generates one or more sets of peaks. A given set of peaks (Fig. 3-29b) consists of all the charged fragments that were generated by breaking the same type of bond (but at different points in the peptide). One set of peaks includes only the fragments in which the charge was retained on the amino-terminal side of the broken bonds; another includes only the fragments in which the charge was retained on the carboxyl-terminal side of the broken bonds. Each successive peak in a given set has one less amino acid than the peak before. The difference in mass from peak to peak identifies the amino acid that was lost in each case, thus revealing the sequence of the peptide. The only ambiguities involve leucine and isoleucine, which have the same mass. Although multiple sets of peaks usually are generated, the two most prominent sets generally consist of charged fragments derived from breakage of the peptide bonds. The amino acid sequence derived from one set can be confirmed by the other, improving the confidence in the sequence information obtained.

The analysis of complex mixtures of proteins—even entire cellular proteomes—is facilitated by liquid chromatography (LC) that is integrated into the instrument (**LC-MS/MS**). The organism of interest is generally one in which the genomic sequence is known. Cellular proteins are first isolated in an extract, then digested into relatively short peptides by a protease such as trypsin. The very complex mixture of peptides is subjected to chromatography, so that resolved peptides are introduced to the mass spectrometer successively. Transfer from the liquid phase to the gas phase is facilitated by MALDI or ESI. Each peptide is analyzed for amino acid sequence, and that sequence is compared to the known genomic sequence available in databases to identify the protein it came from. Because more peptides are generated from the more common proteins in the mixture, the exercise also provides a measure of protein abundance. MS/MS scans of dozens of different peptides can be generated in less than a second. The entire proteome of a yeast cell can be analyzed in less than an hour.

Mass spectrometry provides a wealth of information for proteomics research, enzymology, and protein chemistry in general. The accurately measured molecular mass of a protein is critical to its identification. Changes in the cellular proteome can be monitored as a function of metabolic state or environmental conditions. Mass changes in the peptides scanned during a proteome analysis can reveal protein modifications of all kinds. Amino acid sequencing can reveal changes in protein sequence that result from the editing of messenger RNA in eukaryotes (Chapter 26). These methods, along with modern DNA sequencing processes (Chapter 8), are all part of a robust toolbox used to probe biological information at many levels.

Small Peptides and Proteins Can Be Chemically Synthesized

Many peptides are potentially useful as pharmacologic agents, and their production is of considerable commercial importance. In addition to its commercial applications, the synthesis of specific peptide portions of larger proteins is an increasingly important tool for the study of protein structure and function. There are three ways to obtain a peptide: (1) purification from tissue, a task often made difficult by the vanishingly low concentrations of some peptides; (2) genetic engineering (Chapter 9); and (3) direct chemical synthesis. Powerful techniques now make direct chemical synthesis an attractive option in many cases.

The complexity of proteins makes the traditional synthetic approaches of organic chemistry impractical for peptides with more than four or five amino acid residues. One problem is the difficulty of purifying the product after each step.

The major breakthrough in this technology was provided by R. Bruce Merrifield in 1962. His innovation was to synthesize a peptide while keeping one end attached to a solid support. The support is an insoluble polymer (resin) contained within a column, similar to that used for chromatographic procedures. The peptide is built up on this support one amino acid at a time, through a standard set of reactions in a repeating cycle (**Fig. 3-30**). At each successive step in the cycle, protective chemical groups block unwanted reactions.

The technology for chemical peptide synthesis has been automated. An important limitation of the process is the efficiency of each chemical cycle. Incomplete reaction at one stage can lead to formation of an impurity (in the form of a shorter peptide) in the next. The chemistry has been optimized to permit the synthesis of proteins of 100 amino acid residues in a few days in reasonable yield. A very similar approach is used to synthesize nucleic acids (see Fig. 8-33). It is worth noting that this technology, impressive as it is, still pales when compared with biological processes. The same 100-residue protein would be synthesized with exquisite fidelity in about 5 seconds in a bacterial cell.

Methods for the efficient ligation (joining together) of peptides allow the assembly of synthetic peptides into larger polypeptides and proteins. Novel forms of proteins can be created with precisely positioned chemical groups, including those that might not normally be found in a cellular protein. This provides one approach to test theories of enzyme catalysis, to create proteins with altered chemical properties, and to design protein sequences that will fold into particular structures. This last application provides the ultimate test of our ability to relate the primary structure of a peptide to the three-dimensional structure that it takes up in solution.

Amino Acid Sequences Provide Important Biochemical Information

P4 Knowledge of the sequence of amino acids in a protein can offer insights into its three-dimensional structure and its function, cellular location, and evolution. Most of these insights are derived by searching for similarities between a protein of interest and previously studied proteins. Comparison of a newly obtained sequence with sequence data in international repositories often reveals relationships both surprising and enlightening.

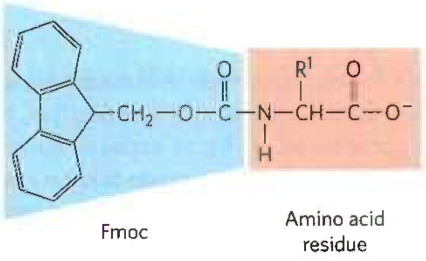
We do not understand in detail exactly how the amino acid sequence determines three-dimensional structure, nor can we always predict function from sequence. However, protein families that have some shared structural or functional features can be readily identified on the basis of amino acid sequence similarities. Individual proteins are assigned to families based on the degree of similarity in amino acid sequence. Members of a family are usually identical across 25% or more of their sequences, and proteins in these families generally share at least some structural and functional characteristics. Some families, however, are defined by identities involving only a few amino acid residues that are critical to a certain function. A number of similar substructures, or "domains" (to be defined more fully in Chapter 4), occur in many functionally unrelated proteins. These domains often fold into structural configurations that have an unusual degree of stability or that are specialized for a certain environment. Evolutionary relationships can also be inferred from the structural and functional similarities within protein families.

Certain amino acid sequences serve as signals that determine the cellular location, chemical modification, and half-life of a protein. Special signal sequences, usually at the amino terminus, are used to target certain proteins for export from the cell; other proteins are targeted for distribution to the nucleus, the cell surface, the cytosol, or other cellular locations. Other sequences act as attachment sites for prosthetic groups, such as sugar groups in glycoproteins and lipids in lipoproteins. Some of these signals are well characterized and are easily recognized in the sequence of a newly characterized protein (Chapter 27).

KEY CONVENTION Much of the functional information encapsulated in protein sequences comes in the form of **consensus sequences.** This term is applied to such sequences in DNA, RNA, or protein. When a series of related nucleic acid sequences or protein sequences are compared, a consensus sequence is the one that reflects the most common base or amino acid at each position. Parts of the sequence that have particularly good agreement often represent evolutionarily conserved functional domains. Mathematical tools available online can generate consensus sequences or identify them in sequence databases. **Box 3-2** illustrates common conventions for displaying consensus sequences. ■

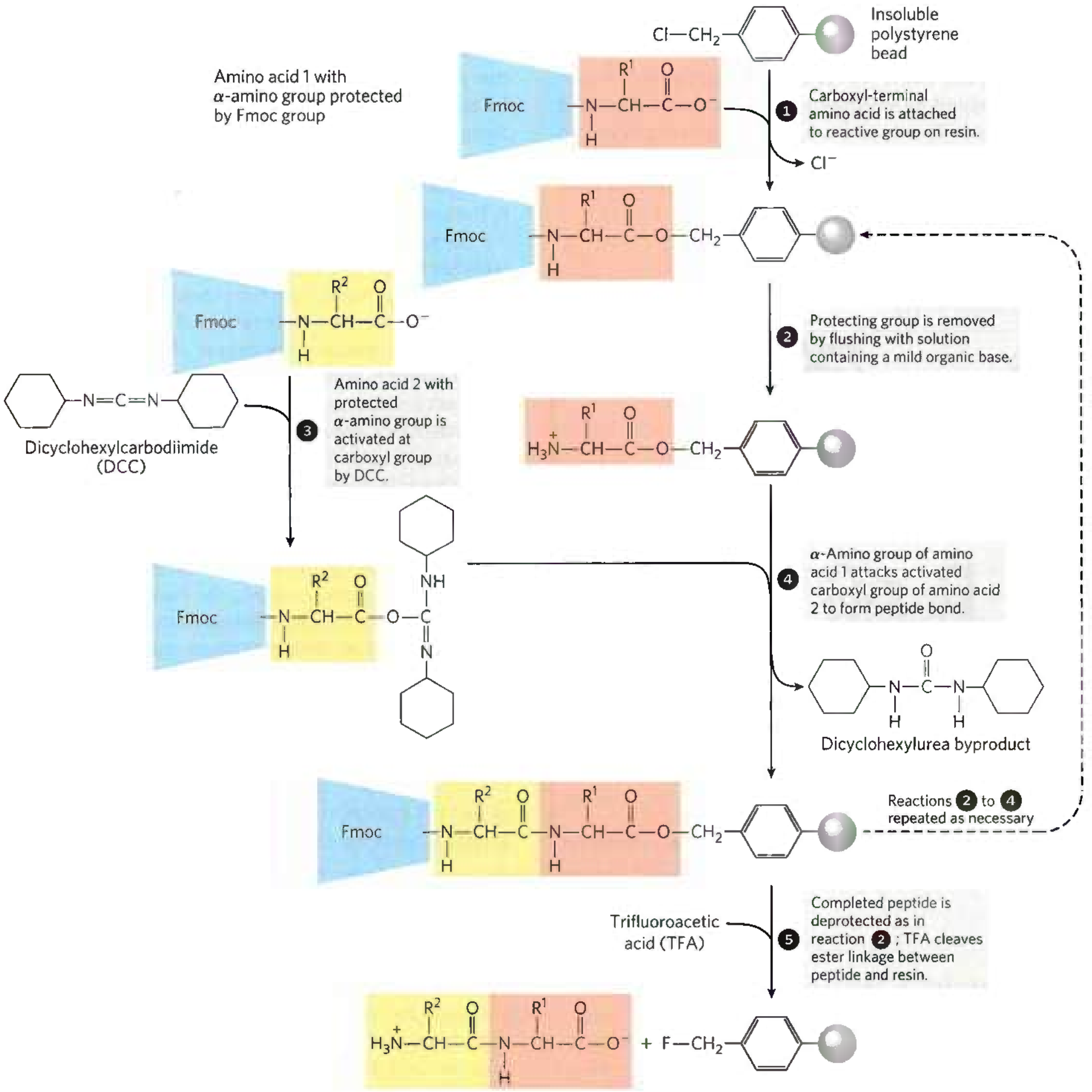
Protein Sequences Help Elucidate the History of Life on Earth

The simple string of letters denoting the amino acid sequence of a protein holds a surprising wealth of information, which is being unlocked by applying the tools of **bioinformatics** to genomic and protein sequence data.



Fmoc
Amino acid residue
R^1

FIGURE 3-30 Chemical synthesis of a peptide on an insoluble polymer support. Reactions ① through ④ are necessary for the formation of each peptide bond. The 9-fluorenylmethoxycarbonyl (Fmoc) group (shaded blue) prevents unwanted reactions at the α-amino group of the residue (shaded light red). Chemical synthesis proceeds from the carboxyl terminus to the amino terminus, the reverse of the direction of protein synthesis in vivo.



Insoluble polystyrene bead
Amino acid 1 with α-amino group protected by Fmoc group
Fmoc
①
Carboxyl-terminal amino acid is attached to reactive group on resin.
Cl^-
②
Protecting group is removed by flushing with solution containing a mild organic base.
Dicyclohexylcarbodiimide (DCC)
③
Amino acid 2 with protected α-amino group is activated at carboxyl group by DCC.
④
α-Amino group of amino acid 1 attacks activated carboxyl group of amino acid 2 to form peptide bond.
Dicyclohexylurea byproduct
Reactions ② to ④ repeated as necessary
Trifluoroacetic acid (TFA)
⑤
Completed peptide is deprotected as in reaction ②; TFA cleaves ester linkage between peptide and resin.

P4 Each protein's function relies on its three-dimensional structure, which in turn is determined largely by its primary structure. Thus, the biochemical information conveyed by a protein sequence is limited only by our understanding of structural and functional principles. The constantly evolving tools of bioinformatics make it possible to identify functional segments in new proteins and also help to establish both their sequence and their structural relationships to proteins already in the databases. On a different level of inquiry, protein sequences

BOX 3-2

Consensus Sequences and Sequence Logos

Consensus sequences can be represented in several ways. To illustrate two types of conventions, we use two examples of consensus sequences (Fig. 1): an ATP-binding structure called a P loop (see Fig. 12-2) and a Ca^{2+}-binding structure called an EF hand (see Fig. 12-17). The rules described here are adapted from those used by the sequence comparison website PROSITE (http://prosite.expasy.org/sequence_logo.html), using the standard one-letter codes for the amino acids.

In one type of consensus sequence designation, shown at the top of (a) and (b) in Figure 1, each position is separated from its neighbor by a hyphen. A position where any amino acid is allowed is designated x. Ambiguities are indicated by listing the acceptable amino acids for a given position between square brackets. For example, in (a), [AG] means Ala or Gly. If all but a few amino acids are allowed at one position, the amino acids that are *not* allowed are listed between curly brackets. For example, in (b), {W} means any amino acid except Trp. Repetition of an element of the pattern is indicated by following that element with a number or range of numbers between parentheses. In (a), for example, x(4) means x-x-x-x; x(2,4) means x-x, or x-x-x, or x-x-x-x. When a pattern is restricted to either the amino terminus or carboxyl terminus of a sequence, that pattern starts with < or ends with >, respectively (not so for either example here). A period ends the pattern. Applying these rules to the consensus sequence in (a), either A or G can be found at the first position. Any amino acid can occupy the next four positions, followed by an invariant G and an invariant K. The last position is either S or T.

Sequence logos provide a more informative and graphic representation of an amino acid (or nucleic acid) multiple sequence alignment. Each logo consists of a stack of symbols for each position in the sequence. The overall height of the stack (in bits) indicates the degree of sequence conservation at that position, whereas the height of each symbol (letter) in the stack indicates the relative frequency of that amino acid (or nucleotide). For amino acid sequences, the colors denote the characteristics of the amino acid: polar (G, S, T, Y, C, Q, N), green; basic (K, R, H), blue; acidic (D, E), red; and hydrophobic (A, V, L, I, P, W, F, M), black. The classification of amino acids in this scheme is somewhat different from the classification in Table 3-1 and Figure 3-5. The amino acids with aromatic side chains are subsumed into the nonpolar (F, W) and polar (Y) classifications. Glycine, always hard to classify, is assigned to the polar group. Note that when multiple amino acids are acceptable at a particular position, they rarely occur with equal probability. One or a few usually predominate. The logo representation makes the predominance clear, and a conserved sequence in a protein is made obvious. However, the logo obscures some amino acid residues that may be allowed at a position, such as the Cys that occasionally occurs at position 8 of the EF hand in (b).

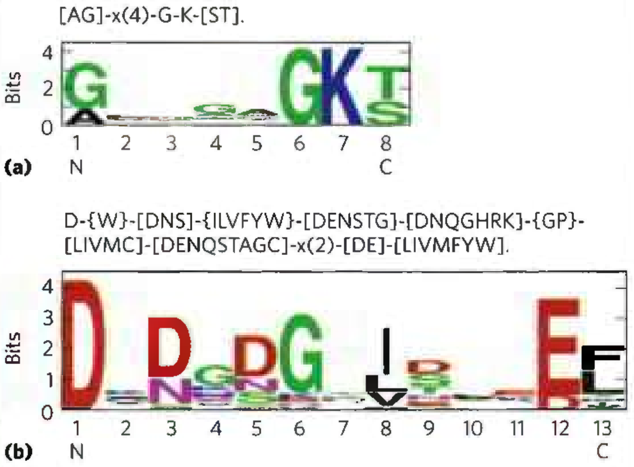


FIGURE 1 Representations of two consensus sequences. (a) P loop, an ATP-binding structure; (b) EF hand, a Ca^{2+}-binding structure. [Sequence data for (a) from document ID PDOC00017 and for (b) from document ID PDOC00018, www.expasy.org/prosite, N. Hulo et al., *Nucleic Acids Res.* 34:D227, 2006. Sequence logos created with WebLogo, http://weblogo.berkeley.edu, G. E. Crooks et al., *Genome Res.* 14:1188, 2004.]

are beginning to tell us how the proteins evolved and, ultimately, how life evolved on this planet.

The field of molecular evolution is often traced to Emile Zuckerkandl and Linus Pauling, whose work in the mid-1960s advanced the use of nucleotide and protein sequences to explore evolution. The premise is deceptively straightforward. If two organisms are closely related, the sequences of their genes and proteins should be similar. The sequences increasingly diverge as the evolutionary distance between two organisms increases. The promise of this approach began to be realized in the 1970s, when Carl Woese used ribosomal RNA sequences to define the Archaea as a group of living organisms distinct from the Bacteria and Eukarya. The information in genome and protein sequence databases can be used to trace biological history if we can learn to read the genetic hieroglyphics.

Evolution has not taken a simple linear path. **P4** For a given protein, the amino acid residues essential for the activity of the protein are conserved over evolutionary time. The residues that are less important to function may vary over time—that is, one amino acid may substitute for another—and these variable residues can provide the information to trace evolution. Some proteins have more variable amino acid residues than others. For these and other reasons, different proteins evolve at different rates.

Another complicating factor in tracing evolutionary history is the rare transfer of a gene or a group of genes from one organism to another, a process called **horizontal gene transfer**. The transferred genes may be similar to the genes they were derived from in the original organism, whereas most other genes in the two organisms may be only distantly related. An example of horizontal gene transfer is the recent rapid spread of antibiotic-resistance genes in bacterial populations. The proteins derived from these transferred genes would not be good candidates for the study of bacterial evolution, because they share only a very limited evolutionary history with their "host" organisms.

The study of molecular evolution generally focuses on families of closely related proteins. In most cases, the families chosen for analysis have essential functions in cellular metabolism that must have been present in the earliest viable cells, thus greatly reducing the chance that they were introduced relatively recently by horizontal gene transfer. For example, a protein called EF-1α (elongation factor 1α) is involved in the synthesis of proteins in all eukaryotes. A similar protein, EF-Tu, with the same function, is found in bacteria. Similarities in sequence and function indicate that EF-1α and EF-Tu are members of a family of proteins that share a common ancestor. The members of protein families are called **homologous proteins**, or **homologs**. The concept of a homolog can be further refined. If two proteins in a family (that is, two homologs) are present in the same species, they are referred to as **paralogs**. Homologs from different species are called **orthologs**. The process of tracing evolution involves first identifying suitable families of homologous proteins and then using them to reconstruct evolutionary paths.

Homologs are identified using computer programs that can directly compare specific protein sequences or that can search databases to identify any protein with an amino acid that matches within defined parameters. The electronic search process can be thought of as sliding one sequence past the other until a section with a good match is found. Within this sequence alignment, a positive score is assigned for each position where the two sequences are identical, and a negative score is introduced wherever gaps need to be introduced in one sequence or the other to bring them into register. The overall score provides a measure of the quality of the alignment (**Fig. 3-31**). The program selects the alignment with the optimal score that maximizes identical amino acid residues while minimizing the introduction of gaps.

Finding identical amino acids is often inadequate in attempts to identify related proteins or, more importantly, to determine how closely related the proteins are on an evolutionary time scale. A more useful analysis also considers the chemical properties of substituted amino acids. Many of the amino acid differences within a protein family may be conservative—that is, an amino acid residue is replaced by a residue that has similar chemical properties. For example, a Glu residue may substitute in one family member for the Asp residue found in another; both amino acids are negatively charged. Logically, such a conservative substitution should receive a higher score in a sequence alignment than a nonconservative substitution does—for example, in replacement of the Asp residue with a hydrophobic Phe residue.

P4 For most efforts to find homologies and explore evolutionary relationships, protein sequences are superior to nucleic acid sequences that do not encode a protein or functional RNA. For a nucleic acid, with its four different types of residues, random alignment of nonhomologous sequences will generally yield matches for at least 25% of the positions. Introduction of a few gaps can often increase the fraction of matched residues to 40% or more, and the probability of chance alignment of unrelated sequences becomes quite high. The 20 different amino acid residues in proteins greatly lower the probability of uninformative chance alignments of this type.

The programs used to generate a sequence alignment are complemented by methods that test the reliability of the alignments. A common computerized test is to shuffle the amino acid sequence of one of the proteins being compared in order to produce a random sequence, then to instruct the program to align the shuffled sequence with the other, unshuffled one. Scores are assigned to the new alignment, and the shuffling and alignment process is repeated many times. The

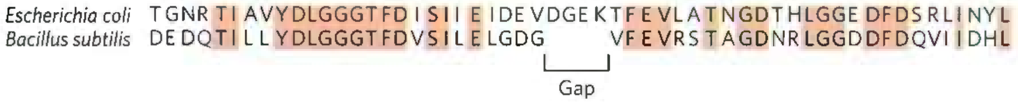
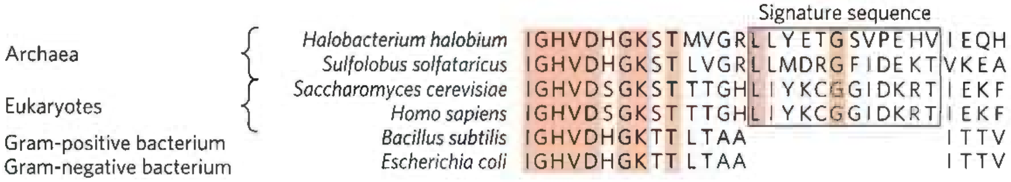

Escherichia coli TGNRTIAVYDLGGGTFDISIIEIDEVDGEKTFEVLATNGDTHLGGEDFDSRLINYL
Bacillus subtilis DEDQTILLYDLGGGTFDVSILELGDG VFEVRSTAGDNRLGGDDFDQVIIDHL

FIGURE 3-31 Aligning protein sequences with the use of gaps. Shown here is the sequence alignment of a short section of the Hsp70 proteins (a widespread class of protein-folding chaperones) from two well-studied bacterial species, *E. coli* and *Bacillus subtilis*. Introduction of a gap in the *B. subtilis* sequence allows a better alignment of amino acid residues on either side of the gap. Identical amino acid residues are shaded. [Information from R. S. Gupta, *Microbiol. Mol. Biol. Rev.* 62:1435, 1998, Fig. 2.]



			Signature sequence	
Archaea	*Halobacterium halobium*	IGHVDHGKSTMVGR	LLYETGSVPEHV	IEQH
	Sulfolobus solfataricus	IGHVDHGKSTLVGR	LLMDRGFIDEKT	VKEA
Eukaryotes	*Saccharomyces cerevisiae*	IGHVDSGKSTTTGH	LIYKCGGIDKRT	IEKF
	Homo sapiens	IGHVDSGKSTTTGH	LIYKCGGIDKRT	IEKF
Gram-positive bacterium	*Bacillus subtilis*	IGHVDHGKTTLTAA		ITTV
Gram-negative bacterium	*Escherichia coli*	IGHVDHGKTTLTAA		ITTV

FIGURE 3-32 A signature sequence in the EF-1α/EF-Tu protein family. The signature sequence (boxed) is a 12-residue insertion near the amino terminus of the sequence. Residues that align in all species are shaded. Both archaea and eukaryotes have the signature, although the sequences of the insertions are distinct for the two groups. The variation in the signature sequence reflects the significant evolutionary divergence that has occurred at this site since it first appeared in a common ancestor of both groups. [Information from R. S. Gupta, *Microbiol. Mol. Biol. Rev.* 62:1435, 1998, Fig. 7.]

original alignment, before shuffling, should have a score significantly higher than any of those within the distribution of scores generated by the random alignments; this increases the confidence that the sequence alignment has identified a pair of homologs. Note that the absence of a significant alignment score does not necessarily mean that no evolutionary relationship exists between two proteins. As we shall see in Chapter 4, three-dimensional structural similarities sometimes reveal evolutionary relationships where sequence homology has been wiped away by time.

To use a protein family to explore evolution, researchers identify family members with similar molecular functions in the widest possible range of organisms. The sequence divergence in these protein families allows segregation of organisms into classes based on their evolutionary relationships. Certain segments of a protein sequence may be found in the organisms of one taxonomic group but not in other groups; these segments can be used as **signature sequences** for the group in which they are found. An example of a signature sequence is an insertion of 12 amino acids near the amino terminus of the EF-1α/EF-Tu proteins in all archaea and eukaryotes but not in bacteria (**Fig. 3-32**). This particular signature is one of many biochemical clues that can help establish the evolutionary relatedness of eukaryotes and archaea.

P4 By considering the sequences of multiple proteins, researchers can construct elaborate evolutionary trees. **Figure 3-33** presents one such tree for 10,462 bacterial species, based on the sequences of 120 proteins ubiquitous in bacteria. In Figure 3-33, the free end points of lines are called "external nodes"; each represents an extant species, and each is so labeled. The points where two lines come together, the "internal nodes," represent extinct ancestor species. In most representations (including Fig. 3-33), the lengths of the lines connecting the nodes reflect amino acid substitutions in the selected proteins that separate one species from another. The use of 120 different proteins permits calibration and a more accurate determination of the time required for the various species to diverge.

As more sequence information is made available in databases, we move toward one of the core goals of biology — creating a detailed tree of life that describes the evolution and relationship of every organism on Earth. The story is a work in progress. The questions being asked and answered are fundamental to how humans view themselves and the world around them.

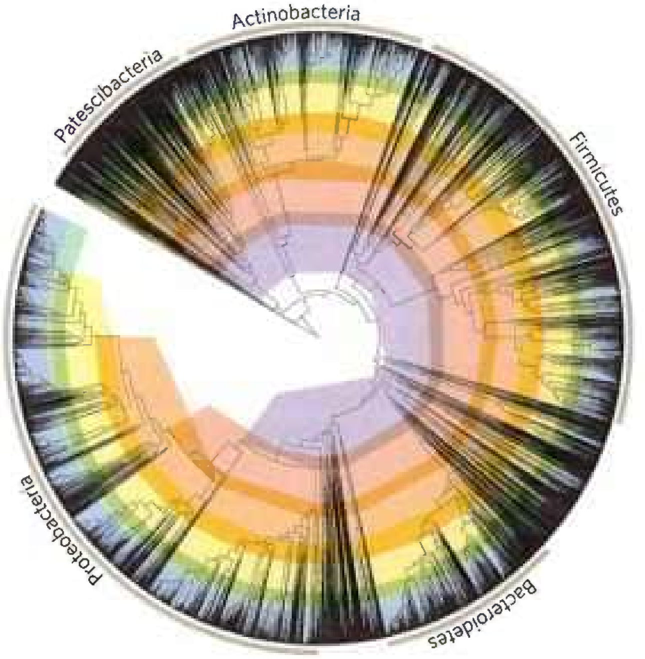


FIGURE 3-33 Evolutionary tree derived from amino acid sequence comparisons. This tree includes data from 10,462 bacterial species. The leaf nodes (points of intersection with the outer circle) represent extant species. Inner nodes (points where lines emanating from the leaf nodes come together) represent extinct ancestral species. Line lengths correspond to evolutionary time, as measured by sequence divergence in 120 proteins common to all bacteria. The major bacterial phyla are denoted by lines encompassing parts of the outer circle. [Information from D. H. Parks, *Nat. Biotechnol.* 36:996, 2018, Fig. 1c.]

SUMMARY 3.4 *The Structure of Proteins: Primary Structure*

- Differences in protein function result from differences in amino acid composition and sequence. The chemical properties of particular amino acid residues are often critical to the function of a protein.

- Most amino acid sequences are deduced from genomic sequences and by mass spectrometry. Methods derived from classical approaches to protein sequencing remain important in protein chemistry.
- Short proteins and peptides (up to about 100 residues) can be chemically synthesized. The peptide is built up, one amino acid residue at a time, while tethered to a solid support.
- Protein sequences are a rich source of information about protein structure and function. Bioinformatics can analyze changes in the amino acid sequences of homologous proteins over time to trace the evolution of life on Earth.

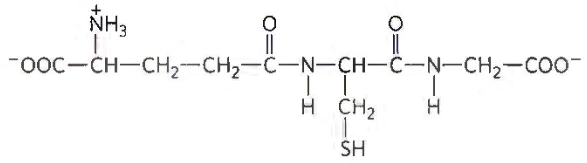
KEY TERMS

Terms in bold are defined in the glossary.

amino acids	70
residue	70
R group	71
chiral center	71
enantiomers	71
absolute configuration	72
D, L system	72
polarity	73
absorbance, *A*	75
zwitterion	77
amphoteric	77
ampholyte	77
isoelectric pH (isoelectric point, pI)	79
peptide	80
protein	80
peptide bond	81
condensation	81
hydrolysis	81
oligopeptide	81
polypeptide	81
amino-terminal residue	81
carboxyl-terminal residue	81
oligomeric protein	82
protomer	82
conjugated protein	83
prosthetic group	83
crude extract	84
fraction	84
fractionation	84
dialysis	84
column chromatography	84
ion-exchange chromatography	84
cation-exchange resin	86
anion-exchange resin	86
size-exclusion chromatography	86
affinity chromatography	86
high-performance liquid chromatography (HPLC)	86
electrophoresis	87
sodium dodecyl sulfate (SDS)	88
isoelectric focusing	88
specific activity	90
primary structure	90
secondary structure	90
tertiary structure	90
quaternary structure	90
proteases	93
mass spectrometry	93
proteome	94
analyte	94
matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS)	94
electrospray ionization mass spectrometry (ESI MS)	94
tandem mass spectrometry (MS/MS)	94
consensus sequence	96
bioinformatics	96
horizontal gene transfer	99
homologous proteins	99
homologs	99
paralogs	99
orthologs	99
signature sequence	100

PROBLEMS

1. **Amino Acid Constituents of Glutathione** Glutathione is an important peptide antioxidant found in cells from bacteria to humans.

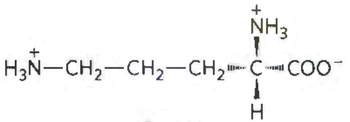


$$^{-}OOC-\underset{}{\overset{\overset{+}{N}H_3}{CH}}-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-\underset{H}{N}-\underset{\underset{SH}{CH_2}}{CH}-\overset{O}{\overset{\|}{C}}-\underset{H}{N}-CH_2-COO^{-}$$

Glutathione

Identify the three amino acid constituents of glutathione. What is unusual about glutathione's structure?

2. **Absolute Configuration of Ornithine** Ornithine is an amino acid that is not a building block of proteins. Instead, ornithine is an important intermediate in the urea cycle, the metabolic process that facilitates the excretion of ammonia waste products in animals.



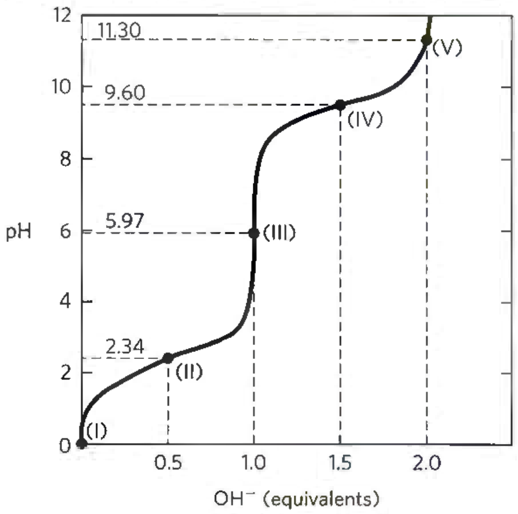
$$H_3\overset{+}{N}-CH_2-CH_2-CH_2-\underset{H}{\overset{\overset{+}{N}H_3}{C}}-COO^{-}$$

Ornithine

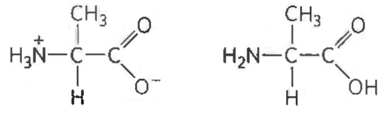
What is the absolute configuration of the ornithine molecule shown here?

3. **Relationship between the Titration Curve and the Acid-Base Properties of Glycine** A researcher titrated a 100 mL solution of 0.1 M glycine at pH 1.72 with 2 M NaOH solution. She then monitored the pH and plotted the results in the graph shown. The key points in the titration are designated I to V. For each of the following statements, *identify* the appropriate key point in the titration. Note that statement (k) applies to more than one key point in the titration.

(a) The pH is equal to the pK_a of the carboxyl group.
(b) The pH is equal to the pK_a of the protonated amino group.
(c) The predominant glycine species is $^+H_3N-CH_2-COOH$.
(d) The predominant glycine species is $^+H_3N-CH_2-COO^-$.
(e) Glycine exists as a 50:50 mixture of $^+H_3N-CH_2-COOH$ and $^+H_3N-CH_2-COO^-$.
(f) The *average* net charge of glycine is $+\frac{1}{2}$.
(g) Half of the amino groups are ionized.
(h) The *average* net charge of glycine is 0.
(i) The *average* net charge of glycine is –1.
(j) This is the isoelectric point for glycine.
(k) Glycine has its maximum buffering capacity at these regions.



4. Charge States of Alanine at Its pI At a pH equal to the isoelectric point (pI) of alanine, the *net* charge on alanine is zero. Two structures can be drawn that have a net charge of zero, but the predominant form of alanine at its pI is zwitterionic.

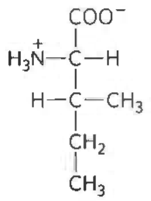

Zwitterionic Uncharged

(a) Why is alanine predominantly zwitterionic at its pI?
(b) What fraction of alanine is in the completely uncharged form at its pI?

5. Ionization State of Histidine Each ionizable group of an amino acid can exist in one of two states, charged or neutral. The electric charge on the functional group is determined by the relationship between its pK_a and the pH of the solution. This relationship is described by the Henderson-Hasselbalch equation.
(a) Histidine has three ionizable functional groups. Write the equilibrium equations for its three ionizations, and assign the proper pK_a for each ionization. Draw the structure of histidine in each ionization state. What is the net charge on the histidine molecule in each ionization state?
(b) Draw the structures of the predominant ionization state of histidine at pH 1, 4, 8, and 12. Note that you can approximate the ionization state by treating each ionizable group independently.
(c) What is the net charge of histidine at pH 1, 4, 8, and 12? For each pH, will histidine migrate toward the anode (+) or toward the cathode (−) when placed in an electric field?

6. Separation of Amino Acids by Ion-Exchange Chromatography We can analyze mixtures of amino acids by first separating the mixture into its components through ion-exchange chromatography. Amino acids placed on a cation-exchange resin (see Fig. 3-17a) containing sulfonate ($—SO_3^-$) groups flow down the column at different rates because of two factors that influence their movement: (1) ionic attraction between the sulfonate residues on the column and positively charged functional groups on the amino acids, and (2) aggregation of nonpolar amino acid side chains with the hydrophobic backbone of the polystyrene resin. Note that the ionic attraction is more significant than hydrophobicity for this column media. For each pair of amino acids listed, determine which will be eluted first from the cation-exchange column by a pH 7.0 buffer.
(a) Glutamate and lysine
(b) Arginine and methionine
(c) Aspartate and valine
(d) Glycine and leucine
(e) Serine and alanine

7. Naming the Stereoisomers of Isoleucine Consider the structure of the amino acid isoleucine.



(a) How many chiral centers does isoleucine have?
(b) How many optical isomers does isoleucine have?
(c) Draw perspective formulas for all the optical isomers of isoleucine.

8. Comparing the pK_a Values of Alanine and Polyalanine The titration curve of alanine shows the ionization of two functional groups with pK_a values of 2.34 and 9.69, corresponding to the ionization of the carboxyl and the protonated amino groups, respectively. The titration of di-, tri-, and larger oligopeptides of alanine also shows the ionization of only two functional groups, although the experimental pK_a values are different. The table summarizes the trend in pK_a values.

Amino acid or peptide	pK_1	pK_2
Ala	2.34	9.69
Ala–Ala	3.12	8.30
Ala–Ala–Ala	3.39	8.03
Ala–$(Ala)_n$–Ala, $n \geq 4$	3.42	7.94

(a) Draw the structure of Ala–Ala–Ala. Identify the functional groups associated with pK_1 and pK_2.
(b) Why does the value of pK_1 *increase* with each additional Ala residue in the oligopeptide?
(c) Why does the value of pK_2 *decrease* with each additional Ala residue in the oligopeptide?

9. Bonds Form by Condensation The peptide bond is an amide, generated by eliminating the elements of water from the two amino acids so joined. From which groups are the three atoms of water eliminated?

10. The Size of Proteins Calculate the approximate molecular weight of a protein composed of 682 amino acid residues in a single polypeptide chain.

11. Relationship between the Number of Amino Acid Residues and Protein Mass Experimental results describing a protein's amino acid composition are useful to estimate the molecular weight of the entire protein. A quantitative amino acid analysis reveals that bovine cytochrome *c* contains 2% cysteine (M_r 121) by weight.
(a) Calculate the *approximate* molecular weight in daltons of bovine cytochrome *c* if the number of cysteine residues is 2.

Bovine chymotrypsinogen has a molecular mass of 25.6 kDa. Amino acid analysis shows that this enzyme is 4.7% Gly (M_r 75.1).
(b) Calculate how many glycine residues are present in a molecule of bovine chymotrypsinogen.

12. Subunit Composition of a Protein A protein has a molecular mass of 400 kDa when measured by size-exclusion chromatography. When subjected to gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), the protein gives three bands with molecular masses of 180, 160, and 60 kDa. When electrophoresis is carried out in the presence of SDS and dithiothreitol, three bands again form, this time with molecular masses of 160, 90, and 60 kDa. How many subunits does the protein have, and what is the molecular mass of each?

13. Net Electric Charge of Peptides A peptide has the sequence Glu–His–Trp–Ser–Gly–Leu–Arg–Pro–Gly.
(a) Calculate the net charge of the molecule at pH 3, 8, and 11. (Incorporation into a peptide can alter pK_a values somewhat,

but for this exercise, use pK_a values for side chains and terminal amino and carboxyl groups as given in Table 3-1.)
(b) Estimate the pI for this peptide.

14. Isoelectric Point of Histones Histones are proteins found in eukaryotic cell nuclei, tightly bound to DNA, which has many phosphate groups. The pI of histones is very high, about 10.8. What amino acid residues must be present in relatively large numbers in histones? In what way do these residues contribute to the strong binding of histones to DNA?

15. Solubility of Polypeptides One method for separating polypeptides makes use of their different solubilities. The solubility of large polypeptides in water depends on the relative polarity of their R groups, particularly on the number of ionized groups: the more ionized groups there are, the more soluble the polypeptides are. Which of each pair of polypeptides is more soluble at the indicated pH?

(a) $(Gly)_{20}$ or $(Glu)_{20}$ at pH 7.0
(b) $(Lys–Val)_3$ or $(Phe–Cys)_3$ at pH 7.0
(c) $(Ala–Ser–Gly)_5$ or $(Asn–Ser–His)_5$ at pH 6.0
(d) $(Ala–Asp–Phe)_5$ or $(Asn–Ser–His)_5$ at pH 3.0

16. Purification of an Enzyme A biochemist discovers and purifies a new enzyme, generating the purification table shown.

Procedure	Total protein (mg)	Activity (units)
1. Crude extract	10,000	68,000
2. Precipitation (salt)	5,000	65,000
3. Precipitation (pH)	4,000	56,000
4. Ion-exchange chromatography	70	49,000
5. Affinity chromatography	12	42,000
6. Size-exclusion chromatography	8	40,000

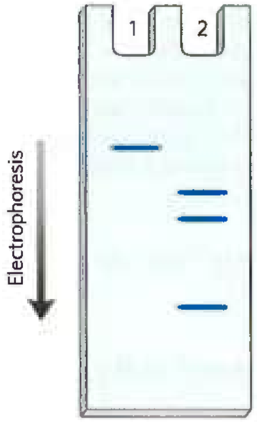
(a) From the information given in the table, calculate the specific activity of the enzyme after each purification procedure.
(b) Which of the purification procedures used for this enzyme is most effective (i.e., gives the greatest relative increase in purity)?
(c) Which of the purification procedures is least effective?
(d) Is there any indication based on the results shown in the table that the enzyme after step 6 is now pure? What else could be done to estimate the purity of the enzyme preparation?

17. De-salting a Protein by Dialysis A purified protein is in a Hepes (*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)) buffer at pH 7 with 500 mM NaCl. A dialysis membrane tube holds a 1 mL sample of the protein solution. The sample in the dialysis membrane floats in a beaker containing 1 L of the same Hepes buffer, but with 0 mM NaCl, for dialysis. Small molecules and ions (such as Na^+, Cl^-, and Hepes) can diffuse across the dialysis membrane, but the protein cannot.
(a) Calculate the concentration of NaCl in the protein sample, once the dialysis has come to equilibrium. Assume that no volume changes occur in the sample during the dialysis.
(b) Calculate the final NaCl concentration in the protein sample after dialysis in 250 mL of the same Hepes buffer, with 0 mM NaCl, twice in succession.

18. Predicting Cation Exchange Elution Order Suppose a column is filled with a cation-exchange resin at pH 7.0. In what order would the given peptides elute from the column if each has the same number of residues?

Peptide A: Ala 30%, Asp 10%, Lys 10%, Ser 15%, Pro 25%, Cys 10%
Peptide B: Ile 25%, Asp 20%, Arg 5%, Tyr 15%, His 5%, Thr 30%
Peptide C: Ala 40%, Glu 5%, Arg 20%, Ser 5%, His 5%, Trp 25%

19. Protein Analysis by Gel Electrophoresis Chymotrypsin is a protease with a molecular mass of 25.6 kDa. The figure shows a stained SDS polyacrylamide gel with a single band in lane 1 and three bands of lower molecular weight in lane 2. Lane 1 contains a preparation of chymotrypsin, and lane 2 contains chymotrypsin pretreated with performic acid. Why does performic acid treatment of chymotrypsin generate three bands in lane 2?



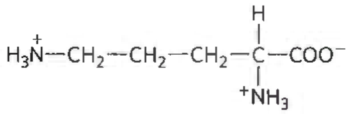
20. Sequence Determination of Leucine Enkephalin Suppose a researcher isolates a peptide from brain tissue that binds to the same receptor as opiate drugs. This peptide is an opioid leucine enkephalin, a class of endogenous peptides that act within the brain to lower pain sensation. The researcher performs a series of procedures to determine the peptide's sequence. First, she completely hydrolyzes the peptide by boiling it in 18% w/w HCl solution. Analysis of the hydrolysis products indicates the presence of Gly, Leu, Phe, and Tyr, in a 2:1:1:1 molar ratio. Second, she treats the peptide with 1-dimethylaminoaphthalene-5-sulfonyl chloride (dansyl chloride) before subjecting it to complete hydrolysis. Chromatography indicates the presence of the dansylamino acid derivative of tyrosine. No free tyrosine is present.
(a) Given the empirical composition and the results from the dansyl chloride reaction, where is Tyr located in the peptide?

Finally, the researcher incubates the peptide with chymotrypsin for two hours at 10 °C and analyzes the products using chromatography. Complete digestion of the peptide with chymotrypsin followed by chromatography yields free tyrosine and leucine, plus a tripeptide containing Phe and Gly in a 1:2 ratio.
(b) Give the final sequence for the peptide based on the results of the acid hydrolysis, dansyl chloride reaction, and the chymotryptic digestion.

21. Analysis of a Protein by Mass Spectrometry Investigators purify a protein produced by yeast grown under standard growth conditions. They incubate the protein with trypsin and sequence the peptides produced using mass spectrometry. One of the detected peptides, called peptide X, has the sequence Ala–Ser–Ala–Gly–Lys–Glu–Leu–Ile–Phe–Gln. The investigators then isolate the same protein, but this time from yeast grown under the stress of ultraviolet irradiation. When the sample is analyzed, a peptide with the mass of peptide X is no longer found. Instead, detection reveals a new peptide with the same sequence, except for an amino acid that replaces Ser and has a molecular mass of 167 Da. The investigators conclude that the protein has been altered in response to stress, and that the serine residue in the analyzed peptide has been modified. An unmodified series residue has a molecular mass of 87 Da. What modification might account for the change in the peptide's mass?

22. Structure of a Peptide Antibiotic from *Bacillus brevis* Extracts from the bacterium *Bacillus brevis* contain a peptide with antibiotic properties. This peptide forms complexes with metal ions and seems to disrupt ion transport across the cell membranes of other bacterial species, leading to bacterial death. The structure of the peptide has been determined from a series of observations.

(a) Complete acid hydrolysis of the peptide, followed by amino acid analysis, yielded equimolar amounts of Leu, Orn, Phe, Pro, and Val. Orn is ornithine, an amino acid not present in proteins but present in some peptides. Ornithine has the structure

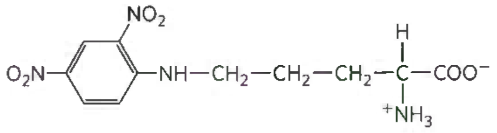


$$H_3\overset{+}{N}-CH_2-CH_2-CH_2-\underset{^{+}NH_3}{\overset{H}{C}}-COO^-$$

(b) The molecular weight of the peptide is approximately 1,200 Da.

(c) The peptide failed to undergo hydrolysis when treated with the enzyme carboxypeptidase. This enzyme catalyzes the hydrolysis of the carboxyl-terminal residue of a polypeptide unless the residue is Pro or, for some reason, does not contain a free carboxyl group.

(d) Treatment of the intact peptide with 1-fluoro-2,4-dinitrobenzene, followed by complete hydrolysis and chromatography, yielded only free amino acids and the derivative shown here.



(Hint: The 2,4-dinitrophenyl derivative involves the amino group of a side chain rather than the α-amino group.)

(e) Partial hydrolysis of the peptide followed by chromatographic separation and sequence analysis yielded these di- and tripeptides (the amino-terminal amino acid is always the first amino acid):

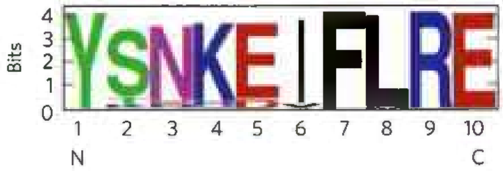
Leu–Phe Phe–Pro Orn–Leu Val–Orn
Val–Orn–Leu Phe–Pro–Val Pro–Val–Orn

Given this information, deduce the amino acid sequence of the peptide antibiotic. Show your reasoning. When you have arrived at a structure, demonstrate that it is consistent with *each* experimental observation.

23. Efficiency in Peptide Synthesis A peptide with the primary structure Lys–Arg–Pro–Leu–Ile–Asp–Gly–Ala must be synthesized by the methods developed by Merrifield. Calculate the percentage of the peptides synthesized that will be full length and have the correct sequence if the addition of each amino acid residue is 96% efficient. Do the calculation a second time but assume a 99% efficiency for each cycle.

24. Sequence Comparisons Proteins called molecular chaperones (described in Chapter 4) assist in the process of protein folding. One class of chaperones, found in organisms from bacteria to mammals, is heat shock protein 90 (Hsp90). All Hsp90 chaperones contain a 10 amino acid "signature sequence" that allows ready identification of these proteins in sequence databases. Two representations of this signature sequence are shown here.

Y-x-[NQHD]-[KHR]-[DE]-[IVA]-F-[LM]-R-[ED].



(a) In this sequence, which amino acid residues are invariant (conserved across all species)?

(b) At which position(s) are amino acids limited to those with positively charged side chains? For each position, which amino acid is more commonly found?

(c) At which positions are substitutions restricted to amino acids with negatively charged side chains? For each position, which amino acid predominates?

(d) There is one position that can be any amino acid, although one amino acid appears much more often than any other. What position is this, and which amino acid appears most often?

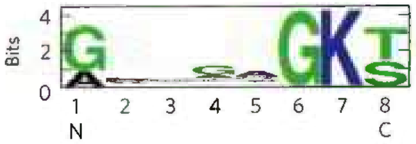
25. Chromatographic Methods Three polypeptides, the sequences of which are represented using the one-letter code for their amino acids, are present in a mixture:

1. ATKNRASCLVPKHGALMFWRHKQLVSDPILQKRQHILVCRNAAG
2. GPYFGDEPLDVHDEPEEG
3. PHLLSAWKGMEGVGKSQSFAALIVILA

(a) Of the three, which one would migrate most slowly during the following:

Anion-exchange chromatography?
Cation-exchange chromatography?
Size-exclusion (gel-filtration) chromatography?

(b) Which peptide contains the ATP-binding motif shown in the sequence logo?



DATA ANALYSIS PROBLEM

26. Determining the Amino Acid Sequence of Insulin Figure 3-24 shows the amino acid sequence of bovine insulin, determined by Frederick Sanger and his coworkers. Most of this work is described in a series of articles published in the *Biochemical Journal* from 1945 to 1955.

In 1945, researchers knew that insulin was a small protein consisting of two or four polypeptide chains linked by disulfide bonds. Sanger's team had developed a few simple methods for studying protein sequences.

Treatment with FDNB. FDNB (1-fluoro-2,4-dinitrobenzene) reacted with free amino (but not amide or guanidinium) groups in proteins to produce dinitrophenyl (DNP) derivatives of amino acids (see Fig. 3-25).

Acid Hydrolysis. Boiling a protein with 10% HCl for several hours hydrolyzed all of its peptide and amide bonds. Short treatments produced short polypeptides; the longer the treatment, the more complete the breakdown of the protein into its amino acids.

Oxidation of Cysteines. Treatment of a protein with performic acid cleaved all the disulfide bonds and converted all Cys residues to cysteic acid residues (see Fig. 3-26).

Paper Chromatography. This more primitive version of thin-layer chromatography (see Fig. 10-25) separated compounds based on their chemical properties, allowing identification of single amino acids and, in some cases, dipeptides. Thin-layer chromatography also separates larger peptides.

As reported in his first paper (1945), Sanger reacted insulin with FDNB and hydrolyzed the resulting protein. He found many free amino acids, but only three DNP–amino acids: α-DNP-glycine (DNP group attached to the α-amino group), α-DNP-phenylalanine, and ε-DNP-lysine (DNP attached to the ε-amino group). Sanger interpreted these results as showing that insulin had two protein chains: one with Gly at its amino terminus and one with Phe at its amino terminus. One of the two chains also contained a Lys residue, not at the amino terminus. Sanger named the chain beginning with a Gly residue "A" and the chain beginning with Phe "B."

(a) Explain how Sanger's results support his conclusions.

(b) Are the results consistent with the known structure of bovine insulin (see Fig. 3-24)?

In a later paper (1949), Sanger described how he used these techniques to determine the first few amino acids (amino-terminal end) of each insulin chain. To analyze the B chain, for example, he carried out the following steps:

1. Oxidized insulin to separate the A and B chains.
2. Prepared a sample of pure B chain with paper chromatography.
3. Reacted the B chain with FDNB.
4. Gently acid-hydrolyzed the protein so that some small peptides would be produced.
5. Separated the DNP-peptides from the peptides that did not contain DNP groups.
6. Isolated four of the DNP-peptides, which were named B1 through B4.
7. Strongly hydrolyzed each DNP-peptide to give free amino acids.
8. Identified the amino acids in each peptide with paper chromatography.

The results were as follows:

B1: α-DNP-phenylalanine only
B2: α-DNP-phenylalanine; valine
B3: aspartic acid; α-DNP-phenylalanine; valine
B4: aspartic acid; glutamic acid;
α-DNP-phenylalanine; valine

(c) Based on these data, what are the first four (amino-terminal) amino acids of the B chain? Explain your reasoning.

(d) Does this result match the known sequence of bovine insulin (Fig. 3-24)? Explain any discrepancies.

Sanger and colleagues used these and related methods to determine the entire sequence of the A and B chains. Their sequence for the A chain was as follows:

$$\overset{1}{\text{Gly}}\text{–Ile–Val–Glx–}\overset{5}{\text{Glx}}\text{–Cys–Cys–Ala–Ser–}\overset{10}{\text{Val}}\text{–}$$
$$\text{Cys–Ser–Leu–Tyr–}\overset{15}{\text{Glx}}\text{–Leu–Glx–Asx–Tyr–}\overset{20}{\text{Cys}}\text{–Asx}$$

Because acid hydrolysis had converted all Asn to Asp and all Gln to Glu, these residues had to be designated Asx and Glx, respectively (exact identity in the peptide unknown). Sanger solved this problem by using protease enzymes that cleave peptide bonds, but not the amide bonds in Asn and Gln residues, to prepare short peptides. He then determined the number of amide groups present in each peptide by measuring the NH_4^+ released when the peptide was acid-hydrolyzed. Some of the results for the A chain are shown below. The peptides may not have been completely pure, so the numbers were approximate—but good enough for Sanger's purposes.

Peptide name	Peptide sequence	Number of amide groups in peptide
Ac1	Cys–Asx	0.7
Ap15	Tyr–Glx–Leu	0.98
Ap14	Tyr–Glx–Leu–Glx	1.06
Ap3	Asx–Tyr–Cys–Asx	2.10
Ap1	Glx–Asx–Tyr–Cys–Asx	1.94
Ap5pa1	Gly–Ile–Val–Glx	0.15
Ap5	Gly–Ile–Val–Glx–Glx–Cys–Cys–Ala–Ser–Val–Cys–Ser–Leu	1.16

(e) Based on these data, determine the amino acid sequence of the A chain. Explain how you reached your answer. Compare your answer with Figure 3-24.

References

Sanger, F. 1945. The free amino groups of insulin. *Biochem. J.* 39:507–515.

Sanger, F. 1949. The terminal peptides of insulin. *Biochem. J.* 45:563–574.

Chapter 4

THE THREE-DIMENSIONAL STRUCTURE OF PROTEINS

4.1 Overview of Protein Structure *107*

4.2 Protein Secondary Structure *111*

4.3 Protein Tertiary and Quaternary Structures *116*

4.4 Protein Denaturation and Folding *128*

4.5 Determination of Protein and Biomolecular Structures *136*

Proteins are big molecules. The covalent backbone of a typical protein contains hundreds of individual bonds. Because free rotation is possible around many of these bonds, the protein can, in principle, assume a virtually uncountable number of spatial arrangements, or **conformations**. In reality, however, each protein has a specific chemical or structural function, which suggests that each has a unique three-dimensional structure (**Fig. 4-1**). How stable is this structure, what factors guide its formation, and what holds it together? By the late 1920s, several proteins had been crystallized, including hemoglobin (M_r 64,500) and the enzyme urease (M_r 483,000). Given that, generally, the ordered array of molecules in a crystal can form only if the molecular units are identical, crystallization was evidence that even very large proteins are discrete chemical entities with unique structures. However, we now know that protein structure is always malleable, and in sometimes surprising ways. Changes in structure can be as important to a protein's function as the structure itself.

In this chapter, we examine the structure of proteins. We emphasize five principles:

P1 **Protein structures are stabilized by noncovalent interactions and forces.** Formation of a thermodynamically favorable structure depends on the influences of the hydrophobic effect, hydrogen bonds, ionic interactions, and van der Waals forces. Natural protein structures are constrained by peptide bonds, whose configurations can be described by the dihedral angles ϕ and ψ.

P2 **Protein segments can adopt regular secondary structures such as the α helix and the β conformation.** These structures are defined by particular values of ϕ and ψ and their formation is impacted by the amino acid composition of the segment. All of the ϕ and ψ values for a given protein structure can be visualized using a Ramachandran plot.

P3 **Tertiary structure describes the well-defined, three-dimensional fold adopted by a protein.** Protein structures are often built by combinatorial use of common protein folds or motifs. Quaternary structure describes the interactions between components of a multisubunit assembly.

P4 **Tertiary structure is determined by amino acid sequence.** Even though protein folding is complex, some denatured proteins can spontaneously refold into their active conformation based only on the chemical properties of their constituent amino acids. Cellular proteostasis involves numerous pathways that regulate the folding, unfolding, and degradation of proteins. Many human diseases arise from protein misfolding and defects in proteostasis.

P5 **The three-dimensional structures of proteins can be defined.** Structural biologists use a variety of instruments and computational methods to solve biomolecular structures. The choice of method may depend on factors such as the size of the protein being studied, its properties, or the desired resolution of the final structure.

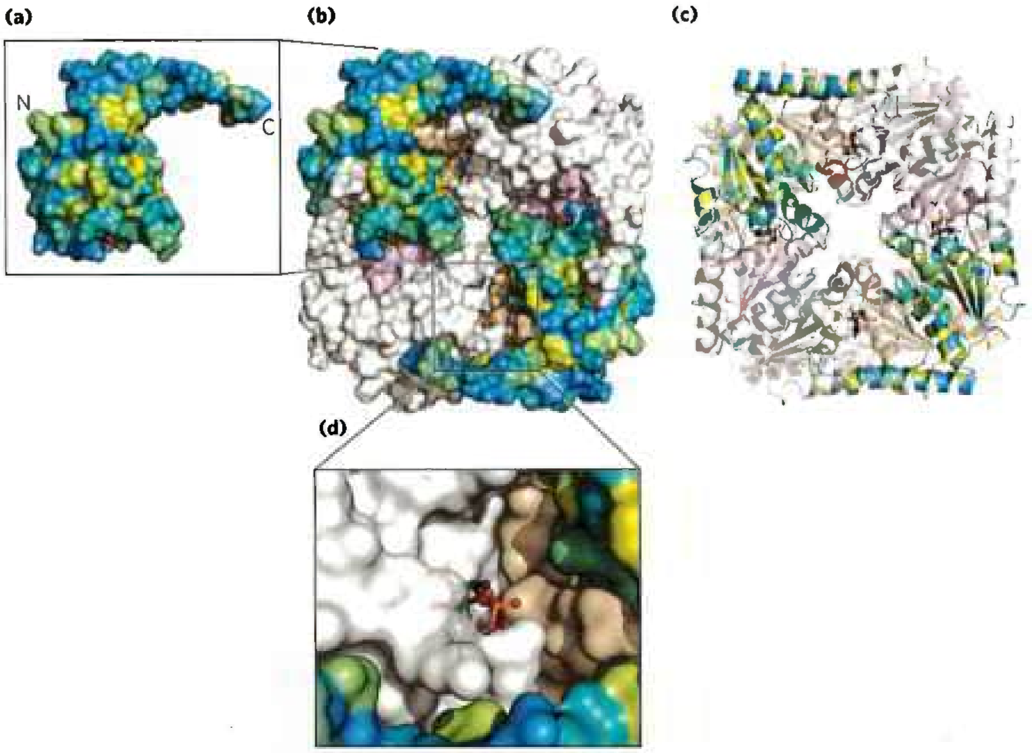


FIGURE 4-1 Relationship between protein structure and function. (a) The PurE enzyme from *Escherichia coli* catalyzes a reaction that forms carbon–carbon bonds in de novo purine biosynthesis. PurE is a small (17 kDa) single-domain protein. In this view, the protein surface of PurE has been modeled and colored by hydrophobicity: yellow for hydrophobic surfaces, blue for hydrophilic surfaces, and shades of green for those in between. It is apparent that the protein folds so that many of its polar groups are accessible to solvent. (b, c) The enzymatically active form of PurE is an octamer; eight PurE protomers combine to create a square-shaped quaternary structure with eight active sites. The structure in (b) is a surface representation; (c) is a ribbon diagram that traces the peptide backbone. Two protomers are colored by surface hydrophobicity. Others are shown in single colors (two each in gray, tan, and pink). (d) Each active site is formed using segments of three different protomers. A molecule of the reaction product carboxyaminoimidazole ribonucleotide bound at the active site is shown as a stick structure. [Data from PDB ID 2NSL, A. A. Hoskins et al., *Biochemistry* 46:2842, 2007.]

4.1 Overview of Protein Structure

The possible conformations of a protein or protein segment include any structural state it can achieve without breaking covalent bonds. A change in conformation could occur, for example, by rotation about single bonds. However, of the many conformations that are theoretically possible in a protein containing hundreds of single bonds, one or a few generally predominate under biological conditions. The need for multiple stable conformations reflects the changes that must take place in most proteins as they bind to other molecules or catalyze reactions. The conformations existing under a given set of conditions are usually the ones that are thermodynamically the most stable—that is, having the lowest free energy (G). Proteins in any of their functional, folded conformations are often called **native** proteins.

For the vast majority of proteins, a particular structure or small set of structures is critical to function. However, in many cases, parts of proteins lack discernible structure. These protein segments are intrinsically disordered. In some cases, entire proteins are intrinsically disordered, yet are fully functional.

What determines the most stable conformations of a typical protein? We can build an understanding of protein conformation stepwise from the discussion of primary structure in Chapter 3 through a consideration of secondary, tertiary, and quaternary structures. To this approach we must add emphasis on common and classifiable folding patterns, variously called supersecondary structures, folds, or motifs, which provide an important organizational context to this complex endeavor.

A Protein's Conformation Is Stabilized Largely by Weak Interactions

Stability is the tendency of a protein to maintain a native conformation. Native proteins are only marginally stable; the ΔG separating the folded and unfolded states in typical proteins under physiological conditions is in the range of only 5 to 65 kJ/mol. A given polypeptide chain can theoretically assume countless conformations, and as a result, the unfolded state of a protein is characterized by a high degree of conformational entropy. This entropy, along with the hydrogen-bonding interactions of many groups in the polypeptide chain with the solvent (water), tends to maintain the unfolded state. **P1** The chemical interactions that counteract these effects and stabilize the native conformation include disulfide (covalent) bonds and the weak (noncovalent) interactions and forces described in Chapter 2: hydrogen bonds, the hydrophobic effect, and ionic interactions.

Covalent disulfide bonds are strong, but they are also uncommon. The environment within most cells is highly reducing due to high concentrations of reductants such as glutathione, and most sulfhydryls will remain in the reduced state. Outside the cell, the environment is often more oxidizing, and disulfide formation is more likely to occur. In eukaryotes, disulfide bonds are found primarily in secreted, extracellular proteins (for example, the hormone insulin). Disulfide bonds are also uncommon in bacterial proteins. However, thermophilic bacteria, as well as the archaea, typically have many proteins with stabilizing disulfide bonds; this is presumably an adaptation to life at high temperatures.

For all proteins of all organisms, weak interactions are especially important in the folding of polypeptide chains into their secondary and tertiary structures. The association of multiple polypeptides to form quaternary structures also relies on these weak interactions.

About 200 to 460 kJ/mol are required to break a single covalent bond, whereas weak interactions can be disrupted by a mere 0.4 to 30 kJ/mol. Individual covalent bonds, such as disulfide bonds linking separate parts of a single polypeptide chain, are clearly much stronger than individual weak interactions. Yet, because they are so numerous, the weak interactions predominate as a stabilizing force in protein structure. **P1** In general, the protein conformation with the lowest free energy (that is, the most stable conformation) is the one with the maximum number of weak interactions.

The stability of a protein is not simply the sum of the free energies of formation of the many weak interactions within it. For every hydrogen bond formed in a protein during folding, a hydrogen bond (of similar strength) between the same group and water was broken. The net stability contributed by a given hydrogen bond, or the *difference* in free energies of the folded and unfolded states, may be close to zero. Ionic interactions may be either stabilizing or destabilizing. We must therefore look elsewhere to understand why a particular native conformation is favored.

Packing of Hydrophobic Amino Acids Away from Water Favors Protein Folding

P1 On carefully examining the contribution of weak interactions to protein stability, we find that the **hydrophobic effect** generally predominates. Pure water contains a network of hydrogen-bonded H_2O molecules. No other molecule has the hydrogen-bonding potential of water, and the presence of other molecules in an aqueous solution disrupts the hydrogen bonding of water. When water surrounds a hydrophobic molecule, the optimal arrangement of hydrogen bonds results in a highly structured shell, or **solvation layer**, of water around the molecule (see Fig. 2-7). The increased order of the water molecules in the solvation layer correlates with an unfavorable decrease in the entropy of the water. However, when nonpolar groups cluster together, the extent of the solvation layer decreases, because each group no longer presents its entire surface to the solution. The result is a favorable increase in entropy. As described in Chapter 2, this increase in entropy is the major thermodynamic driving force for the association of hydrophobic groups in aqueous solution. Hydrophobic amino acid side chains therefore tend to cluster in a protein's interior, away from water (think of an oil droplet in water). The amino acid sequences of most proteins thus include a significant content of hydrophobic amino acid side chains (especially Leu, Ile, Val, Phe, and Trp). These are positioned so that they are clustered when the protein is folded, forming a hydrophobic protein core.

Under physiological conditions, the formation of hydrogen bonds in a protein is driven largely by this same entropic effect. Polar groups can generally form hydrogen bonds with water and hence are soluble in water. However, the number of hydrogen bonds per unit mass is generally greater for pure water than for any other liquid or solution, and there are limits to the solubility of even the most polar molecules as their presence causes a net decrease in hydrogen bonding per unit mass. Therefore, a solvation layer forms to some extent even around polar molecules. Although the energy of formation of an intramolecular hydrogen bond between two polar groups in a macromolecule is largely canceled by the elimination of such interactions between these polar groups and water, the release of structured water as intramolecular associations form provides an entropic driving force for folding. Most of the net change in free energy as nonpolar amino acid side chains aggregate within a protein is therefore derived from the increased entropy in the surrounding aqueous solution resulting from the burial of hydrophobic surfaces. This more than counterbalances the large loss of conformational entropy as a polypeptide is constrained into its folded conformation.

Polar Groups Contribute Hydrogen Bonds and Ion Pairs to Protein Folding

The hydrophobic effect is clearly important in stabilizing conformation; the interior of a structured protein is generally a densely packed core of hydrophobic amino acid side chains. It is also important that any polar or charged groups in the protein interior have suitable partners for hydrogen bonding or ionic interactions. One hydrogen bond seems to contribute little to the stability of a native structure, but the presence of hydrogen-bonding groups without partners in the hydrophobic core of a protein can be so *destabilizing* that conformations containing these groups are often thermodynamically untenable. The favorable free-energy change resulting from the combination of several such groups with partners in the surrounding solution can be greater than the free-energy difference between the folded and unfolded states. In addition, hydrogen bonds between groups in a protein form cooperatively (formation of one makes formation of the next one more likely) in repeating secondary structures that optimize hydrogen bonding, as described below. In this way, hydrogen bonds often have an important role in guiding the protein-folding process.

P1 The interaction of oppositely charged groups that form an ion pair, or salt bridge, can have either a stabilizing or destabilizing effect on protein structure. As in the case of hydrogen bonds, charged amino acid side chains interact with water and salts when the protein is unfolded, and the loss of those interactions must be considered when researchers evaluate the effect of a salt bridge on the overall stability of a folded protein. However, the strength of a salt bridge increases as it moves to an environment of lower dielectric constant, ε (p. 46): from the polar aqueous solvent (ε near 80) to the nonpolar protein interior (ε near 4). Salt bridges, especially those that are partly or entirely buried, can thus provide significant stabilization to a protein structure. This trend explains the increased occurrence of buried salt bridges in the proteins of thermophilic organisms. Ionic interactions also limit structural flexibility and confer a uniqueness to a particular protein structure that the clustering of nonpolar groups via the hydrophobic effect cannot provide.

Individual van der Waals Interactions Are Weak but Combine to Promote Folding

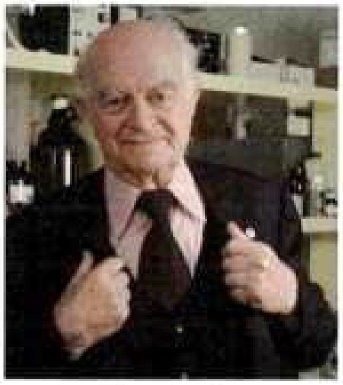
In the tightly packed atomic environment of a protein, one more type of weak interaction can have a significant effect: van der Waals interactions (p. 49). Van der Waals interactions are dipole-dipole interactions involving the permanent electric dipoles in groups such as carbonyls, transient dipoles derived from fluctuations of the electron cloud surrounding any atom, and dipoles induced by interaction of one atom with another that has a permanent or transient dipole. As atoms approach each other, these dipole-dipole interactions provide an attractive intermolecular force that operates over only a limited intermolecular distance (0.3 to 0.6 nm). P1 Individually, van der Waals interactions contribute little to overall protein stability. However, in a well-packed protein, or in an interaction between a protein and another protein or other molecule at a complementary surface, the number of such interactions can be substantial.

Most of the structural patterns outlined in this chapter reflect two simple rules: (1) hydrophobic residues are largely buried in the protein interior, away from water, and (2) the number of hydrogen bonds and ionic interactions within the protein is maximized, thus reducing the number of unpaired hydrogen-bonding and ionic groups. Proteins within membranes (which we examine in Chapter 11) and proteins that are intrinsically disordered or have intrinsically disordered segments follow different rules. This reflects their particular function or environment, but weak interactions are still critical structural elements. For example, soluble but intrinsically disordered protein segments are often enriched in amino acid side chains that are charged (especially Arg, Lys, Glu) or small (Gly, Ala), providing little or no opportunity for the formation of a stable hydrophobic core.

The Peptide Bond Is Rigid and Planar

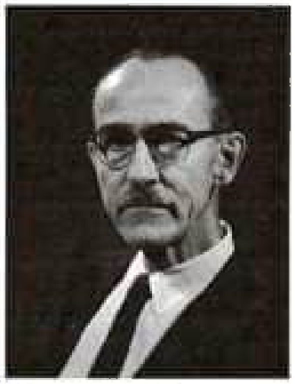
Covalent bonds, too, place important constraints on the conformation of a polypeptide. In the late 1930s, Linus Pauling and Robert Corey embarked on a series of studies that laid the foundation for our current understanding of protein structure. They began with a careful analysis of the peptide bond.

The α carbons of adjacent amino acid residues are separated by three covalent bonds, arranged as C_α—C—N—C_α. X-ray diffraction studies of crystals of amino acids and of simple dipeptides and tripeptides showed that the peptide C—N bond is somewhat shorter than the C—N bond in a simple amine and that the atoms associated with the peptide bond are coplanar. This indicated a resonance or partial sharing of two pairs of electrons between the carbonyl oxygen and the amide nitrogen (**Fig. 4-2a**). The oxygen has a partial negative charge and the hydrogen bonded to the nitrogen has a net partial positive charge, setting up a small electric dipole. The six atoms of the **peptide group** lie in a single plane, with the oxygen atom of the carbonyl group trans to the hydrogen atom of the amide nitrogen. From these findings Pauling and Corey concluded that the peptide C—N bonds, because of their partial double-bond character, cannot rotate freely. Rotation is permitted about the N—C_α and the C_α—C bonds. P1 The backbone of a polypeptide chain can thus be pictured as a series of rigid planes, with consecutive planes sharing a common point of rotation at C_α (Fig. 4-2b). The rigid peptide bonds limit the range of conformations possible for a polypeptide chain.



Linus Pauling, 1901–1994

[Nancy R. Schiff/Getty Images.]



Robert Corey, 1897–1971

[Courtesy California Institute of Technology Archives.]

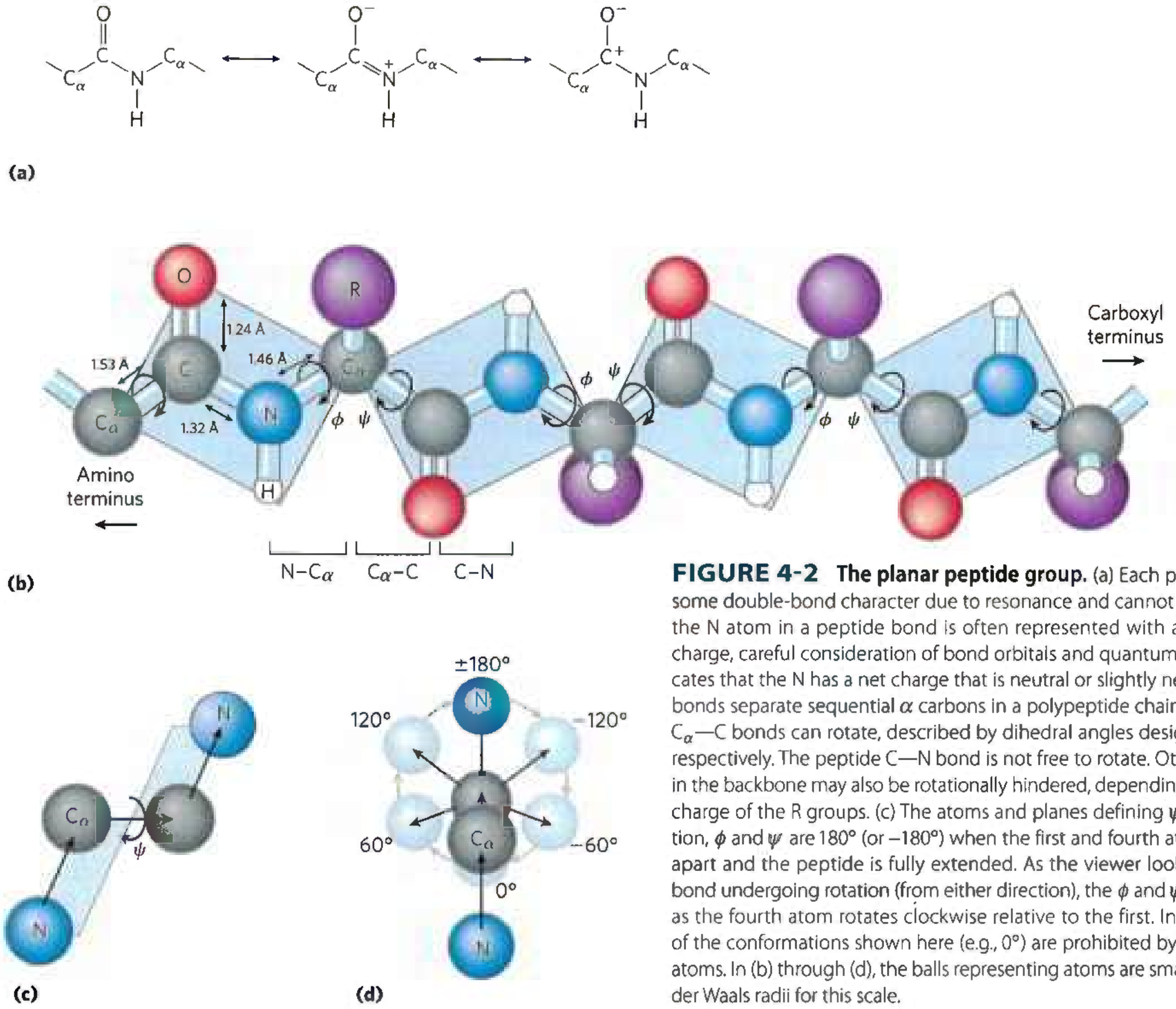


FIGURE 4-2 The planar peptide group. (a) Each peptide bond has some double-bond character due to resonance and cannot rotate. Although the N atom in a peptide bond is often represented with a partial positive charge, careful consideration of bond orbitals and quantum mechanics indicates that the N has a net charge that is neutral or slightly negative. (b) Three bonds separate sequential α carbons in a polypeptide chain. The N—C_α and C_α—C bonds can rotate, described by dihedral angles designated φ and ψ, respectively. The peptide C—N bond is not free to rotate. Other single bonds in the backbone may also be rotationally hindered, depending on the size and charge of the R groups. (c) The atoms and planes defining ψ. (d) By convention, φ and ψ are 180° (or −180°) when the first and fourth atoms are farthest apart and the peptide is fully extended. As the viewer looks out along the bond undergoing rotation (from either direction), the φ and ψ angles increase as the fourth atom rotates clockwise relative to the first. In a protein, some of the conformations shown here (e.g., 0°) are prohibited by steric overlap of atoms. In (b) through (d), the balls representing atoms are smaller than the van der Waals radii for this scale.

Peptide conformation is defined by three dihedral angles (also known as torsion angles) called φ (phi), ψ (psi), and ω (omega), reflecting rotation about each of the three repeating bonds in the peptide backbone. A dihedral angle is the angle at the intersection of two planes. In the case of peptides, the planes are defined by bond vectors in the peptide backbone. Two successive bond vectors describe a plane. Three successive bond vectors describe two planes (the central bond vector is common to both; Fig. 4-2c), and the angle between these two planes is what we measure to describe peptide conformation.

KEY CONVENTION The important dihedral angles in a peptide are defined by the three bond vectors connecting four consecutive main-chain (peptide backbone) atoms (Fig. 4-2c): φ involves the C—N—C_α—C bonds (with the rotation occurring about the N—C_α bond), and ψ involves the N—C_α—C—N bonds. Both φ and ψ are defined as ±180° when the polypeptide is fully extended and all peptide groups are in the same plane (Fig. 4-2d). As one looks down the central bond vector in the direction of the vector arrow (as depicted in Fig. 4-2c for ψ), the dihedral angles increase as the distal (fourth) atom is rotated clockwise (Fig. 4-2d). From the ±180° position, the dihedral angle increases from −180° to 0°, at which point the first and fourth atoms are eclipsed. The rotation can be continued from 0° to +180° (same position as −180°) to bring the structure back to the starting point. The third dihedral angle, ω, is not often considered. It involves the C_α—C—N—C_α bonds. The central bond in this case is the peptide bond, where rotation is constrained. The peptide bond is almost always (99.6% of the time) in the trans configuration, constraining ω to a value of ±180°. For a rare cis peptide bond, ω = 0°. ■

In principle, φ and ψ can have any value between −180° and +180°, but many values are prohibited by steric interference between atoms in the polypeptide backbone and amino acid side chains. The conformation in which both φ and ψ are 0° (Fig. 4-2d) is prohibited for this reason; this conformation is merely a reference point for describing the dihedral angles. Backbone angle preferences in a polypeptide represent yet another constraint on the overall folded structure of a protein.

SUMMARY 4.1 *Overview of Protein Structure*

- A typical protein usually has one or more stable three-dimensional conformations that reflect its function. Some proteins have segments that are intrinsically disordered but are nonetheless essential for function.
- Whereas nonpeptide covalent bonds, particularly disulfide bonds, can play a role in stabilization of some structures, proteins are stabilized largely by multiple weak, noncovalent interactions and forces.
- The hydrophobic effect, derived from the increase in entropy of the surrounding water when nonpolar molecules or groups are clustered together, makes the major contribution to stabilizing the globular form of most soluble proteins.
- Hydrogen bonds and ionic interactions are optimized in the thermodynamically most stable structures.
- Van der Waals interactions involve attractive forces between molecular dipoles that occur over short distances. Individually these interactions are weak, but they combine in well-packed protein structures to provide significant effects and stabilization.
- The nature of the covalent bonds in the polypeptide backbone places constraints on structure. The peptide bond has a partial double-bond character that keeps the entire six-atom peptide group in a rigid planar configuration. The $\mathrm{N{-}C_\alpha}$ and $\mathrm{C_\alpha{-}C}$ bonds can rotate to define the dihedral angles ϕ and ψ, respectively, although permitted values of ϕ and ψ are limited by steric clashes and other constraints.

4.2 Protein Secondary Structure

The term **secondary structure** refers to any chosen segment of a polypeptide chain and describes the local spatial arrangement of its main-chain atoms, without regard to the positioning of its side chains or its relationship to other segments. A *regular* secondary structure occurs when each dihedral angle, ϕ and ψ, remains the same or nearly the same throughout the segment. P2 A few types of secondary structure are particularly stable and occur widely in proteins. The most prominent are the α helix and β conformation; another common type is the β turn. Secondary structures without a regular pattern are sometimes referred to as undefined or as random coils. Random coil, however, does not properly describe the structure of these segments. The path of most of the polypeptide backbone in a typical protein is not random; rather, it is highly specific to the structure and function of that particular protein. Our discussion here focuses on the regular structures that are most common.

The α Helix Is a Common Protein Secondary Structure

Pauling and Corey were aware of the importance of hydrogen bonds in orienting polar chemical groups such as the $\mathrm{C{=}O}$ and $\mathrm{N{-}H}$ groups of the peptide bond. They also had the experimental results of William Astbury, who in the 1930s had conducted pioneering x-ray studies of proteins. Astbury demonstrated that the protein that makes up hair and porcupine quills (the fibrous protein α-keratin) has a regular structure that repeats every 5.15 to 5.20 Å. (The angstrom, Å, named after the physicist Anders J. Ångström, is equal to 0.1 nm. Although not an SI unit, it is used universally by structural biologists to describe atomic distances—it is approximately the length of a typical $\mathrm{C{-}H}$ bond.) With this information and their data on the peptide bond, and with the help of precisely constructed models, Pauling and Corey set out to determine the likely conformations of protein molecules.

The first breakthrough came in 1948. Pauling, at that time a visiting lecturer at Oxford University, became ill and retired to his apartment for several days of rest. Bored with the reading available, Pauling grabbed some paper and pencils to work out a plausible stable structure that could be taken up by a polypeptide chain. The model he developed, and later confirmed in work with Corey and coworker Herman Branson, was the simplest arrangement the polypeptide chain can assume that maximizes the use of internal hydrogen bonding. It is a helical structure, and Pauling and Corey called it the **α helix** (**Fig. 4-3**). In this structure, the polypeptide backbone is tightly wound around an imaginary axis drawn longitudinally through the middle of the helix, and the R groups of the amino acid residues protrude outward from the helical backbone (Figs 4-3b, c). The repeating unit is a single turn of the helix, which extends about 5.4 Å along the long axis, slightly greater than the periodicity that Astbury observed on x-ray analysis of hair keratin. The backbone atoms of the amino acid residues in the prototypical α helix have a characteristic set of dihedral angles that define the conformation of the α helix (**Table 4-1**), and each helical turn includes 3.6 amino acid residues. The α-helical segments in proteins often deviate slightly from these dihedral angles, and they even vary somewhat within a single, continuous segment so as to produce subtle bends or kinks in the helical axis.

Pauling and Corey considered both right-handed and left-handed variants of the α helix. The subsequent elucidation of the three-dimensional structure of myoglobin and other proteins showed that the right-handed α helix is the common form (**Box 4-1**). Extended left-handed α helices are theoretically less stable and have not been observed in proteins. The α helix proved to be the predominant structure in α-keratins. More generally, about one-fourth of all amino acid residues in proteins are found in α helices, the exact fraction varying greatly from one protein to another.

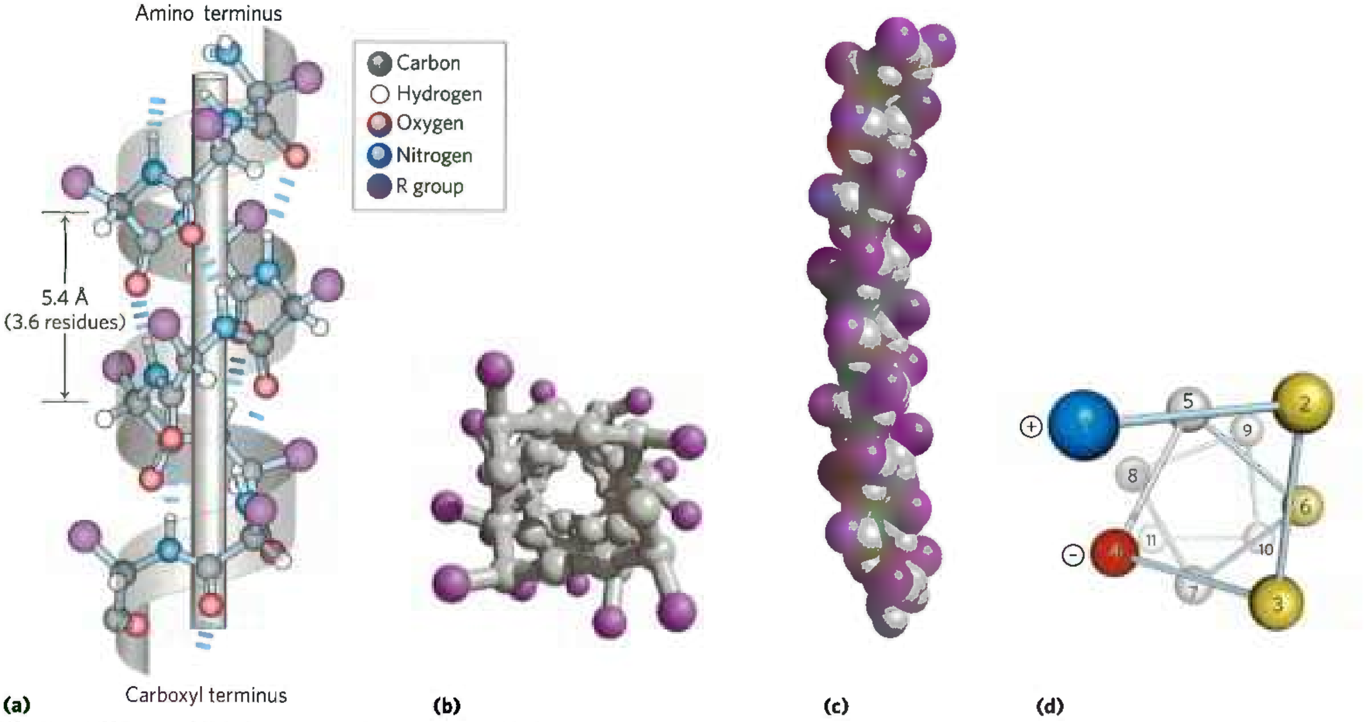


FIGURE 4-3 Models of the α helix, showing different aspects of its structure. (a) Ball-and-stick model showing the intrachain hydrogen bonds. The repeat unit is a single turn of the helix, 3.6 residues. (b) The α helix viewed from one end, looking down the longitudinal axis. Note the positions of the R groups, represented by purple spheres. This ball-and-stick model, which emphasizes the helical arrangement, gives the false impression that the helix is hollow, because the balls do not represent the van der Waals radii of the individual atoms. (c) As this space-filling model shows, the atoms in the center of the α helix are in very close contact. (d) Helical wheel projection of an α helix. This representation can be colored to identify surfaces with particular properties. The yellow residues, for example, could be hydrophobic and conform to an interface between the helix shown here and another part of the same or another polypeptide. The red (negative) and blue (positive) residues illustrate the potential for interaction of oppositely charged side chains separated by two residues in the helix. [(b, c) Data from PDB ID 4TNC, K. A. Satyshur et al., *J. Biol. Chem.* 263:1628, 1988.]

TABLE 4-1 Idealized ϕ and ψ Angles for Common Secondary Structures in Proteins

Structure	ϕ	ψ
α Helix	−57°	−47°
β Conformation		
Antiparallel	−139°	+135°
Parallel	−119°	+113°
Collagen triple helix	−51°	+153°
β Turn type I		
i + 1[a]	−60°	−30°
i + 2[a]	−90°	0°
β Turn type II		
i + 1	−60°	+120°
i + 2	+80°	0°

Note: In real proteins, dihedral angles often vary somewhat from these idealized values.

[a] The i + 1 and i + 2 angles are those for the second and third amino acid residues in the β turn, respectively.

Why does the α helix form more readily than many other possible conformations? The answer lies, in part, in its optimal use of intrahelical hydrogen bonds. The structure is stabilized by a hydrogen bond between the hydrogen atom attached to the electronegative nitrogen atom of a peptide linkage and the electronegative carbonyl oxygen atom of the fourth amino acid on the amino-terminal side of that peptide bond (Fig. 4-3a). Within the α helix, every peptide bond (except those close to each end of the helix) participates in such hydrogen bonding. Each successive turn of the α helix is held to adjacent turns by three to four hydrogen bonds, conferring significant stability on the overall structure. At the ends of an α-helical segment, there are always three or four amide carbonyl or amino groups that cannot participate in this helical pattern of hydrogen bonding. These may be exposed to the surrounding solvent, where they hydrogen-bond with water, or other parts of the protein may cap the helix to provide the needed hydrogen-bonding partners.

WORKED EXAMPLE 4-1 *Secondary Structure and Protein Dimensions*

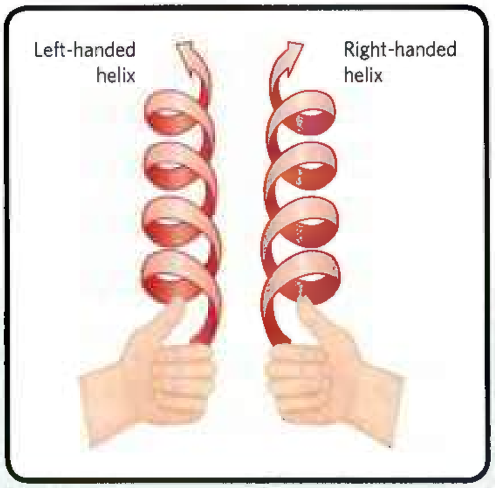
What is the length, in both Å and nm, of a polypeptide with 80 amino acid residues in a single, continuous α helix?

SOLUTION: An idealized α helix has 3.6 residues per turn, and the rise along the helical axis is 5.4 Å. Thus, the rise along the axis for each amino acid residue is 1.5 Å. The length of the polypeptide is therefore 80 residues × 1.5 Å/residue = 1.2×10^2 Å or 12 nm.

BOX 4-1 METHODS

Knowing the Right Hand from the Left

There is a simple method for determining whether a helical structure is right-handed or left-handed. Make fists of your two hands with thumbs outstretched and pointing away from you. Looking at your right hand, think of a helix spiraling up your right thumb in the direction in which the other four fingers are curled as shown (clockwise). The resulting helix is right-handed. Your left hand will demonstrate a left-handed helix, which rotates in the counterclockwise direction as it spirals up your thumb.



Amino Acid Sequence Affects Stability of the α Helix

Not all polypeptides can form a stable α helix. Each amino acid residue in a polypeptide has an intrinsic propensity to form an α helix, reflecting the properties of the R group and how they affect the capacity of the adjoining main-chain atoms to take up the characteristic ϕ and ψ angles. Alanine shows the greatest tendency to form α helices in most experimental model systems.

The position of an amino acid residue relative to its neighbors is also important. Interactions between amino acid side chains can stabilize or destabilize the α-helical structure. For example, if a polypeptide chain has a long block of Glu residues, this segment of the chain will not form an α helix at pH 7.0. The negatively charged carboxyl groups of adjacent Glu residues repel each other so strongly that they prevent formation of the α helix. For the same reason, if there are many adjacent Lys and/or Arg residues, with positively charged R groups at pH 7.0, they also repel each other and prevent formation of the α helix. The size and shape of Asn, Ser, Thr, and Cys residues can also destabilize an α helix if they are close together in the chain.

The twist of an α helix ensures that critical interactions occur between an amino acid side chain and the side chain three (and sometimes four) residues away on either side of it. This is made clear when the α helix is depicted as a helical wheel (Fig. 4-3d). Positively charged amino acids are often found three residues away from negatively charged amino acids, permitting the formation of an ion pair. Two aromatic amino acid residues are often similarly spaced, resulting in a juxtaposition stabilized by the hydrophobic effect.

A constraint on the formation of the α helix is the presence of Pro or Gly residues, which have the least likelihood of forming α helices. In proline, the nitrogen atom is part of a rigid ring (see Fig. 4-7), and rotation about the N—C_α bond is not possible. Thus, a Pro residue introduces a destabilizing kink in an α helix. In addition, the nitrogen atom of a Pro residue in a peptide linkage has no substituent hydrogen to participate in hydrogen bonds with other residues. For these reasons, proline is found only rarely in an α helix. Glycine occurs infrequently in α helices for a different reason: it has more conformational flexibility than the other amino acid residues. Polymers of glycine tend to take up coiled structures quite different from an α helix.

A final factor affecting the stability of an α helix is the identity of the amino acid residues near the ends of the α-helical segment of the polypeptide. A small electric dipole exists in each peptide bond (Fig. 4-2a). These dipoles are aligned through the hydrogen bonds of the helix, resulting in a net dipole along the helical axis that increases with helix length (**Fig. 4-4**). The partial positive and negative charges of the helix dipole reside on the peptide amino and carbonyl groups near the amino-terminal and carboxyl-terminal ends, respectively. For this reason, negatively charged amino acids are often found near the amino terminus of the helical segment, where they have a stabilizing interaction with the positive charge of the helix dipole; a positively charged amino acid at the amino-terminal end is destabilizing. The opposite is true at the carboxyl-terminal end of the helical segment.

In summary, five types of constraints affect the stability of an α helix: (1) the intrinsic propensity of an amino acid residue to form an α helix; (2) the interactions between R groups, particularly those spaced three (or four) residues apart; (3) the bulkiness of adjacent R groups; (4) the occurrence of Pro and Gly residues; and (5) interactions between amino acid residues at the ends of the helical segment and the electric dipole inherent to the α helix. **P2** The tendency of a given segment of a

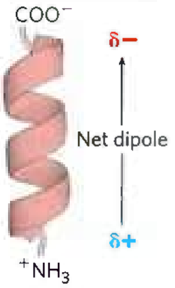


FIGURE 4-4 Helix dipole. The electric dipole of a peptide bond (see Fig. 4-2a) is transmitted along an α-helical segment through the intrachain hydrogen bonds, resulting in an overall helix dipole.

polypeptide chain to form an α helix therefore depends on the identity and sequence of amino acid residues within the segment.

The β Conformation Organizes Polypeptide Chains into Sheets

In 1951, Pauling and Corey predicted a second type of repetitive structure, the **β conformation**. This is a more extended conformation of polypeptide chains, and its structure is again defined by backbone atoms arranged according to a characteristic set of dihedral angles (Table 4-1). P2 In the β conformation, the backbone of the polypeptide chain is extended into a zigzag rather than helical structure (**Fig. 4-5**). A single protein segment in the β conformation is often called a β strand. The arrangement of several strands side by side, all in the β conformation, is called a **β sheet**. The zigzag structure of the individual polypeptide segments gives rise to a pleated appearance of the overall sheet. Hydrogen bonds form between backbone atoms of adjacent segments of polypeptide chain within the sheet. The individual segments that form a β sheet are usually nearby on the polypeptide chain but can also be quite distant from each other in the linear sequence of the polypeptide; they may even be in different polypeptide chains. The R groups of adjacent amino acids protrude from the zigzag structure in opposite directions, creating the alternating pattern seen in the side view in Figure 4-5.

The adjacent polypeptide chains in a β sheet can be either parallel or antiparallel (having the same or opposite amino-to-carboxyl orientations, respectively). The structures are somewhat similar, although the repeat period is shorter for the parallel conformation (6.5 vs. 7.0 Å for antiparallel) and the hydrogen-bonding patterns are different. The interstrand hydrogen bonds are essentially in-line (see Fig. 2-5) in the antiparallel β sheet, whereas they are distorted or not in-line for the parallel variant. In natural proteins, antiparallel β sheets are found twice as frequently as parallel β sheets. The idealized structures exhibit the bond angles given in Table 4-1; these values vary somewhat in real proteins, resulting in structural variation, as seen above for α helices.

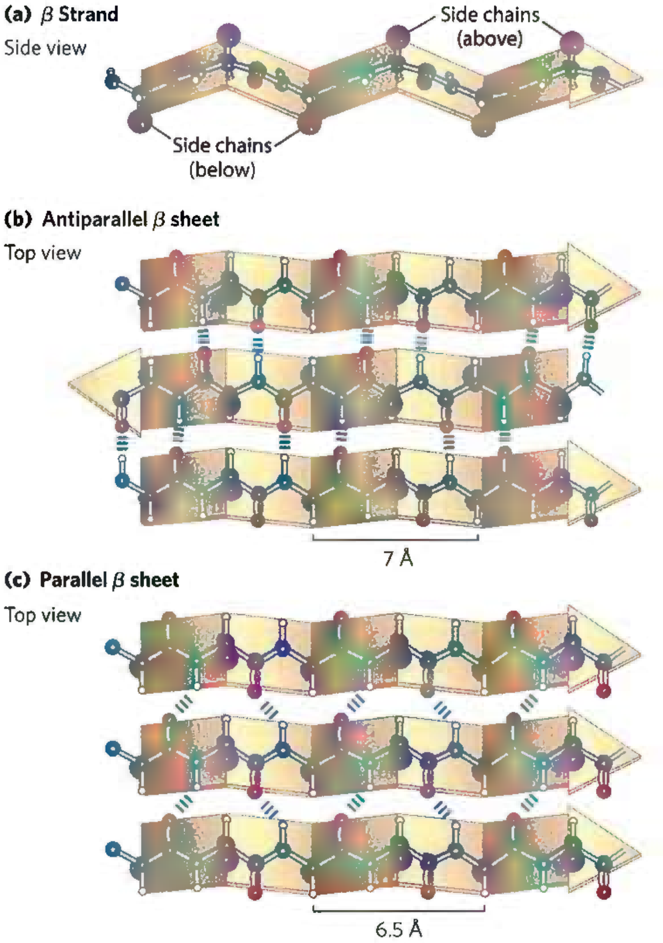


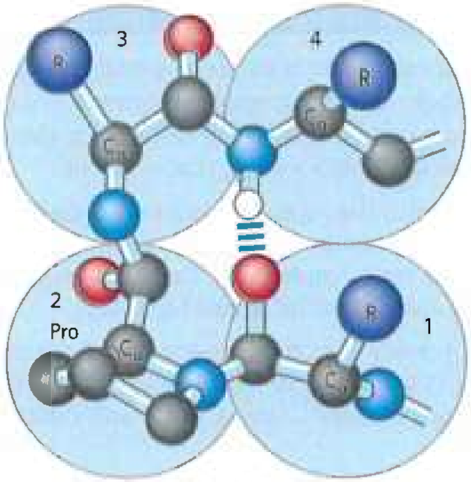
FIGURE 4-5 The β conformation of polypeptide chains. These (a) side and (b, c) top views reveal the R groups extending out from the β sheet and emphasize the pleated shape formed by the planes of the peptide bonds. (An alternative name for this structure is β-pleated sheet.) Hydrogen-bond cross-links between adjacent chains are also shown. The amino-terminal to carboxyl-terminal orientations of adjacent chains (arrows) can be the opposite or the same, forming (b) an antiparallel β sheet or (c) a parallel β sheet.

β Turns Are Common in Proteins

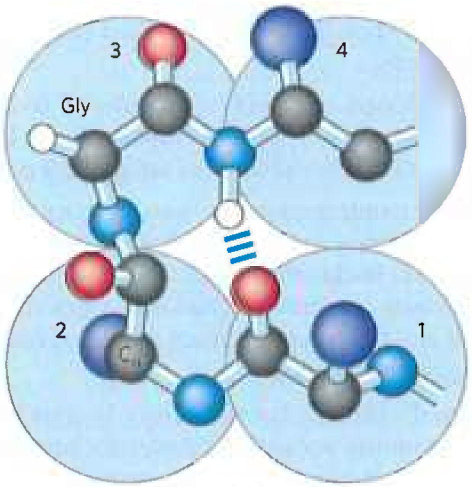
In globular proteins, which have a compact folded structure, some amino acid residues are in turns or loops where the polypeptide chain reverses direction (**Fig. 4-6**). P2 These are the connecting elements that link successive runs of α helix or β conformation. Particularly common are **β turns** that connect the ends of two adjacent segments of an antiparallel β sheet. The structure is a 180° turn involving four amino acid residues, with the carbonyl oxygen of the first residue forming a hydrogen bond with the amino-group hydrogen of the fourth. The peptide groups of the central two residues do not participate in any interresidue hydrogen bonding. Several types of β turns have been described, each defined by the ϕ and ψ angles of the bonds that link the four amino acid residues that make up the particular turn (Table 4-1). Gly and Pro residues often occur in β turns, the former because it is small and flexible, the latter because peptide bonds involving the imino nitrogen of proline readily assume the cis configuration (**Fig. 4-7**), a form that is particularly amenable to a tight turn. The two types of β turns shown in Figure 4-6 are the most common. Beta turns are often found near the surface of a protein, where the peptide groups of the central two amino acid residues in the turn can hydrogen-bond with water. Considerably less common is the γ turn, a three-residue turn with a hydrogen bond between the first and third residues.

Common Secondary Structures Have Characteristic Dihedral Angles

The α helix and the β conformation are the major repetitive secondary structures in a wide variety of proteins, although other repetitive structures exist in some specialized proteins (an example is collagen; see Fig. 4-12).



Type I β turn



Type II β turn

FIGURE 4-6 Structures of β turns. Type I and type II β turns are most common, distinguished by the ϕ and ψ angles taken up by the peptide backbone in the turn (see Table 4-1). Type I turns occur more than twice as frequently as type II. Note the hydrogen bond between the peptide groups of the first and fourth residues of the bends. (Individual amino acid residues are framed by large blue circles. Not all H atoms are shown in these depictions.)

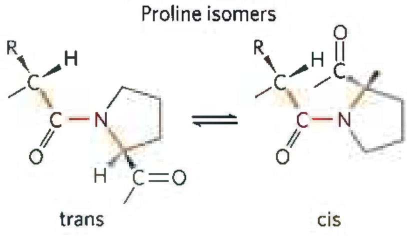


FIGURE 4-7 Trans and cis isomers of a peptide bond involving the imino nitrogen of proline. Of the peptide bonds between amino acid residues other than Pro, more than 99.95% are in the trans configuration. For peptide bonds involving the imino nitrogen of proline, however, about 6% are in the cis configuration; many of these occur at β turns.

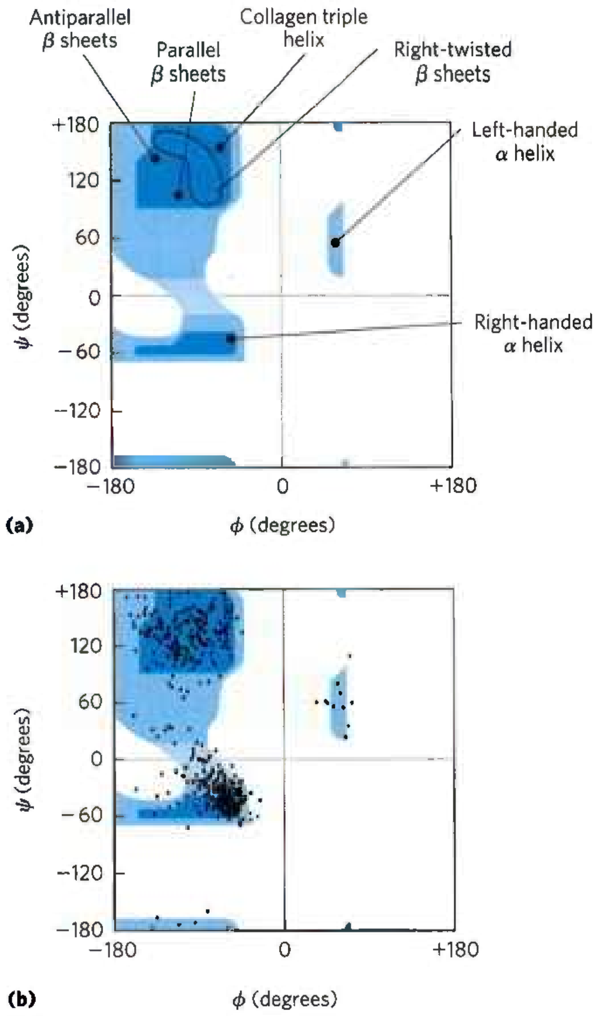


FIGURE 4-8 Ramachandran plots showing a variety of structures. (a) The values of ϕ and ψ for various allowed conformations and secondary structures are shown. Peptide conformations deemed possible are those that involve little or no steric interference, based on calculations using known van der Waals radii and dihedral angles modeled as a hard sphere. Other types of Ramachandran plots make different assumptions. The areas shaded dark blue represent conformations that involve no steric overlap and are thus fully allowed. Medium blue indicates conformations permitted if atoms are allowed to approach each other by an additional 0.1 nm, a slight clash. The lightest blue indicates conformations that are permissible if a very modest flexibility (a few degrees) is allowed in the ω dihedral angle that describes the peptide bond itself (generally constrained to 180°). The white regions are conformations that are not allowed. Although left-handed α helices extending over several amino acid residues are theoretically possible, they have not been observed in proteins. The asymmetry of the plot results from the L stereochemistry of the amino acid residues. (b) The values of ϕ and ψ for all the amino acid residues except Gly in the enzyme pyruvate kinase (isolated from rabbit) are overlaid on the plot of allowed conformations. The small, flexible Gly residues were excluded because they frequently fall outside the expected (blue) ranges. [(a) Information from T. E. Creighton, *Proteins*, p. 166. © 1984 by W. H. Freeman and Company. (b) Data from Hazel Holden, University of Wisconsin–Madison, Department of Biochemistry.]

Every type of secondary structure can be completely described by the dihedral angles ϕ and ψ associated with each residue. **P2** **Ramachandran plots**, introduced by G. N. Ramachandran, are useful tools for visualizing all of the ϕ and ψ angles observed in a particular protein structure and are often used to test the quality of three-dimensional protein structures. In a Ramachandran plot, the dihedral angles that define the α helix and the β conformation fall within a relatively restricted range of sterically allowed structures (**Fig. 4-8a**). Most values of ϕ and ψ taken from known protein structures fall into the expected regions, with high concentrations near the α helix and β conformation values, as predicted (Fig. 4-8b). The only amino acid residue often found in a conformation outside these regions is glycine. Because its side chain is small, a Gly residue can take part in many conformations that are sterically forbidden for other amino acids.

Common Secondary Structures Can Be Assessed by Circular Dichroism

Any form of structural asymmetry in a molecule gives rise to differences in absorption of left-handed versus right-handed circularly polarized light. Measurement of this difference is called **circular dichroism (CD) spectroscopy**. An ordered structure, such as a folded protein, gives rise to an absorption spectrum that can have peaks or regions with both positive and negative values. For proteins, spectra are obtained in the far UV region (190 to 250 nm). In this region, the light-absorbing entity, or chromophore, is the peptide bond; a signal is obtained when the peptide bond is in a folded environment. The difference in molar extinction coefficients (see Box 3-1) for left-handed and right-handed, circularly polarized light ($\Delta\varepsilon$) is plotted as a function of wavelength. The α helix and β conformations have characteristic CD spectra (**Fig. 4-9**). Using CD spectra, biochemists can determine whether proteins are properly folded, estimate the fraction of the protein that is folded in either of the common secondary structures, and monitor transitions between the folded and unfolded states.

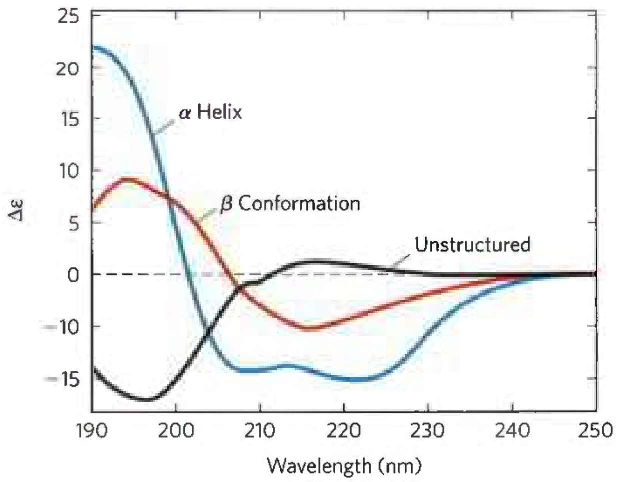


FIGURE 4-9 Circular dichroism spectroscopy. These spectra show polylysine entirely as α helix, as β conformation, or in an unstructured, denatured state. The *y* axis unit is a simplified version of the units most commonly used in CD experiments. Since the curves are different for α helix, β conformation, and unstructured, the CD spectrum for a given protein can provide a rough estimate for the fraction of the protein made up of the two most common secondary structures. The CD spectrum of the native protein can serve as a benchmark for the folded state, useful for monitoring denaturation or conformational changes brought about by changes in solution conditions.

SUMMARY 4.2 *Protein Secondary Structure*

- Secondary structure is the local spatial arrangement of the main-chain atoms in a selected segment of a polypeptide chain; it can be completely defined by the ϕ and ψ angles of all the amino acids in that segment.
- In the α helix, the repeating unit is a single helical turn of ~5.4 Å or 3.6 amino acids. The common form found in proteins is right-handed with the amino acid R groups protruding away from the helical backbone.
- The propensity of a protein segment to form an α helix depends on the composition of its amino acids and amino acid positions relative to one another and relative to the helical dipole.
- In the β conformation, amino acids are extended in a zigzag fashion. When several β strands are arranged adjacent to one another, they can form either parallel or antiparallel β sheets.
- Turns or loops connect segments of α helix or β strands. β turns, which often contain Gly or Pro residues, tend to connect segments of antiparallel β sheets.
- The Ramachandran plot is a visual description of the combinations of ϕ and ψ dihedral angles that are permitted in a peptide backbone and those that are not permitted due to steric constraints. Dihedral angles that define the α helix and the β conformation are found only within certain regions of the plot.
- Circular dichroism spectroscopy is a method for assessing common secondary structure and monitoring folding in proteins based on absorption of circularly polarized UV light.

4.3 Protein Tertiary and Quaternary Structures

P3 The overall three-dimensional arrangement of all atoms in a protein is referred to as the protein's **tertiary structure**. Whereas the term "secondary structure" refers to the spatial arrangement of amino acid residues that are adjacent in a segment of a polypeptide, tertiary structure includes *longer-range* aspects of amino acid sequence. Amino acids that are far apart in the polypeptide sequence and are in different types of secondary structure may interact within the completely folded structure of a protein. Interacting segments of polypeptide chains are held in their characteristic tertiary positions by several kinds of weak interactions (and sometimes by covalent bonds such as disulfide cross-links) between the segments. Some proteins contain two or more separate polypeptide chains, or subunits, which may be identical or different. The arrangement of these protein subunits in three-dimensional complexes constitutes **quaternary structure**.

In considering these higher levels of structure, it is useful to designate the major groups into which many proteins can be classified: **fibrous proteins**, with polypeptide chains arranged in long strands or sheets; **globular proteins**, with polypeptide chains folded into a spherical or globular shape; **membrane proteins**, with polypeptide chains embedded in hydrophobic lipid membranes; and **intrinsically disordered proteins**, with polypeptide chains lacking stable tertiary structures. We focus here on fibrous, globular, and intrinsically disordered proteins; membrane proteins are discussed in Chapter 11. These three groups are structurally distinct. Fibrous proteins usually consist of a single type of secondary structure, and their tertiary structure is relatively simple. Globular proteins often contain several types of secondary structure. Intrinsically disordered proteins can lack secondary structure entirely. The groups also differ functionally: the structures that provide support, shape, and external protection to vertebrates are made of fibrous proteins. Most enzymes are globular proteins, whereas regulatory proteins can be globular, disordered, or contain both globular and disordered segments.

Fibrous Proteins Are Adapted for a Structural Function

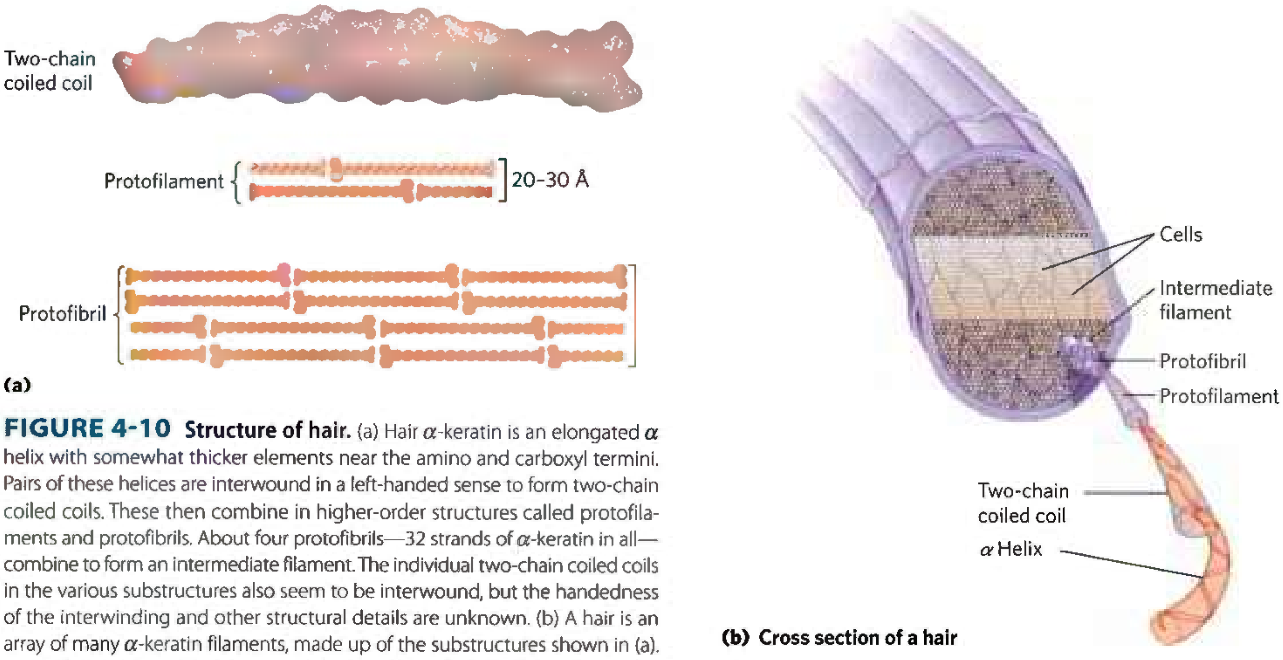
α-Keratin, collagen, and silk fibroin nicely illustrate the relationship between protein structure and biological function (**Table 4-2**). **P2** Fibrous proteins share properties that give strength and/or flexibility to the structures in which they occur. In each case, the fundamental structural unit is a simple repeating element of secondary structure. All fibrous proteins are insoluble in water, a property conferred by a high concentration of hydrophobic amino acid residues both in the interior of the protein and on its surface. These hydrophobic surfaces are largely buried, as many similar polypeptide chains are packed together to form elaborate supramolecular complexes. The underlying structural simplicity of fibrous proteins makes them particularly useful for illustrating some of the fundamental principles of protein structure discussed previously.

α-Keratin The α-keratins have evolved for strength. Found only in mammals, these proteins constitute almost the entire dry weight of hair, wool, nails, claws, quills, horns, and hooves and much of the outer layer of skin. The α-keratins are part of a broader family of proteins called intermediate filament (IF) proteins. Other IF proteins are found in the cytoskeletons of animal cells. All IF proteins have a structural function and share the structural features exemplified by the α-keratins.

The α-keratin helix is a right-handed α helix, the same helix found in many other proteins. Francis Crick and Linus Pauling, in the early 1950s, independently suggested that the α helices of keratin were arranged as a coiled coil. Two strands of α-keratin, oriented in parallel (with their amino termini at the same end), are wrapped about each other to form a supertwisted coiled coil. The supertwisting amplifies the strength of the overall structure, just as strands are twisted to make a strong rope (**Fig. 4-10**). The twisting

TABLE 4-2 Secondary Structures and Properties of Some Fibrous Proteins

Structure	Characteristics	Examples of occurrence
α Helix, cross-linked by disulfide bonds	Tough, insoluble protective structures of varying hardness and flexibility	α-Keratin of hair, feathers, nails
β Conformation	Soft, flexible filaments	Silk fibroin
Collagen triple helix	High tensile strength, without stretch	Collagen of tendons, bone matrix



(b) Cross section of a hair

FIGURE 4-10 Structure of hair. (a) Hair α-keratin is an elongated α helix with somewhat thicker elements near the amino and carboxyl termini. Pairs of these helices are interwound in a left-handed sense to form two-chain coiled coils. These then combine in higher-order structures called protofilaments and protofibrils. About four protofibrils—32 strands of α-keratin in all—combine to form an intermediate filament. The individual two-chain coiled coils in the various substructures also seem to be interwound, but the handedness of the interwinding and other structural details are unknown. (b) A hair is an array of many α-keratin filaments, made up of the substructures shown in (a). [(a) Information from PDB ID 3TNU, C. H. Lee et al., *Nature Struct. Mol. Biol.* 19:707, 2012.]

Box 4-2 **MEDICINE**

Why Sailors, Explorers, and College Students Should Eat Their Fresh Fruits and Vegetables

> . . . from this misfortune, together with the unhealthiness of the country, where there never falls a drop of rain, we were stricken with the "camp-sickness," which was such that the flesh of our limbs all shrivelled up, and the skin of our legs became all blotched with black, mouldy patches, like an old jack-boot, and proud flesh came upon the gums of those of us who had the sickness, and none escaped from this sickness save through the jaws of death. The signal was this: when the nose began to bleed, then death was at hand.
>
> —*The Memoirs of the Lord of Joinville*, ca. 1300*

This excerpt describes the plight of Louis IX's scurvy-weakened army before it was destroyed by the Egyptians toward the end of the Seventh Crusade (1248–1254). What was the nature of the malady afflicting these thirteenth-century soldiers?

Scurvy is caused by lack of vitamin C, or ascorbic acid (ascorbate). Vitamin C is required for, among other things, the hydroxylation of proline and lysine in collagen; scurvy is a deficiency disease characterized by general degeneration of connective tissue. Manifestations of advanced scurvy include numerous small hemorrhages caused by fragile blood vessels; tooth loss, poor wound healing, and the reopening of old wounds; bone pain and degeneration; and eventually heart failure. Milder cases of vitamin C deficiency are accompanied by fatigue, irritability, and an increased severity of respiratory tract infections. Most animals make large amounts of vitamin C, converting glucose to ascorbate in four enzymatic steps. But in the course of evolution, humans and some other animals—gorillas, guinea pigs, and fruit bats—have lost the last enzyme in this pathway and must obtain ascorbate in their diet. Vitamin C is available in a wide range of fruits and vegetables. Until 1800, however, it was often absent in the dried foods and other food supplies stored for winter or for extended travel.

Scurvy came to wide public notice during the European voyages of discovery from 1500 to 1800. In fact, during the first circumnavigation of the globe (1519–1522) by Ferdinand Magellan, more than 80% of his crew were lost to scurvy. Winter outbreaks of scurvy in Europe were gradually eliminated in the nineteenth century as the cultivation of the potato, introduced from South America, became widespread.

In 1747, James Lind, a Scottish surgeon in the Royal Navy, carried out the first controlled clinical study in recorded history. During an extended voyage on the 50-gun warship HMS *Salisbury*, Lind selected 12 sailors suffering from scurvy and separated them into groups of two. All 12 received the same diet, except that each group was given a different remedy for scurvy from among those recommended at the time. The sailors given lemons and oranges recovered and returned to duty. Lind's *Treatise on the Scurvy* was published in 1753, but inaction persisted in the Royal Navy for another 40 years. In 1795, the British admiralty finally mandated

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*From Ethel Wedgwood, *The Memoirs of the Lord of Joinville: A New English Version*, E. P. Dutton and Company, 1906.

---

of the axis of an α helix to form a coiled coil explains the discrepancy between the 5.4 Å per turn predicted for an α helix by Pauling and Corey and the 5.15 to 5.2 Å repeating structure observed in the x-ray diffraction of hair (see end-of-chapter problem 2). The helical path of the supertwists is left-handed, opposite in sense to the α helix. The surfaces where the two α helices touch are made up of hydrophobic amino acid residues, their R groups meshed together in a regular interlocking pattern. This permits a close packing of the polypeptide chains within the left-handed supertwist. Not surprisingly, α-keratin is rich in the hydrophobic residues Ala, Val, Leu, Ile, Met, and Phe.

An individual polypeptide in the α-keratin coiled coil has a relatively simple tertiary structure, dominated by an α-helical secondary structure with its helical axis twisted in a left-handed superhelix. The intertwining of the two α-helical polypeptides is an example of quaternary structure. Coiled coils of this type are common structural elements in filamentous proteins and in the muscle protein myosin (see Fig. 5-26). The quaternary structure of α-keratin can be quite complex. Many coiled coils can be assembled into large supramolecular complexes, such as the arrangement of α-keratin that forms the intermediate filament of hair (Fig. 4-10b).

The strength of fibrous proteins is enhanced by covalent cross-links between polypeptide chains in the multihelical "ropes" and between adjacent chains in a supramolecular assembly. In α-keratins, the cross-links stabilizing quaternary structure are disulfide bonds. In the hardest and toughest α-keratins, such as those of rhinoceros horn, up to 18% of the residues are cysteines involved in disulfide bonds.

Collagen Like the α-keratins, **collagen** has evolved to provide strength. It is found in connective tissue such as tendons, cartilage, the organic matrix of bone, and the cornea of the eye. In fact, collagen is the most abundant protein in mammals, usually comprising 25% to 35% of total protein content. The collagen helix is a unique secondary structure, quite distinct from the α helix. It is left-handed and has three amino acid residues per turn (**Fig. 4-11** and Table 4-1). Collagen is also a coiled coil, but one with distinct tertiary and quaternary structures: three separate polypeptides, called α chains (not to be confused with α helices), are twisted about each other. The superhelical twisting is right-handed in collagen, opposite in sense to the left-handed helix of the α chains.

There are many types of vertebrate collagen. Typically, they contain about 35% Gly, 11% Ala, and 21% Pro and 4-Hyp (4-hydroxyproline, an uncommon amino acid; see Fig. 3-8a). The food product gelatin is derived from collagen. It has little nutritional value as a

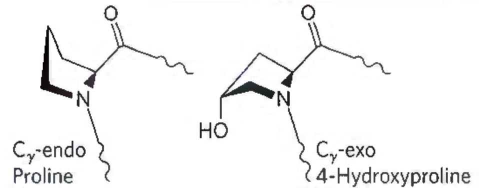


FIGURE 1 The C_γ-endo conformation of proline and the C_γ-exo conformation of 4-hydroxyproline.

a ration of concentrated lime or lemon juice for all British sailors (hence the name "limeys"). Scurvy continued to be a problem in some other parts of the world until 1932, when Hungarian scientist Albert Szent-Györgyi, and W. A. Waugh and C. G. King at the University of Pittsburgh, isolated and synthesized ascorbic acid.

So why is ascorbate so necessary to good health? Of particular interest to us here is its role in the formation of collagen. As noted in the text, collagen is constructed of the repeating tripeptide unit Gly–X–Y, where X and Y are generally Pro or 4-Hyp—the proline derivative L-hydroxyproline, which plays an essential role in the folding of collagen and in maintaining its structure. The proline ring is normally found as a mixture of two puckered conformations, called C_γ-endo and C_γ-exo (Fig. 1). The collagen helix structure requires the Pro/4-Hyp residue in the Y positions to be in the C_γ-exo conformation, and it is this conformation that is enforced by the hydroxyl substitution at C-4 in 4-Hyp. In the absence of vitamin C, cells cannot hydroxylate the Pro at the Y positions. This leads to collagen instability and the connective tissue problems seen in scurvy.

The hydroxylation of specific Pro residues in procollagen, the precursor of collagen, requires the action of the α-ketoglutarate-dependent enzyme prolyl 4-hydroxylase. In the normal prolyl 4-hydroxylase reaction, one molecule of α-ketoglutarate and one of O_2 bind to the enzyme. The α-ketoglutarate is oxidatively decarboxylated to form CO_2 and succinate. The remaining oxygen atom is then used to hydroxylate an appropriate Pro residue in procollagen. No ascorbate is needed in this reaction. However, prolyl 4-hydroxylase also catalyzes an oxidative decarboxylation of α-ketoglutarate that is not coupled to proline hydroxylation. During this reaction, the Fe^{2+} becomes oxidized, inactivating the enzyme and preventing the proline hydroxylation. Ascorbate is needed to reduce the iron and restore enzyme activity so that proline hydroxylation of procollagen can continue.

Scurvy remains a problem today, not only in remote regions where nutritious food is scarce but, surprisingly, also among young adults with poor eating habits in large cities. A 2009 study of more than 1,100 men and women between the ages of 20 and 29 in Toronto, Canada, found that 1 in 7 young adults had vitamin C deficiency due to unmet dietary needs. Moreover, lower vitamin C levels were associated with higher measures of obesity and blood pressure and fewer servings a day of healthy foods. Just like eighteenth-century sailors, twenty-first-century young adults need to eat their fruits and vegetables!

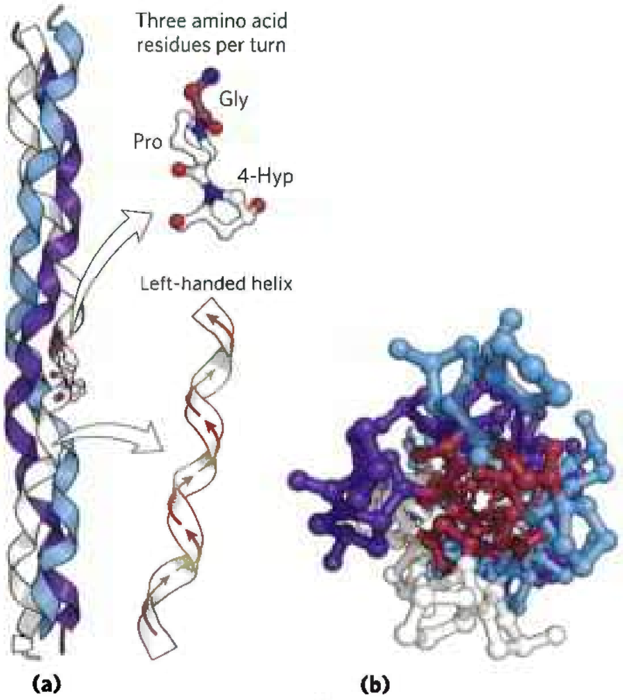


FIGURE 4-11 **Structure of collagen.** (a) The α chain of collagen has a repeating secondary structure unique to this protein. The repeating tripeptide sequence Gly–X–Y, where X is often Pro and Y is often 4-Hyp, adopts a left-handed helical structure with three residues per turn. Three of these helices (shown here in white, blue, and purple) wrap around one another with a right-handed twist. (b) The three-stranded collagen superhelix shown from one end, in a ball-and-stick representation. Gly residues are shown in red. Glycine, because of its small size, is required at the tight junction where the three chains are in contact. The balls in this illustration do not represent the van der Waals radii of the individual atoms. The center of the three-stranded superhelix is not hollow, as it appears here, but very tightly packed. [Data from PDB ID 1CGD, J. Bella et al., *Structure* 3:893, 1995.]

protein, because collagen is extremely low in many amino acids that are essential in the human diet. The unusual amino acid content of collagen is related to structural constraints unique to the collagen helix. The amino acid sequence in collagen is generally a repeating tripeptide unit, Gly–X–Y, where X is often Pro and Y is often 4-Hyp. Only Gly residues can be accommodated at the very tight junctions between the individual α chains (Fig. 4-11b). The Pro and 4-Hyp residues permit the sharp twisting of the collagen helix. The amino acid sequence and the supertwisted quaternary structure of collagen allow a very close packing of its three polypeptides. 4-Hydroxyproline has a special role in the structure of collagen—and in human history (**Box 4-2**).

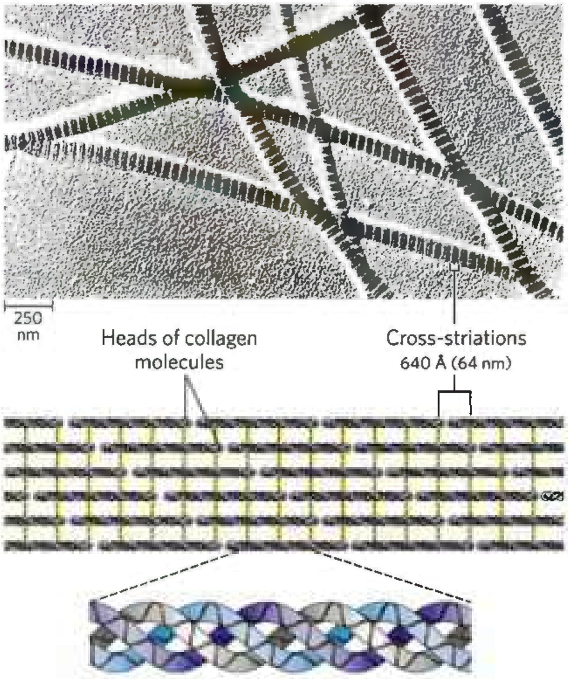
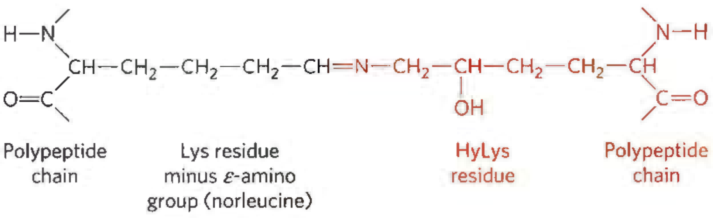

250 nm
Heads of collagen molecules
Cross-striations 640 Å (64 nm)
Section of collagen molecule

FIGURE 4-12 Structure of collagen fibrils. Collagen (M_r 300,000) is a rod-shaped molecule, about 3,000 Å long and only 15 Å thick. Its three helically intertwined α chains may have different sequences; each chain has about 1,000 amino acid residues. Collagen fibrils are made up of collagen molecules aligned in a staggered fashion and cross-linked for strength. The specific alignment and degree of cross-linking vary with the tissue and produce characteristic cross-striations in an electron micrograph. In the example shown here, alignment of the head groups of every fourth molecule produces striations 640 Å (64 nm) apart. [J. Gross/Biozentrum, University of Basel/Science Source.]

The tight wrapping of the α chains in the collagen triple helix provides tensile strength greater than that of a steel wire of equal cross section. Collagen fibrils (**Fig. 4-12**) are supramolecular assemblies consisting of triple-helical collagen molecules (sometimes referred to as tropocollagen molecules) associated in a variety of ways to provide different degrees of tensile strength. The α chains of collagen molecules and the collagen molecules of fibrils are cross-linked by unusual types of covalent bonds involving Lys, HyLys (5-hydroxylysine), or His residues that are present at a few of the X and Y positions. These links create uncommon amino acid residues such as dehydrohydroxylysinonorleucine. The increasingly rigid and brittle character of aging connective tissue results from accumulated covalent cross-links in collagen fibrils.


H—N
CH—CH₂—CH₂—CH₂—CH=N—CH₂—CH—CH₂—CH₂—CH
O=C
OH
N—H
C=O
Polypeptide chain
Lys residue minus ε-amino group (norleucine)
HyLys residue
Polypeptide chain

Dehydrohydroxylysinonorleucine

A typical mammal has more than 30 structural variants of collagen, particular to certain tissues and each somewhat different in sequence and function. Some human genetic defects in collagen structure illustrate the close relationship between amino acid sequence and three-dimensional structure in this protein. Osteogenesis imperfecta is characterized by abnormal bone formation in babies; at least eight variants of this condition, with different degrees of severity, occur in the human population. Ehlers-Danlos syndrome is characterized by loose joints, and at least six variants occur in humans. The composer Niccolò Paganini (1782–1840) was famed for his seemingly impossible dexterity in playing the violin. He suffered from a variant of Ehlers-Danlos syndrome that rendered him effectively double-jointed. In both disorders, some variants can be lethal, whereas others cause lifelong problems.

All of the variants of both conditions result from the substitution of an amino acid residue with a larger R group (such as Cys or Ser) for a single Gly residue in an α chain in one or another of the collagen proteins (a different Gly residue in each disorder). These single-residue substitutions have a catastrophic effect on collagen function because they disrupt the Gly–X–Y repeat that gives collagen its unique helical structure. Given its role in the collagen triple helix (Fig. 4-11), Gly cannot be replaced by another amino acid residue without substantial deleterious effects on collagen structure.

Fibroin The protein of silk, fibroin, is produced by insects and spiders. Its polypeptide chains are predominantly in the β conformation. Fibroin is rich in Ala and Gly residues, permitting a close packing of β sheets and an interlocking arrangement of R groups (**Fig. 4-13**). The overall structure is stabilized by extensive hydrogen bonding between all peptide linkages in the polypeptides of each β sheet and by the optimization of van der Waals interactions between sheets. Silk does not stretch, because the β conformation is already highly extended (Fig. 4-5). However, the structure is flexible, because the sheets are held together by numerous weak interactions rather than by covalent bonds such as the disulfide bonds in α-keratins.

Structural Diversity Reflects Functional Diversity in Globular Proteins

In a globular protein, different segments of the polypeptide chain (or multiple polypeptide chains) fold back on each other, generating a more compact shape than is seen in the fibrous proteins (**Fig. 4-14**). The folding also provides the structural diversity necessary for proteins to carry out a wide array of biological functions. Globular proteins include enzymes, transport proteins, motor proteins, regulatory proteins, immunoglobulins, and proteins with many other functions.

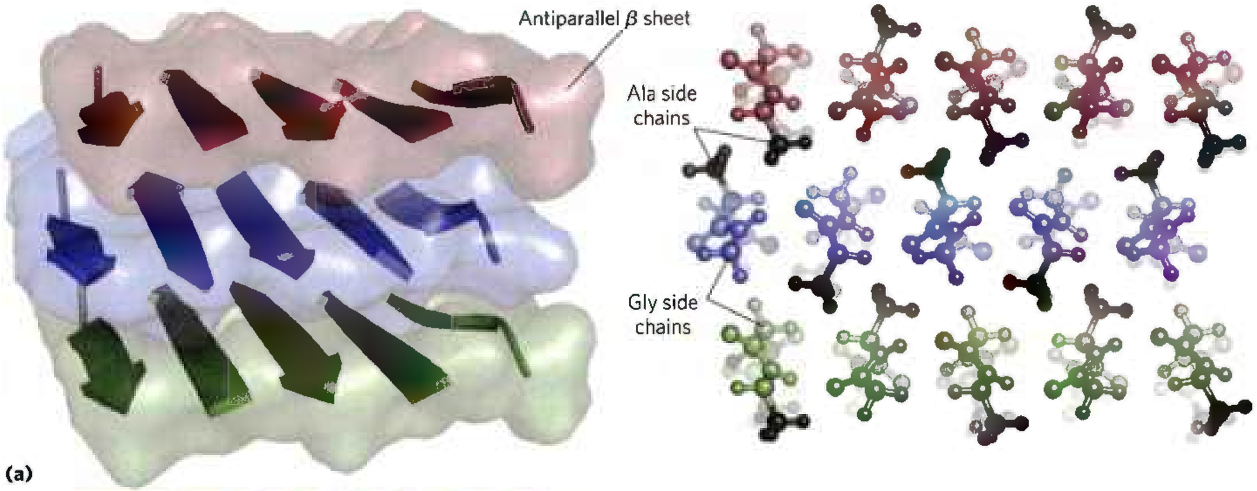


FIGURE 4-13 Structure of silk. The fibers in silk cloth and in a spider web are made up primarily of the protein fibroin. (a) Fibroin consists of layers of antiparallel β sheets rich in Ala and Gly residues. The small side chains interdigitate and allow close packing of the sheets, as shown in the ball-and-stick view. The segments shown here would be just a small part of the fibroin strand. (b) Strands of silk emerge from the spinnerets of a spider in this colorized scanning electron micrograph. [(a) Data from PDB ID 1SLK, S. A. Fossey et al., *Biopolymers* 31:1529, 1991. (b) Tina Weatherby Carvalho/MicroAngela.]

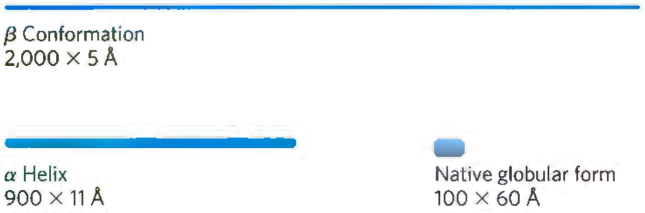


FIGURE 4-14 Globular protein structures are compact and varied. Human serum albumin (M_r 64,500) has 585 residues in a single chain. Given here are the approximate dimensions its single polypeptide chain would have if it occurred entirely in extended β conformation or as an α helix. Also shown is the size of the protein in its native globular form, as determined by x-ray crystallography; the polypeptide chain must be very compactly folded to fit into these dimensions.

Our discussion of globular proteins begins with the principles gleaned from the first protein structures to be elucidated. This is followed by a detailed description of protein substructure and comparative categorization. Such discussions are possible only because of the vast amount of information available online from publicly accessible databases, particularly the Protein Data Bank, or PDB (**Box 4-3**).

Myoglobin Provided Early Clues about the Complexity of Globular Protein Structure

The first breakthrough in understanding the three-dimensional structure of a globular protein came from x-ray diffraction studies of myoglobin carried out by John Kendrew and his colleagues in the 1950s. Myoglobin is a relatively small (M_r 16,700), oxygen-binding protein of muscle cells. It functions both to store oxygen and to facilitate oxygen diffusion in rapidly contracting muscle tissue. Myoglobin contains a single polypeptide chain of 153 amino acid residues of known sequence and a single iron protoporphyrin, or heme, group. The same heme group that is found in myoglobin is found in hemoglobin, the oxygen-binding protein of erythrocytes, and is responsible for the deep red-brown color of both myoglobin and hemoglobin. Myoglobin is particularly abundant in the muscles of diving mammals such as whales, seals, and porpoises—so abundant that the muscles of these animals are brown. Storage and distribution of oxygen by muscle myoglobin permits diving mammals to remain submerged for long periods. The activities of myoglobin and other globin molecules are investigated in greater detail in Chapter 5.

BOX 4-3

The Protein Data Bank

The number of known three-dimensional protein structures is now more than 100,000 and doubles every couple of years. This wealth of information is revolutionizing our understanding of protein structure, the relationship of structure to function, and the evolutionary paths by which proteins arrived at their present state, which can be seen in the family resemblances that come to light as protein databases are sifted and sorted. One of the most important resources available to biochemists is the **Protein Data Bank (PDB**; www.rcsb.org).

The PDB is an archive of experimentally determined three-dimensional structures of biological macromolecules, containing virtually all of the macromolecular structures (such as proteins, RNAs, and DNAs) elucidated to date. Each structure is assigned an identifying label (a four-character identifier called the PDB ID). Such labels are provided in the figure legends for every PDB-derived structure illustrated in this text so that students and instructors can explore the same structures on their own. The data files in the PDB describe the spatial coordinates of each atom for which the position has been determined (many of the cataloged structures are not complete). Additional data files provide information on how the structure was determined and its accuracy. The atomic coordinates can be converted into an image of the macromolecule by using structure visualization software. Students are encouraged to access the PDB and explore structures, using visualization software linked to the database. Macromolecular structure files can also be downloaded and explored on the desktop, using free software such as JSmol.

Figure 4-15 shows several structural representations of myoglobin, illustrating how the polypeptide chain is folded in three dimensions—its tertiary structure. The red group surrounded by protein is heme. The backbone of the myoglobin molecule consists of eight relatively straight segments of α helix interrupted by bends, some of which are β turns. The longest α helix has 23 amino acid residues and the shortest has only 7; all helices are right-handed. More than 70% of the residues in myoglobin are in these α-helical regions. X-ray analysis has revealed the precise position of each of the R groups, which fill up nearly all the space within the folded chain that is not occupied by backbone atoms.

Many important conclusions were drawn from the structure of myoglobin. The positioning of amino acid side chains reflects a structure that is largely stabilized by the hydrophobic effect. Most of the hydrophobic R groups are in the interior of the molecule, hidden from exposure to water. All but two of the polar R groups are located on the outer surface of the molecule, and all are hydrated. The myoglobin molecule is so compact that its interior has room for only four molecules of water. This dense hydrophobic core is typical of globular proteins. The fraction of space occupied by atoms in an organic liquid is 0.4 to 0.6. In a globular protein the fraction is about 0.75, comparable to that in a crystal (in a typical crystal the fraction is 0.70 to 0.78, near the theoretical maximum). In this packed environment, weak interactions strengthen and reinforce each other. For example, the nonpolar side chains in the core are so close together that short-range van der Waals interactions make a significant contribution to stabilizing interactions.

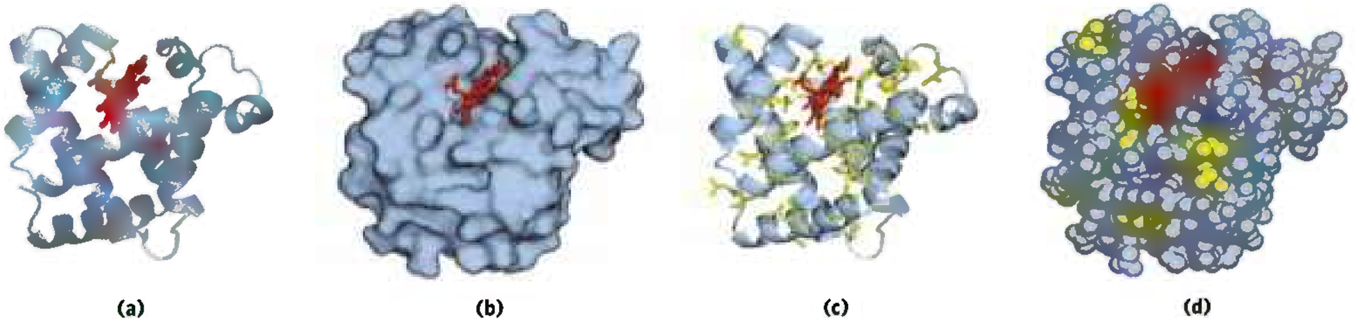


FIGURE 4-15 Tertiary structure of sperm whale myoglobin. Orientation of the protein is similar in (a) through (d); the heme group is shown in red. In addition to illustrating the myoglobin structure, this figure provides examples of several different ways to display protein structure. (a) The polypeptide backbone in a ribbon representation of a type introduced by Jane Richardson, which highlights regions of secondary structure. The α-helical regions are evident. (b) Surface contour image; this is useful for visualizing pockets in the protein where other molecules might bind. (c) Ribbon representation including side chains (yellow) for the hydrophobic residues Leu, Ile, Val, and Phe. (d) Space-filling model with all amino acid side chains. Each atom is represented by a sphere encompassing its van der Waals radius. The hydrophobic residues are again shown in yellow; most are buried in the interior of the protein and thus are not visible. [Data from PDB ID 1MBO, S. E. Phillips, *J. Mol. Biol.* 142:531, 1980.]

Deduction of the structure of myoglobin confirmed some expectations and introduced some new elements of secondary structure. As predicted by Pauling and Corey, all the peptide bonds are in the planar trans configuration. The α helices in myoglobin provided the first direct experimental evidence for the existence of this type of secondary structure. Three of the four Pro residues are found at bends. The fourth Pro residue occurs within an α helix, where it creates a kink necessary for tight helix packing.

The flat heme group rests in a crevice, or pocket, in the myoglobin molecule. Within this pocket, the accessibility of the heme group to solvent is highly restricted. This is important for function, because free heme groups in an oxygenated solution are rapidly oxidized from the ferrous (Fe^{2+}) form, which is active in the reversible binding of O_2, to the ferric (Fe^{3+}) form, which does not bind O_2. As myoglobin structures from many different species were resolved, investigators were able to observe the structural changes that accompany the binding of oxygen or other molecules and thus, for the first time, to understand the correlation between protein structure and function. Hundreds of proteins have now been subjected to similar analysis.

Globular Proteins Have a Variety of Tertiary Structures

Myoglobin illustrates just one of many ways in which a polypeptide chain can fold. **Table 4-3** shows the proportions of α helix and β conformation (expressed as percentage of residues in each type) in several small, single-chain, globular proteins. Each of these proteins has a distinct structure, adapted for its particular biological function, but together they share several important properties with myoglobin. P1 Each is folded compactly, and in each case the hydrophobic amino acid side chains are oriented toward the interior (away from water) and the hydrophilic side chains are on the surface. The structures are also stabilized by a multitude of hydrogen bonds and some ionic interactions.

To understand a complete three-dimensional structure, we need to analyze its folding patterns. We begin by defining two important terms that describe protein structural patterns or elements in a polypeptide chain; then we turn to the folding rules. The first term is **motif**, also called a **fold**. P3 A motif or fold is a recognizable folding pattern involving two or more elements of secondary structure and the connection(s) between them. A motif can be very simple, such as two elements of secondary structure folded against each other, and may represent only a small part of a protein. An example is a **β-α-β loop** (**Fig. 4-16a**). A motif can also be a very elaborate structure involving scores of protein segments folded together, such as the **β barrel** (Fig. 4-16b). In some cases, a single large motif may comprise the entire protein. The terms "motif" and "fold" are often used interchangeably, although "fold" is applied more commonly to somewhat more complex folding patterns. The segment defined as a motif or a fold may or may not be independently stable. We have already encountered a well-studied motif, the coiled coil of α-keratin, which is also found in some other proteins. The distinctive arrangement of eight α helices in myoglobin is replicated in all globins and is called the globin fold. Note that a motif is not a hierarchical structural element falling between secondary and tertiary structure. It is simply a folding pattern.

The second term for describing structural patterns is **domain**. A domain, as defined by Jane Richardson in 1981, is a part of a polypeptide chain that is independently stable or could undergo movements as a single entity with respect to the entire protein. Polypeptides with more than a few hundred amino acid residues often fold into two or more domains, sometimes with different functions. In many cases, a domain from a large protein

TABLE 4-3 Approximate Proportion of α Helix and β Conformation in Some Single-Chain Proteins

	Residues (%)[a]	
Protein (total residues)	**α Helix**	**β Conformation**
Chymotrypsin (247)	14	45
Ribonuclease (124)	26	35
Carboxypeptidase (307)	38	17
Cytochrome *c* (104)	39	0
Lysozyme (129)	40	12
Myoglobin (153)	78	0

Source: Data from C. R. Cantor and P. R. Schimmel, *Biophysical Chemistry, Part I: The Conformation of Biological Macromolecules*, p. 100, W. H. Freeman and Company, 1980.

[a] Portions of the polypeptide chains not accounted for by α helix or β conformation consist of bends and irregularly coiled or extended stretches. Segments of α helix and β conformation sometimes deviate slightly from their normal dimensions and geometry.

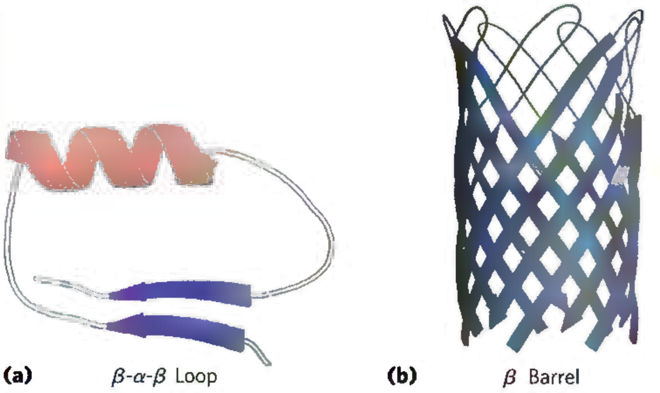

(a) β-α-β Loop (b) β Barrel

FIGURE 4-16 Motifs. (a) A simple motif, the β-α-β loop. (b) A more elaborate motif, the β barrel. This β barrel is a single domain of α-hemolysin (a toxin that kills a cell by creating a hole in its membrane) from the bacterium *Staphylococcus aureus*. [Data from (a) PDB ID 4TIM, M. E. Noble et al., *J. Med. Chem.*, 34:2709, 1991; (b) PDB ID 7AHL, L. Song et al., *Science* 274:1859, 1996.]

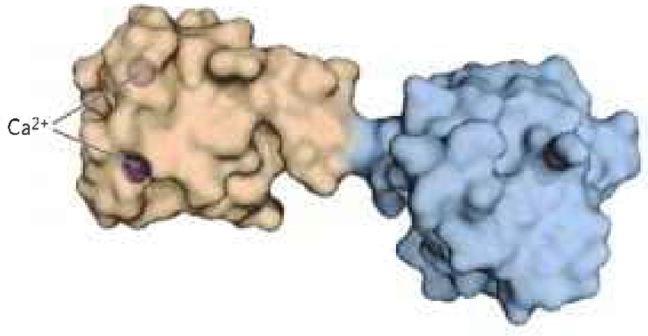
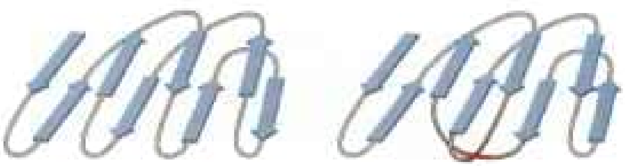
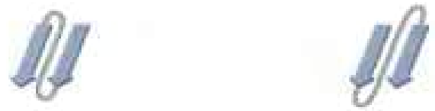


FIGURE 4-17 Structural domains in the polypeptide troponin C. This calcium-binding protein, associated with muscle, has two separate calcium-binding domains, shown here in brown and blue. [Data from PDB ID 4TNC, K. A. Satyshur et al., *J. Biol. Chem.* 263:1628, 1988.]



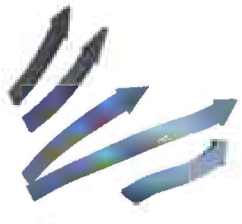
(a) Typical connections in an all-β motif

Crossover connection (not observed)



(b) Right-handed connection between β strands

Left-handed connection between β strands (very rare)



(c) Twisted β sheet

FIGURE 4-18 Stable folding patterns in proteins. (a) Connections between β strands in layered β sheets. The strands here are viewed from one end, with no twisting. The connections at a given end (e.g., near the viewer) rarely cross one another. An example of such a rare crossover is illustrated by the red strands in the structure on the right. (b) Because of the right-handed twist in β strands, connections between strands are generally right-handed. Left-handed connections must traverse sharper angles and are harder to form. (c) This twisted β sheet is from a domain of photolyase (a protein that repairs certain types of DNA damage) from *E. coli*. Connecting loops have been removed so as to focus on the folding of the β sheet. [Data from PDB ID 1DNP, H. W. Park et al., *Science* 268:1866, 1995.]

will retain its native three-dimensional structure even when separated (for example, by proteolytic cleavage) from the remainder of the polypeptide chain. In a protein with multiple domains, each domain may appear as a distinct globular lobe (**Fig. 4-17**); more commonly, extensive contacts between domains make individual domains hard to discern. Different domains often have distinct functions, such as the binding of small molecules or interaction with other proteins. Small proteins usually have only one domain (the domain *is* the protein).

Folding of polypeptides is subject to an array of physical and chemical constraints, and several rules have emerged from studies of common protein-folding patterns.

1. The hydrophobic effect makes a large contribution to the stability of protein structures. Burial of hydrophobic amino acid R groups so as to exclude water requires at least two layers of secondary structure. Simple motifs such as the β-α-β loop (Fig. 4-16a) create two such layers.
2. Where they occur together in a protein, α helices and β sheets generally are found in different structural layers. This is because the backbone of a polypeptide segment in the β conformation (Fig. 4-5) cannot readily hydrogen-bond to an α helix that is adjacent to it.
3. Segments adjacent to each other in the amino acid sequence are usually stacked adjacent to each other in the folded structure. Distant segments of a polypeptide may come together in the tertiary structure, but this is not the norm.
4. The β conformation is most stable when the individual segments are twisted slightly in a right-handed sense. This influences both the arrangement of β sheets derived from the twisted segments and the path of the polypeptide connections between them. Two parallel β strands, for example, must be connected by a crossover strand (**Fig. 4-18a**). In principle, this crossover could have a right-handed or left-handed conformation, but in proteins it is almost always right-handed. Right-handed connections tend to be shorter than left-handed connections and tend to bend through smaller angles, making them easier to form. The twisting of β sheets also leads to a characteristic twisting of the structure formed by many such segments together, as seen in the β barrel (Fig. 4-16b) and the twisted β sheet (Fig. 4-18c), which form the core of many larger structures.

Following these rules, complex motifs can be built up from simple ones. For example, a series of β-α-β loops arranged so that the β strands form a barrel creates a particularly stable and common motif, the **α/β barrel** (**Fig. 4-19**). In this structure, each parallel β segment is attached to its neighbor by an α-helical segment. All connections are right-handed. The α/β barrel is found in many enzymes, often with a binding site (for a cofactor or a substrate) in the form of a pocket near one end of the barrel. Note that domains with similar folding patterns are said to have the same motif, even though their constituent α helices and β sheets may differ in length.

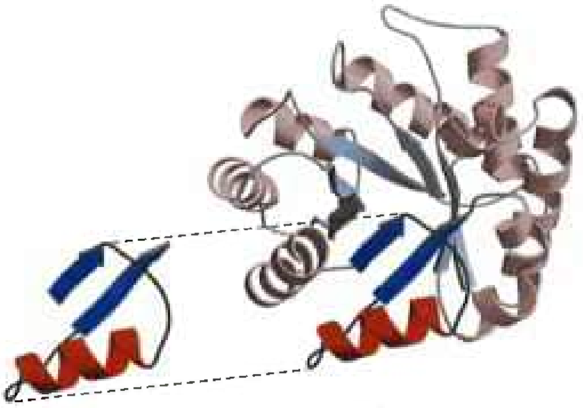


FIGURE 4-19 Constructing large motifs from smaller ones. The α/β barrel is a commonly occurring motif constructed from repetitions of the β-α-β loop motif. This α/β barrel is a domain of pyruvate kinase (a glycolytic enzyme) from rabbit. [Data from PDB ID 1PKN, T. M. Larsen et al., *Biochemistry* 33:6301, 1994.]

Some Proteins or Protein Segments Are Intrinsically Disordered

Although many proteins contain well-folded and stable structures, this is not necessary for the biological function of all proteins. Many proteins or protein segments lack ordered structures in solution. The concept that some proteins function in the absence of a definable three-dimensional structure comes from reassessment of data from many different proteins. As many as a third of all human proteins may be unstructured or may have significant unstructured segments. All organisms have some proteins that fall into this category. Intrinsically disordered proteins have properties that are distinct from those of classical, structured proteins. They often lack a hydrophobic core and instead are characterized by high densities of charged amino acid residues such as Lys, Arg, and Glu. Pro residues are also prominent, as they tend to disrupt ordered structures.

Structural disorder and high charge density can facilitate the function of some proteins as spacers, insulators, or linkers in larger structures. Other disordered proteins are scavengers, binding up ions and small molecules in solution and serving as reservoirs or garbage dumps. However, many intrinsically disordered proteins are at the heart of important protein interaction networks. The lack of an ordered structure can facilitate a kind of functional promiscuity, allowing one protein to interact with multiple or even dozens of partners. Structural disorder allows some inhibitor proteins, such as the mammalian cell division protein p27, to interact with multiple targets in different ways. In solution, p27 lacks definable structure. However, it wraps around and inhibits the action of several enzymes called protein kinases (see Chapter 6) that facilitate cell division. The flexible structure of p27 allows it to accommodate itself to its different target proteins. Human tumor cells, which are cells that have lost the capacity to control cell division normally, generally have reduced levels of p27; the lower the levels of p27, the poorer the prognosis for the cancer patient.

Similarly, intrinsically disordered proteins are often present as hubs or scaffolds at the center of protein networks that constitute signaling pathways (see Fig. 12-30). These proteins, or parts of them, may interact with many different binding partners. They often take on an ordered structure when they interact with other proteins, but the structure they assume may vary with different binding partners. The mammalian protein p53 is also critical in the control of cell division. It contains both structured and unstructured segments, and the different segments interact with dozens of other proteins. An unstructured region of p53 at the carboxyl terminus interacts with at least four different binding partners and assumes a different structure in each of the complexes (**Fig. 4-20**).

Protein Motifs Are the Basis for Protein Structural Classification

More than 150,000 structures are now archived in the Protein Data Bank (PDB; for a deeper explanation, see Box 4-3). An enormous amount of information about protein structural principles, protein function, and protein evolution is contained in these data. Other databases have organized this information and made it more readily accessible. In the Structural Classification of Proteins database, or SCOP2 (http://scop2.mrc-lmb.cam.ac.uk), all of the protein information in the PDB can be searched within four different categories: (1) protein relationships, (2) structural classes, (3) protein types, and (4) evolutionary events. **Figure 4-21** presents examples of protein motifs taken from SCOP2 to illustrate the potential of searching within each category. The figure also introduces another way to represent elements of secondary structure and the relationships among segments of secondary structure in a protein — the **topology diagram**.

The number of folding patterns is not infinite. Among the tens of thousands of distinct protein structures archived in the PDB, only about 1,400 different folds or motifs are classified by the SCOP2 database. Given the many years of progress in structural biology, new motifs are now discovered only rarely. Many examples of recurring domain or motif structures are available, and these reveal that protein tertiary structure is more reliably conserved than amino acid sequence. The comparison of protein structures can thus provide much information about evolution. Proteins with significant similarity in primary structure and/or with similar tertiary structure and function are said to be in the same **protein family**. The protein structures in the PDB belong to about 4,000 different protein families. A strong evolutionary relationship

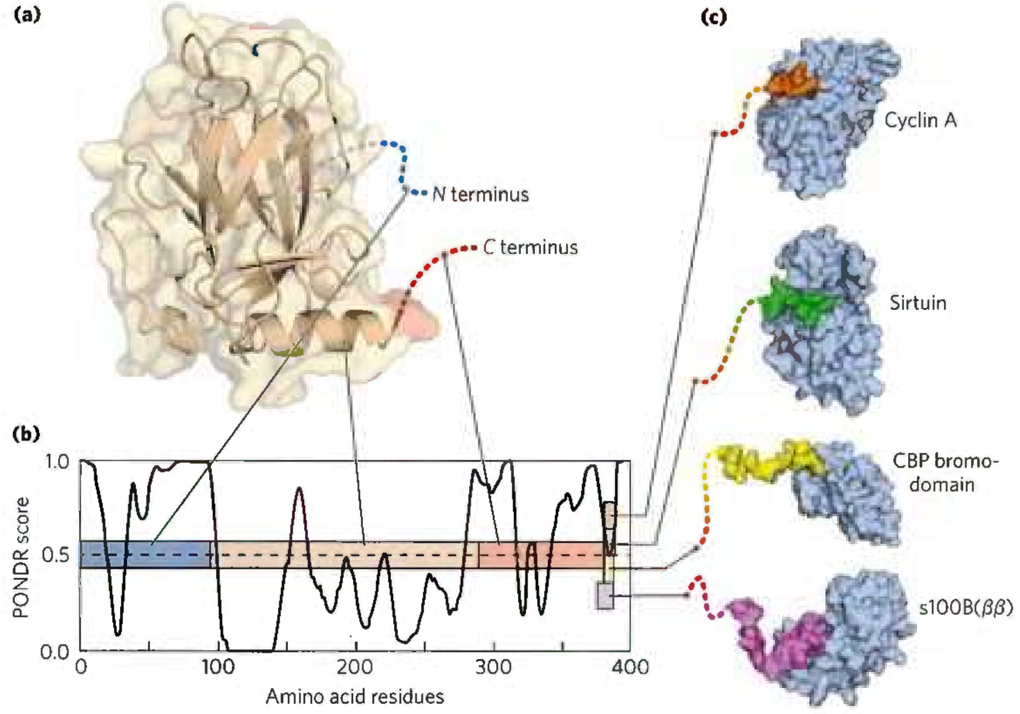


FIGURE 4-20 Binding of the intrinsically disordered carboxyl terminus of p53 protein to its binding partners. (a) The p53 protein is made up of several different segments. Only the central domain is well ordered. (b) The linear sequence of the p53 protein is depicted as a colored bar. The overlaid graph presents a plot of the PONDR (Predictor of Natural Disordered Regions) score versus the protein sequence. PONDR is one of the best available algorithms for predicting the likelihood that a given amino acid residue is in a region of intrinsic disorder, based on the surrounding amino acid sequence and amino acid composition. A score of 1.0 indicates a probability of 100% that a protein will be disordered. In the actual protein structure, the tan central domain is ordered. The amino-terminal (blue) and carboxyl-terminal (red) regions are disordered. (c) The very end of the carboxyl-terminal region has multiple binding partners and folds when it binds to each of them; however, the three-dimensional structure that is assumed when binding occurs is different for each of the interactions shown, and thus this carboxyl-terminal segment (11 to 20 residues) is shown in a different color in each complex. [Information from V. N. Uversky, *Intl. J. Biochem. Cell Biol.* 43:1090, 2011, Fig. 5. (a) Data from PDB ID 1TUP, Y. Cho et al., *Science* 265:346, 1994. (c) Data from Cyclin A: PDB ID 1H26, E. D. Lowe et al., *Biochemistry* 41:15,625, 2002; sirtuin: PDB ID 1MA3, J. L. Avalos et al., *Mol. Cell* 10:523, 2002; CBP bromodomain: PDB ID 1JSP, S. Mujtaba et al., *Mol. Cell* 13:251, 2004; s100B(ββ): PDB ID 1DT7, R. R. Rustandi et al., *Nature Struct. Biol.* 7:570, 2000.]

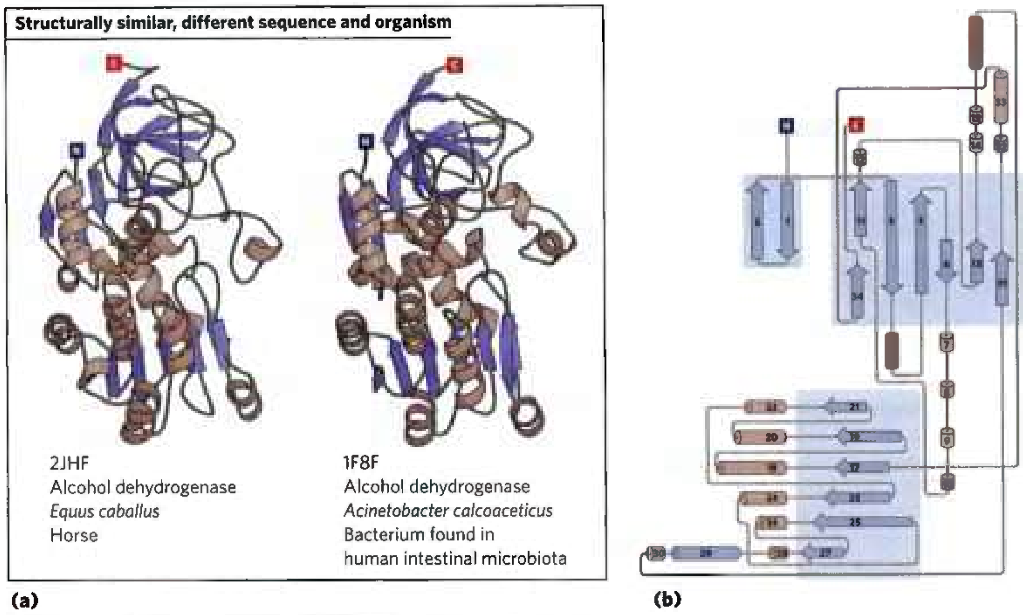
is usually evident within a protein family. For example, the globin family has many different proteins with both structural and sequence similarities to myoglobin (as seen in the proteins used as examples in Figures 4-30 and 4-31 and in Chapter 5). Two or more families that have little similarity in amino acid sequence but make use of the same major structural motif and have functional similarities are grouped into **superfamilies**. An evolutionary relationship among families in a superfamily is considered probable, even though time and functional distinctions—that is, different adaptive pressures—may have erased many of the telltale sequence relationships.

A protein family may be widespread in all three domains of cellular life—the Bacteria, Archaea, and Eukarya—suggesting an ancient origin. Many proteins involved in intermediary metabolism and the metabolism of nucleic acids and proteins fall into this category. Other families may be present in only a small group of organisms, indicating that the structure arose more recently. Tracing the natural history of structural motifs through the use of structural classifications in databases such as SCOP2 provides a powerful complement to sequence analyses in tracing evolutionary relationships. The SCOP2 database is curated manually, with the objective of placing proteins in the correct evolutionary framework based on conserved structural features.

Structural motifs become especially important in defining protein families and superfamilies. Improved protein classification and comparison systems lead inevitably to the elucidation of new functional relationships. Given the central role of proteins in living systems, these structural comparisons can help illuminate every aspect of biochemistry, from the evolution of individual proteins to the evolutionary history of complete metabolic pathways.

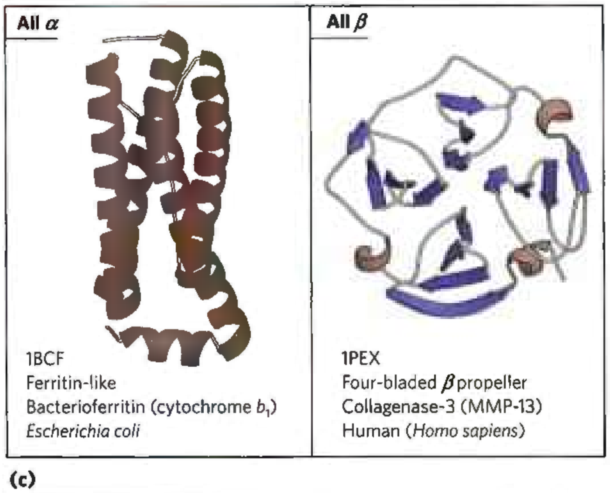
Protein Quaternary Structures Range from Simple Dimers to Large Complexes

P3 Many proteins have multiple polypeptide subunits (from two to hundreds). The association of polypeptide chains can serve a variety of functions. Many multisubunit proteins have regulatory roles; the binding of small molecules may affect the interaction between subunits,



2JHF
Alcohol dehydrogenase
Equus caballus
Horse

1F8F
Alcohol dehydrogenase
Acinetobacter calcoaceticus
Bacterium found in
human intestinal microbiota



1BCF
Ferritin-like
Bacterioferritin (cytochrome b_1)
Escherichia coli

1PEX
Four-bladed β propeller
Collagenase-3 (MMP-13)
Human (*Homo sapiens*)

FIGURE 4-21 Organization of proteins based on motifs. A few of the hundreds of known stable motifs. (a) Structural diagrams of the enzyme alcohol dehydrogenase from two different organisms. Such comparisons illustrate evolutionary relationships that conserve structure as well as function. (b) A topology diagram for the alcohol dehydrogenase from *Acinetobacter calcoaceticus*. Topology diagrams provide a way to visualize elements of secondary structure and their interconnections in two dimensions; the diagrams can be very useful in comparing structural folds or motifs. (c) The Structural Classification of Proteins (SCOP2) database (http://scop2.mrc-lmb.cam.ac.uk) organizes protein folds into four classes: all α, all β, α/β, and $\alpha + \beta$. Examples of all α folds and all β folds are shown with their structural classification data (PDB ID, fold name, protein name, and source organism) from the SCOP2 database. The PDB ID is the unique accession code given to each structure archived in the Protein Data Bank (www.rcsb.org). [Data from (a) PDB ID 2JHF, R. Meijers et al., *Biochemistry* 46:5446, 2007; (a, b) PDB ID 1F8F, J. C. Beauchamp et al. (c) PDB ID 1BCF, F. Frolow et al., *Nature Struct. Biol.* 1:453, 1994; PDB ID 1PEX, F. X. Gomis-Ruth et al., *J. Mol. Biol.* 264:556, 1996.]

causing large changes in the protein's activity in response to small changes in the concentration of substrate or regulatory molecules (Chapter 6). In other cases, separate subunits take on separate but related functions, such as catalysis and regulation. Some associations, such as those seen in the fibrous proteins considered earlier in this chapter and the coat proteins of viruses, serve primarily structural roles. Some very large protein assemblies are the site of complex, multistep reactions. For example, each ribosome, the site of protein synthesis, incorporates dozens of protein subunits along with RNA molecules.

A multisubunit protein can also be referred to as an **oligomer** or **multimer**. If an oligomer has nonidentical subunits, the overall structure of the protein can be asymmetric and quite complicated. However, many oligomers have identical subunits or repeating groups of nonidentical subunits, usually in symmetric arrangements. As noted in Chapter 3, the repeating structural unit in such an oligomeric protein, whether a single subunit or a group of subunits, is called a **protomer**.

The first oligomeric protein to have its three-dimensional structure determined was hemoglobin (M_r 64,500), which contains four polypeptide chains and four heme prosthetic groups, in which the iron atoms are in the ferrous (Fe^{2+}) state (as we shall see in Chapter 5). The protein portion, the globin, consists of two α chains (141 residues each) and two β chains (146 residues each). Note that in this case, α and β do not refer to secondary structures. In a practice that can be confusing to the beginning student, the Greek letters α and β (and γ, δ, and others) are often used to distinguish two different kinds of subunits within a multisubunit protein, regardless of what kinds of secondary structure may predominate in the subunits. Because hemoglobin is four times as large as myoglobin, much more time and effort were required to solve its three-dimensional structure by x-ray analysis, finally achieved by Max Perutz, John Kendrew, and their colleagues in 1959. The subunits of hemoglobin are arranged in symmetric pairs (**Fig. 4-22**), each pair having one α subunit and one β subunit.

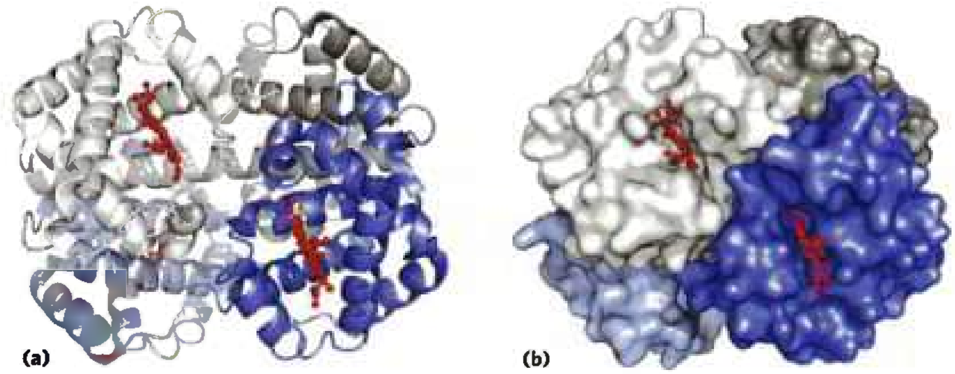


FIGURE 4-22 **Quaternary structure of deoxyhemoglobin.** X-ray diffraction analysis of deoxyhemoglobin (hemoglobin without oxygen molecules bound to the heme groups) shows how the four polypeptide subunits are packed together. (a) A ribbon representation reveals the secondary structural elements of the structure and the positioning of all the heme prosthetic groups. (b) A surface contour model shows the pockets in which the heme prosthetic groups are bound and helps to visualize subunit packing. The α subunits are shown in shades of gray, the β subunits in shades of blue. Note that the heme groups (red) are relatively far apart. [Data from PDB ID 2HHB, G. Fermi et al., *J. Mol. Biol.* 175:159, 1984.]

Max Perutz, 1914–2002 (left), and John Kendrew, 1917–1997 [Hulton Deutsch/Getty Images.]

Hemoglobin can therefore be described either as a tetramer or as a dimer of $\alpha\beta$ protomers. The role these distinct subunits play in hemoglobin function is discussed extensively in Chapter 5.

SUMMARY 4.3 *Protein Tertiary and Quaternary Structures*

- Tertiary structure is the complete three-dimensional structure of a polypeptide chain. Many proteins fall into one of four general classes based on tertiary structure: fibrous, globular, membrane, or disordered.
- Insoluble fibrous proteins, such as those that make up keratin, collagen, and silk, have simple repeating elements of secondary structure. In some fibrous proteins, the individual polypeptide chains interact to form complex quaternary structures like coiled coils for strength and flexibility.
- Globular proteins have more complicated tertiary structures, often containing several types of secondary structure in the same polypeptide chain, and fulfill many different functional roles in the cell.
- The first globular protein structure to be determined, by x-ray diffraction methods, was that of the O_2-binding protein myoglobin. The myoglobin structure revealed for the first time how protein structure and function are connected.
- The complex structures of globular proteins can be analyzed by examination of folding patterns, called motifs or folds. The many thousands of known protein structures are generally assembled from a repertoire of only a few hundred motifs. Domains are regions of a polypeptide chain that can fold stably and independently.
- Some proteins or protein segments are intrinsically disordered, lacking definable three-dimensional structure. These proteins often have distinctive amino acid compositions that allow a more flexible structure, which is critical for their biological function.
- Based on structural similarities, proteins can be organized into families and superfamilies, which are informative about protein function and evolution.
- Quaternary structure results from interactions between the subunits of multisubunit (multimeric) proteins or large supramolecular assemblies. Some multimeric proteins are composed of repeated subunits called protomers.

4.4 Protein Denaturation and Folding

Proteins lead a surprisingly precarious existence. As we have seen, a native protein conformation is only marginally stable. In addition, most proteins must maintain conformational flexibility in order to function. P4 The continual maintenance of the active set of cellular proteins required under a given set of conditions is called **proteostasis**. Cellular proteostasis requires the coordinated function of pathways for protein synthesis and folding, the refolding of proteins that are partially unfolded, and the sequestration and degradation of proteins that have been irreversibly unfolded or are no longer needed. In all cells, these networks involve hundreds of enzymes and specialized proteins.

As seen in **Figure 4-23**, the life of a protein encompasses much more than its synthesis and later degradation. The marginal stability of most proteins can produce a tenuous balance between folded and unfolded states. As proteins are synthesized on ribosomes (Chapter 27), they must fold into their native conformations. Sometimes this occurs spontaneously, but often it requires the assistance of specialized enzymes and complexes called chaperones, which we discuss later in the chapter. Many of these same folding helpers function to refold proteins that become transiently unfolded. Proteins that are not properly folded often have exposed hydrophobic surfaces that render them "sticky," leading to the formation of inactive aggregates. These aggregates may lack their normal function but are not inert; their accumulation in cells lies at the heart of diseases ranging from diabetes to Parkinson disease and Alzheimer disease. Not surprisingly, all cells have elaborate pathways for recycling and/or degrading proteins that are irreversibly misfolded.

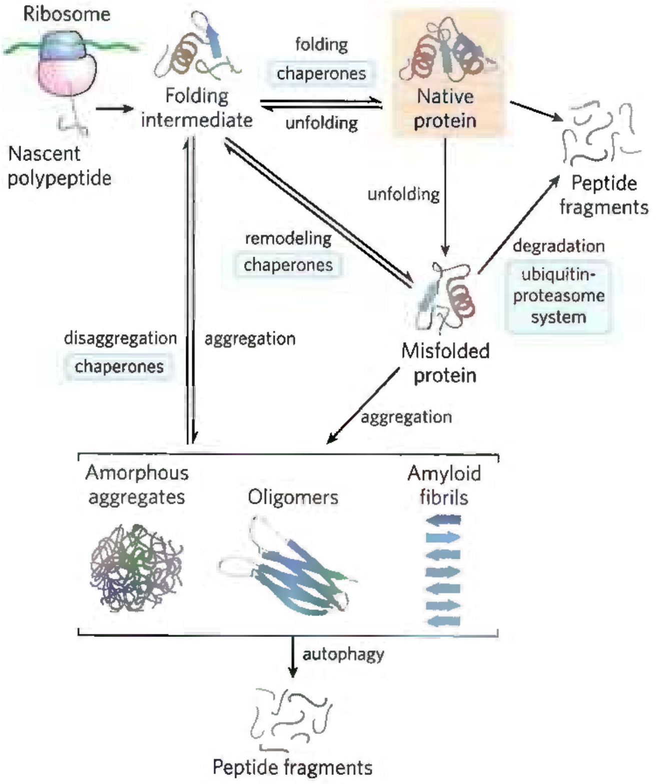


FIGURE 4-23 Pathways that contribute to proteostasis. Three kinds of processes contribute to proteostasis, in some cases with multiple contributing pathways. First, proteins are synthesized on a ribosome. Second, various pathways contribute to protein folding, many of which involve the activity of complexes called chaperones. Chaperones (including chaperonins) also contribute to the refolding of proteins that are partially and transiently unfolded. Finally, proteins that are irreversibly unfolded are subject to sequestration and degradation by several additional pathways. Partially unfolded proteins and protein-folding intermediates that escape the quality-control activities of the chaperones and degradative pathways may aggregate, forming both disordered aggregates and ordered amyloidlike aggregates that contribute to disease and aging processes. [Information from F. U. Hartl et al., *Nature* 475:324, 2011, Fig. 6.]

The transitions between the folded and unfolded states, and the network of pathways that control these transitions, now become our focus.

Loss of Protein Structure Results in Loss of Function

Protein structures have evolved to function in particular cellular or extracellular environments. Conditions different from these environments can result in protein structural changes, large and small. A loss of three-dimensional structure sufficient to cause loss of function is called **denaturation**. The denatured state does not necessarily equate with complete unfolding of the protein and randomization of conformation. Under most conditions, denatured proteins exist in a set of partially folded states.

Most proteins can be denatured by heat, which has complex effects on many weak interactions in a protein (primarily on the hydrogen bonds). If the temperature is increased slowly, a protein's conformation generally remains intact until an abrupt loss of structure (and function) occurs over a narrow temperature range (**Fig. 4-24**). The abruptness of the change suggests that unfolding is a cooperative process: loss of structure in one part of the protein destabilizes other parts. The effects of heat on proteins can be mitigated by structure. The very heat-stable proteins of thermophilic bacteria and archaea have evolved to function at the temperature of hot springs (~100 °C). The folded structures of these proteins are often similar to those of proteins in other organisms, but take some of the principles outlined here to extremes. They often feature high densities of charged residues on their surfaces, even tighter hydrophobic packing in their interiors, and folds rendered less flexible by networks of ion pairs. Each of these features makes these proteins less susceptible to unfolding at high temperatures.

Proteins can also be denatured by extremes of pH, by certain miscible organic solvents such as alcohol or acetone, by certain solutes such as urea and guanidine hydrochloride, or by detergents. Each of these denaturing agents represents a relatively mild treatment in the sense that no covalent bonds in the polypeptide chain are broken. Organic solvents, urea, and detergents act primarily by disrupting the hydrophobic aggregation of nonpolar amino acid side chains that produces the stable core of globular proteins; urea also disrupts hydrogen bonds; and extremes of pH alter the net charge on a protein, causing electrostatic repulsion and the disruption of some hydrogen bonding. The denatured structures

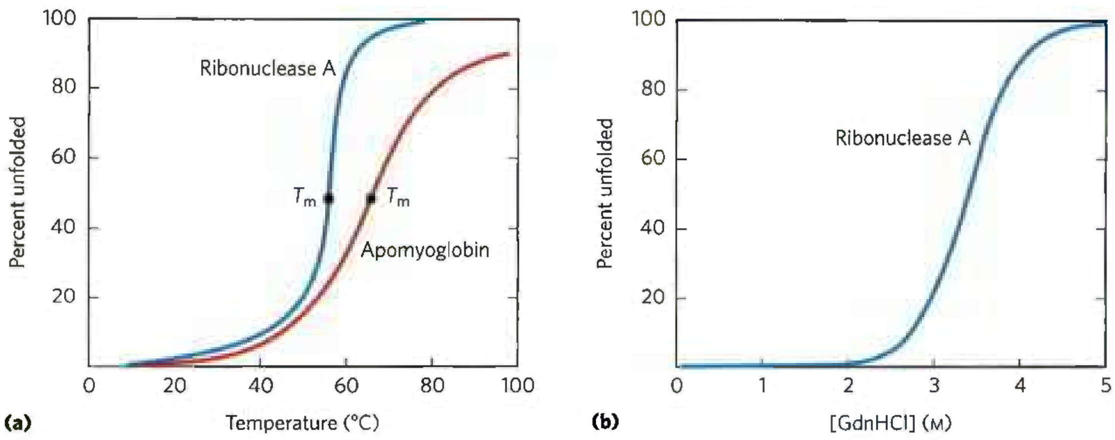


FIGURE 4-24 Protein denaturation. Results are shown for proteins denatured by two different environmental changes. In each case, the transition from the folded state to the unfolded state is abrupt, suggesting cooperativity in the unfolding process. (a) Thermal denaturation of horse apomyoglobin (myoglobin without the heme prosthetic group) and ribonuclease A (with its disulfide bonds intact; see Fig. 4-26). The midpoint of the temperature range over which denaturation occurs is called the melting temperature, or T_m. Denaturation of apomyoglobin was monitored by circular dichroism (see Fig. 4-9), which measures the amount of helical structure in the protein. Denaturation of ribonuclease A was tracked by monitoring changes in the intrinsic fluorescence of the protein, which is affected by changes in the environment of a Trp residue introduced by mutation. (b) Denaturation of disulfide-intact ribonuclease A by guanidine hydrochloride (GdnHCl), monitored by circular dichroism. [Data from (a) R. A. Sendak et al., *Biochemistry* 35:12,978, 1996; I. Nishii et al., *J. Mol. Biol.* 250:223, 1995; (b) W. A. Houry et al., *Biochemistry* 35:10,125, 1996.]

resulting from these various treatments are not necessarily the same.

Denaturation often leads to protein precipitation, a consequence of protein aggregate formation as exposed hydrophobic surfaces associate. The aggregates are often highly disordered. One example is the protein precipitate that can be seen after an egg white is boiled. More-ordered aggregates are also observed in some proteins, as we shall see.

Amino Acid Sequence Determines Tertiary Structure

P4 The tertiary structure of a globular protein is determined by its amino acid sequence. The most important proof of this came from experiments showing that denaturation of some proteins is reversible. Certain globular proteins denatured by heat, extremes of pH, or denaturing reagents will regain their native structure and their biological activity if they are returned to conditions in which the native conformation is stable. This process is called **renaturation**.

A classic example is the denaturation and renaturation of ribonuclease A, demonstrated by Christian Anfinsen in the 1950s. Purified ribonuclease A denatures completely in a concentrated urea solution in the presence of a reducing agent. The reducing agent cleaves the four disulfide bonds to yield eight Cys residues, and the urea disrupts the stabilizing hydrophobic effect, thus freeing the entire polypeptide from its folded conformation. Denaturation of ribonuclease is accompanied by a complete loss of catalytic activity. When the urea and the reducing agent are removed, the denatured ribonuclease spontaneously refolds into its correct tertiary structure, with full restoration of its catalytic activity (**Fig. 4-25**). The refolding of ribonuclease is so accurate that the four

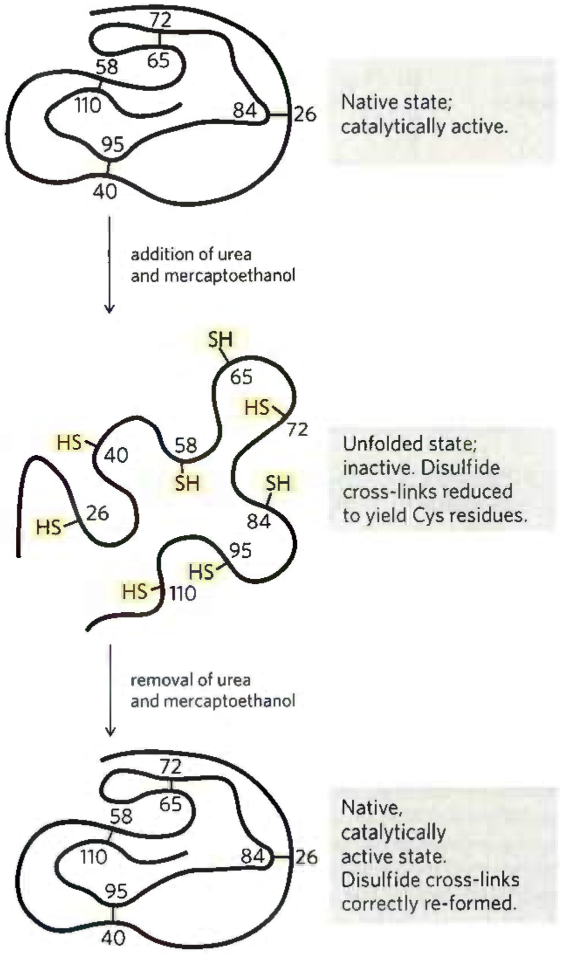

72
58
65
110
84
26
95
40
Native state; catalytically active.
addition of urea and mercaptoethanol
SH
65
HS
72
HS
40
58
SH
SH
HS
26
84
95
HS
HS
110
Unfolded state; inactive. Disulfide cross-links reduced to yield Cys residues.
removal of urea and mercaptoethanol
72
58
65
110
84
26
95
40
Native, catalytically active state. Disulfide cross-links correctly re-formed.

FIGURE 4-25 Renaturation of unfolded, denatured ribonuclease. Urea denatures the ribonuclease, and mercaptoethanol ($HOCH_2CH_2SH$) reduces and thus cleaves the disulfide bonds to yield eight Cys residues. Renaturation involves reestablishing the correct disulfide cross-links.

intrachain disulfide bonds are re-formed in the same positions in the renatured molecule as in the native ribonuclease. Later, similar results were obtained using chemically synthesized, catalytically active ribonuclease A. This eliminated the possibility that some minor contaminant in Anfinsen's purified ribonuclease preparation might have contributed to renaturation of the enzyme, and thus it dispelled any remaining doubt that this enzyme folds spontaneously.

The Anfinsen experiment provided the first evidence that the amino acid sequence of a polypeptide chain contains all the information required to fold the chain into its native, three-dimensional structure. Subsequent work has shown that only a minority of proteins, many of them small and inherently stable, will fold spontaneously into their native form. Even though all proteins have the potential to fold into their native structure, many require some assistance.

Polypeptides Fold Rapidly by a Stepwise Process

In living cells, proteins are assembled from amino acids at a very high rate. For example, *E. coli* cells can make a complete, biologically active protein molecule containing 100 amino acid residues in about 5 seconds at 37 °C. However, the synthesis of peptide bonds on the ribosome is not enough; the protein must fold.

How does the polypeptide chain arrive at its native conformation? Let's assume conservatively that each of the amino acid residues could take up 10 different conformations on average, giving 10^{100} different conformations for the polypeptide. Let's also assume that the protein folds spontaneously by a random process in which it tries out all possible conformations around every single bond in its backbone until it finds its native, biologically active form. If each conformation were sampled in the shortest possible time (~10^{-13} second, or the time required for a single molecular vibration), it would take about 10^{77} years to sample all possible conformations! Clearly, protein folding is not a completely random, trial-and-error process. There must be shortcuts. This problem was first pointed out by Cyrus Levinthal in 1968 and is sometimes called Levinthal's paradox.

The folding pathway of a large polypeptide chain is unquestionably complicated. However, robust algorithms can often predict the structure of smaller proteins on the basis of their amino acid sequences. The major folding pathways are hierarchical. Local secondary structures form first. Certain amino acid sequences fold readily into α helices or β sheets, guided by constraints such as those reviewed in our discussion of secondary structure. Ionic interactions, involving charged groups that are often near one another in the linear sequence of the polypeptide chain, can play an important role in guiding these early folding steps. Assembly of local structures is followed by longer-range interactions between, say, two elements of secondary structure that come together to form stable folded structures. The hydrophobic effect plays a significant role throughout the process, as the aggregation of nonpolar amino acid side chains provides an entropic stabilization to intermediates and, eventually, to the final folded structure. The process continues until complete domains form and the entire polypeptide is folded (**Fig. 4-26**). Notably, proteins dominated by close-range interactions (between pairs of residues generally located near each other in the polypeptide sequence) tend to fold faster than proteins with more complex folding patterns and with many long-range interactions between different segments. As larger proteins with multiple domains are synthesized, domains near the amino terminus (which are synthesized first) may fold before the entire polypeptide has been assembled.

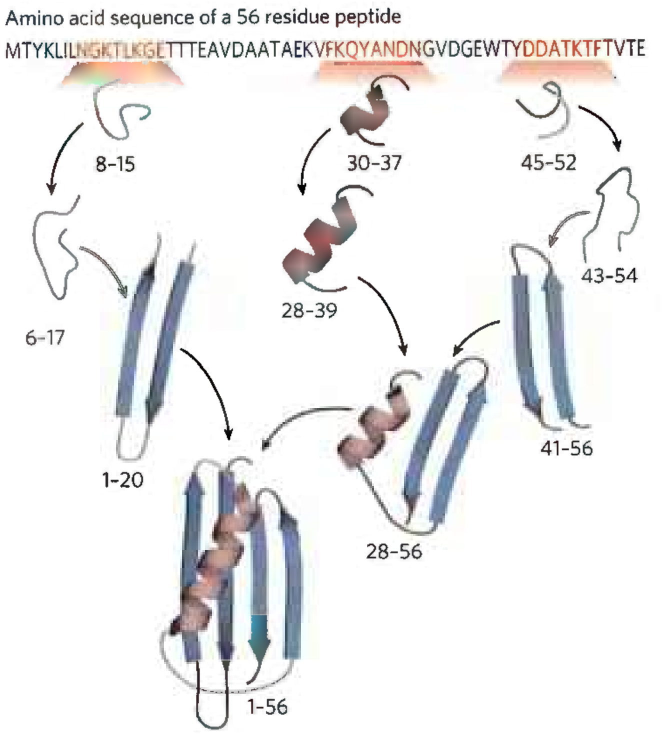

Amino acid sequence of a 56 residue peptide
MTYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTFTVTE
8-15
30-37
45-52
6-17
28-39
43-54
1-20
41-56
28-56
1-56

FIGURE 4-26 A protein-folding pathway as defined for a small protein. A hierarchical pathway is shown, based on computer modeling. Small regions of secondary structure are assembled first and then gradually incorporated into larger structures. The program used for this model has been highly successful in predicting the three-dimensional structure of small proteins from their amino acid sequence. The numbers indicate the amino acid residues in this 56 residue peptide that have acquired their final structure in each of the steps shown. [Information from K. A. Dill et al., *Annu. Rev. Biophys.* 37:289, 2008, Fig. 5.]

Thermodynamically, the folding process can be viewed as a kind of free-energy funnel (**Fig. 4-27**). The unfolded states are characterized by a high degree of conformational entropy and relatively high free energy. As folding proceeds, the narrowing of the funnel reflects the decrease in the conformational space that must be searched as the protein approaches its native state. Small depressions along the sides of the free-energy funnel represent semistable intermediates that can briefly slow the folding process. At the bottom of the funnel, an ensemble

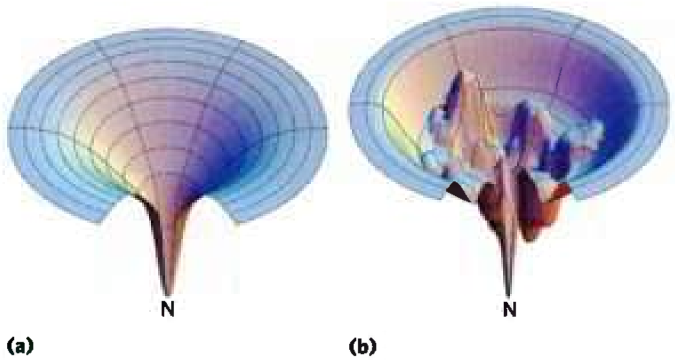


FIGURE 4-27 The thermodynamics of protein folding depicted as free-energy funnels. As proteins fold, the conformational space that can be explored by the structure is constrained. This is modeled as a three-dimensional thermodynamic funnel, with ΔG represented by the depth of the funnel and the native structure (N) at the bottom (lowest free-energy point). The funnel for a given protein can have a variety of shapes, depending on the number and types of folding intermediates in the folding pathways. Any folding intermediate with significant stability and a finite lifetime would be represented as a local free-energy minimum—a depression on the surface of the funnel. (a) A simple but relatively wide and smooth funnel represents a protein that has multiple folding pathways (that is, the order in which different parts of the protein fold is somewhat random), but it assumes its three-dimensional structure with no folding intermediates that have significant stability. (b) This funnel represents a more typical protein that has multiple possible folding intermediates with significant stability on the multiple pathways leading to the native structure. [Information from K. A. Dill et al., *Annu. Rev. Biophys.* 37:289, 2008, Fig. 9.]

of folding intermediates has been reduced to a single native conformation (or one of a small set of native conformations). The funnels can have a variety of shapes, depending on the complexity of the folding pathway, the existence of semistable intermediates, and the potential for particular intermediates to assemble into aggregates of misfolded proteins.

Thermodynamic stability is not evenly distributed over the structure of a protein—the molecule has regions of relatively high stability and others of low or negligible stability. For example, a protein may have two stable domains joined by a segment that is entirely disordered. Regions of low stability may allow a protein to alter its conformation between two or more states. As we shall see in the next two chapters, variations in the stability of regions within a protein are often essential to protein function. Intrinsically disordered proteins or protein segments do not fold at all.

Some Proteins Undergo Assisted Folding

Not all proteins fold spontaneously as they are synthesized in the cell. Folding for many proteins requires **chaperones**, proteins that interact with partially folded or improperly folded polypeptides, facilitating correct folding pathways or providing microenvironments in which folding can occur. Several types of molecular chaperones are found in organisms ranging from bacteria to humans. Two major families of chaperones, both well studied, are the Hsp70 family and the chaperonins.

Proteins in the **Hsp70** family generally have a molecular weight near 70,000 and are more abundant in cells stressed by elevated temperatures (hence, *h*eat *s*hock *p*roteins of M_r 70,000, or Hsp70). Hsp70 proteins bind to regions of unfolded polypeptides that are rich in hydrophobic residues. These chaperones thus "protect" both proteins subject to denaturation by heat and new peptide molecules being synthesized (and not yet folded). Hsp70 proteins also block the folding of certain proteins that must remain unfolded until they have been translocated across a membrane (as described in Chapter 27). Some chaperones also facilitate the quaternary assembly of oligomeric proteins. The Hsp70 proteins bind to and release polypeptides in a cycle that uses energy from ATP hydrolysis and involves several other proteins (including a class called Hsp40). **Figure 4-28** illustrates chaperone-assisted folding as elucidated for the eukaryotic Hsp70 and Hsp40

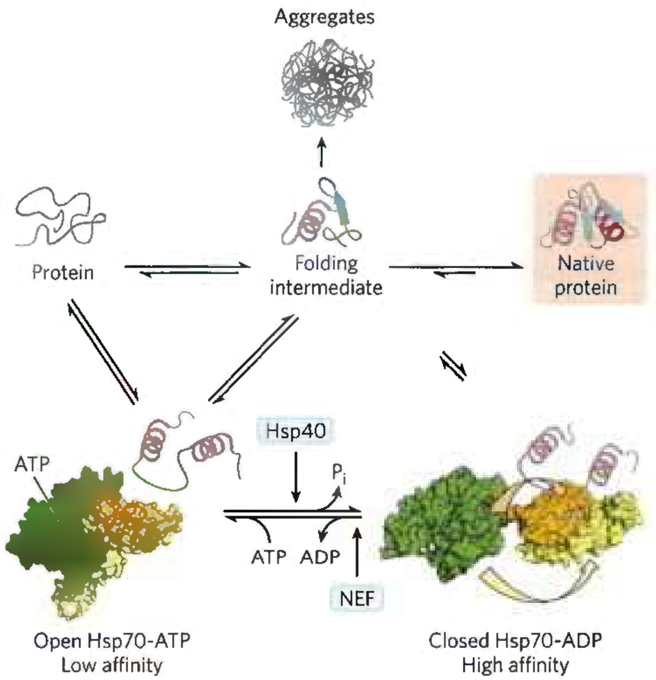


FIGURE 4-28 Chaperones in protein folding. The pathway by which chaperones of the Hsp70 class bind and release polypeptides is illustrated for the eukaryotic chaperones Hsp70 and Hsp40. The chaperones do not actively promote the folding of the substrate protein, but instead prevent aggregation of unfolded peptides. The unfolded or partly folded proteins bind first to the open, ATP-bound form of Hsp70. Hsp40 then interacts with this complex and triggers ATP hydrolysis that produces the closed form of the complex, in which the domains colored orange and yellow come together like the two parts of a jaw, trapping parts of the unfolded protein inside. Dissociation of ADP and recycling of the Hsp70 requires interaction with another type of protein called a nucleotide-exchange factor (NEF). For a population of polypeptide molecules, some fraction of the molecules released after the transient binding of partially folded proteins by Hsp70 will take up the native conformation. The remainder are quickly rebound by Hsp70 or diverted to the chaperonin system. [Information from F. U. Hartl et al., *Nature* 475:324, 2011, Fig. 2. Open Hsp70-ATP: PDB ID 2QXL, Q. Liu and W. A. Hendrickson, *Cell* 131:106, 2007. Closed Hsp70-ADP: Data from PDB ID 2KHO, E. B. Bertelson et al., *Proc. Natl. Acad. Sci. USA* 106:8471, 2009, and PDB ID 1DKZ, X. Zhu et al., *Science* 272:1606, 1996.]

chaperones. The binding of an unfolded polypeptide by an Hsp70 chaperone may break up a protein aggregate or prevent the formation of a new one. When the bound polypeptide is released, it has a chance to resume folding to its native structure. If folding does not occur rapidly enough, the polypeptide may be bound again and the process repeated. Alternatively, the Hsp70-bound polypeptide may be delivered to a chaperonin.

Chaperonins are elaborate protein complexes required for the folding of some cellular proteins that do not fold spontaneously. In *E. coli*, an estimated 10% to 15% of cellular proteins require the resident chaperonin system, called GroEL/GroES, for folding under normal conditions (up to 30% require this assistance when the cells are heat stressed). The analogous chaperonin system in eukaryotes is called Hsp60. The chaperonins first became known when they were found to be necessary for the growth of certain bacterial viruses (hence the designation "Gro"). These chaperone proteins are structured as a series of multisubunit rings, forming two chambers oriented back to back. Inside one of the chambers, a protein is given about 10 seconds to fold. Constraining a protein within the chamber prevents inappropriate protein aggregation and also restricts the conformational space that a polypeptide chain can explore as it folds. The GroEL/GroES folding pathway is discussed in Chapter 27.

Finally, the folding pathways of some proteins require two enzymes that catalyze isomerization reactions. **Protein disulfide isomerase (PDI)** is a widely distributed enzyme that catalyzes the interchange, or shuffling, of disulfide bonds until the bonds of the native conformation are formed. Among its functions, PDI catalyzes the elimination of folding intermediates with inappropriate disulfide cross-links. **Peptide prolyl cis-trans isomerase (PPI)** catalyzes the interconversion of the cis and trans isomers of peptide bonds formed by Pro residues (Fig. 4-7), which can be a slow step in the folding of proteins that contain some Pro peptide bonds in the cis configuration.

Defects in Protein Folding Are the Molecular Basis for Many Human Genetic Disorders

Protein misfolding is a substantial problem in all cells. Despite the many processes that assist in protein folding, a quarter or more of all polypeptides synthesized may be destroyed because they do not fold correctly. P4 In some cases, the misfolding causes or contributes to the development of serious disease.

Many conditions, including type 2 diabetes, Alzheimer disease, Huntington disease, and Parkinson disease, are associated with a misfolding mechanism: a soluble protein that is normally secreted from the cell is secreted in a misfolded state and converted into an insoluble extracellular **amyloid** fiber. The diseases are collectively referred to as **amyloidoses**. The fibers are highly ordered and unbranched, with a diameter of 7 to 10 nm and a high degree of β-sheet structure. The β segments are oriented perpendicular to the axis of the fiber. In some amyloid fibers the overall structure includes two layers of β sheet, such as that shown for amyloid-β peptide in **Figure 4-29**.

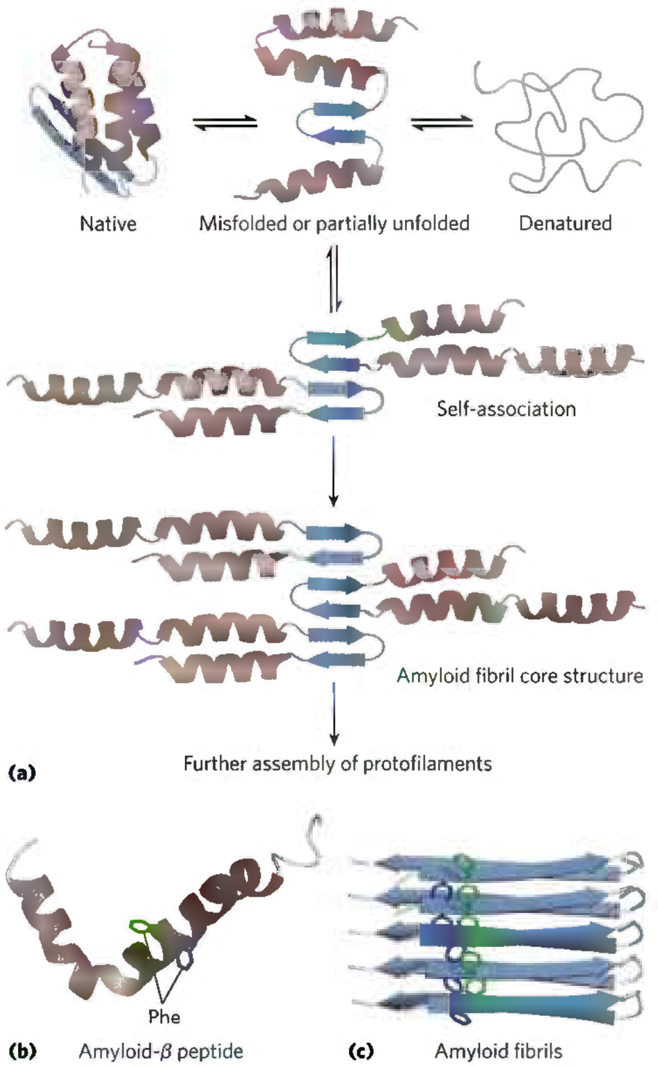

Native
Misfolded or partially unfolded
Denatured
Self-association
Amyloid fibril core structure
(a)
Further assembly of protofilaments
Phe
(b)
Amyloid-β peptide
(c)
Amyloid fibrils

FIGURE 4-29 Formation of disease-causing amyloid fibrils. (a) Protein molecules whose normal structure includes regions of β sheet undergo partial folding. In a small number of the molecules, before folding is complete, the β-sheet regions of one polypeptide associate with the same region in another polypeptide, forming the nucleus of an amyloid. Additional protein molecules slowly associate with the amyloid and extend it to form a fibril. (b) The amyloid-β peptide begins as two α-helical segments of a larger protein. Proteolytic cleavage of this larger protein leaves the relatively unstable amyloid-β peptide, which loses its α-helical structure. It can then assemble slowly into amyloid fibrils (c), which contribute to the characteristic plaques on the exterior of nervous tissue in people with Alzheimer disease. The aromatic side chains shown here play a significant role in stabilizing the amyloid structure. Amyloid is rich in β sheet structure, with the β strands arranged perpendicular to the axis of the amyloid fibril. Amyloid-β peptide takes the form of two layers of extended parallel β sheet. [(a) Information from D. J. Selkoe, *Nature* 426:900, 2003, Fig. 1. (b) Data from PDB ID 1IYT, O. Crescenzi et al., *Eur. J. Biochem.* 269:5642, 2002. (c) Data from PDB ID 2BEG, T. Lührs et al., *Proc. Natl. Acad. Sci. USA* 102:17,342, 2005.]

Box 4-4 **MEDICINE**

Death by Misfolding: The Prion Diseases

A misfolded brain protein seems to be the causative agent of several rare degenerative brain diseases in mammals. Perhaps the best known of these is bovine spongiform encephalopathy (BSE; also known as mad cow disease). Related diseases include kuru and Creutzfeldt-Jakob disease in humans, scrapie in sheep, and chronic wasting disease in deer and elk. These diseases are also referred to as spongiform encephalopathies, because the diseased brain frequently becomes riddled with holes (Fig. 1). Progressive deterioration of the brain leads to a spectrum of neurological symptoms, including weight loss; erratic behavior; problems with posture, balance, and coordination; and loss of cognitive function. The diseases are fatal.

In the 1960s, investigators found that preparations of the disease-causing agents seemed to lack nucleic acids. At this time, Tikvah Alper suggested that the agent was a protein. Initially, the idea seemed heretical. All disease-causing agents known up to that time—viruses, bacteria, fungi, and so on—contained nucleic acids, and their virulence was related to genetic reproduction and propagation. However, four decades of investigations, pursued most notably by Stanley Prusiner, have provided evidence that spongiform encephalopathies are different.

The infectious agent has been traced to a single protein (M_r 28,000), which Prusiner dubbed **prion** protein (PrP). The name was derived from *pro*teinaceous *in*fectious, but

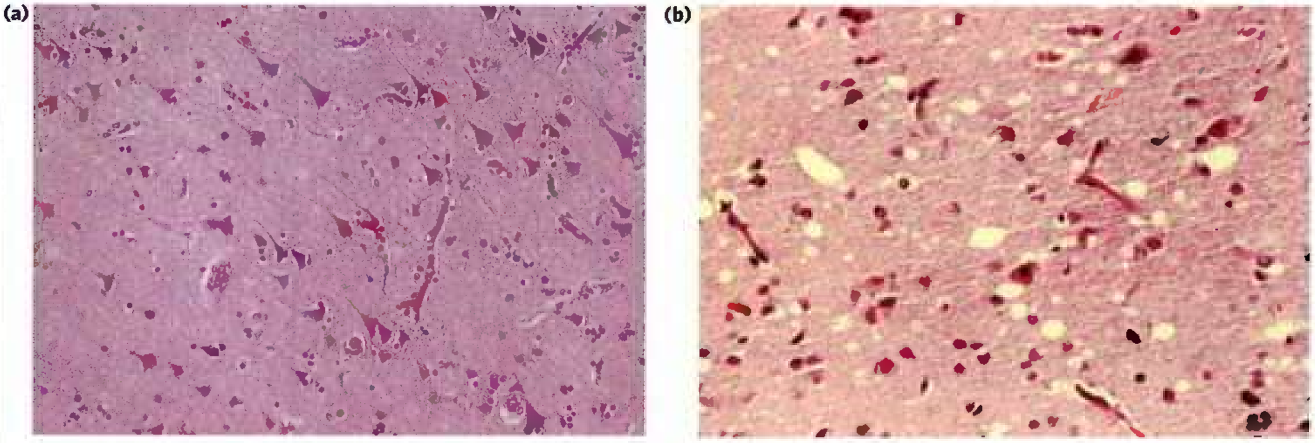


FIGURE 1 (a) Light micrograph of pyramidal cells in the human cerebral cortex. (b) A comparable section from the autopsy of a patient with Creutzfeldt-Jakob disease shows spongiform (vacuolar) degeneration, the most characteristic neurohistological feature. The yellowish vacuoles are intracellular and occur mostly in pre- and postsynaptic processes of neurons. The vacuoles in this section vary in diameter from 20 to 100 μm. [(a) Science Stock Photography/Science Source (b) Ralph C. Eagle, Jr./Science Source.]

Many proteins can take on the amyloid fibril structure as an alternative to their normal folded conformations, and most of these proteins have a concentration of aromatic amino acid residues in a core region of β sheet or α helix. The proteins are secreted in an incompletely folded conformation. The core (or some part of it) folds into a β sheet before the rest of the protein folds correctly, and the β sheets of two or more incompletely folded protein molecules associate to begin forming an amyloid fibril. The fibril grows in the extracellular space. Other parts of the protein then fold differently, remaining on the outside of the β-sheet core in the growing fibril. The effect of aromatic residues in stabilizing the structure is shown in Figure 4-29c. Because most of the protein molecules fold normally, the onset of symptoms in the amyloidoses is often very slow. If a person inherits a mutation such as substitution with an aromatic residue at a position that favors formation of amyloid fibrils, disease symptoms may begin at an earlier age.

The amyloid deposition diseases that trigger neurodegeneration, particularly in older adults, are a special class of localized amyloidoses. Alzheimer disease is associated with extracellular amyloid deposition by neurons, involving the amyloid-β peptide (Fig. 4-29b), derived from a larger transmembrane protein (amyloid-β precursor protein) found in most human tissues. When it is part of the larger protein, the peptide is composed of two α-helical segments spanning the membrane. When the external and internal domains are cleaved off by specific proteases, the relatively unstable amyloid-β peptide leaves the membrane and loses its α-helical structure. It can then take the form of two layers of extended parallel β sheet, which can slowly assemble into amyloid fibrils (Fig. 4-29c). Deposits of these amyloid fibers seem to be the primary cause of Alzheimer disease, but a second type of amyloidlike aggregation, involving a protein called tau, also occurs intracellularly (in neurons) in people with Alzheimer disease. Inherited mutations in

Prusiner thought that "prion" sounded better than "proin." Prion protein is a normal constituent of brain tissue in all mammals. Its role is not known in detail, but it may have a molecular signaling function. Strains of mice lacking the gene for PrP (and thus the protein itself) suffer no obvious ill effects. Illness occurs only when the normal cellular PrP, or PrP$^{\text{C}}$, occurs in an altered conformation called PrP$^{\text{Sc}}$ (Sc denotes scrapie). The structure of PrP$^{\text{C}}$ has two α helices. The structure of PrP$^{\text{Sc}}$ is very different, with much of the structure converted to amyloid-like β sheets (Fig. 2). The interaction of PrP$^{\text{Sc}}$ with PrP$^{\text{C}}$ converts the latter to PrP$^{\text{Sc}}$, initiating a domino effect in which more and more of the brain protein converts to the disease-causing form. The mechanism by which the presence of PrP$^{\text{Sc}}$ leads to spongiform encephalopathy is not understood.

In inherited forms of prion diseases, a mutation in the gene encoding PrP produces a change in one amino acid residue that is believed to make the conversion of PrP$^{\text{C}}$ to PrP$^{\text{Sc}}$ more likely. A complete understanding of prion diseases awaits new information on how prion protein affects brain function. Structural information about PrP is beginning to provide insights into the molecular process that allows the prion proteins to interact so as to alter their conformation (Fig. 2). The significance of prions may extend well beyond spongiform encephalopathies. Evidence is building that prionlike proteins may be responsible for additional neurodegenerative diseases such as multiple system atrophy (MSA), a disease that resembles Parkinson disease.

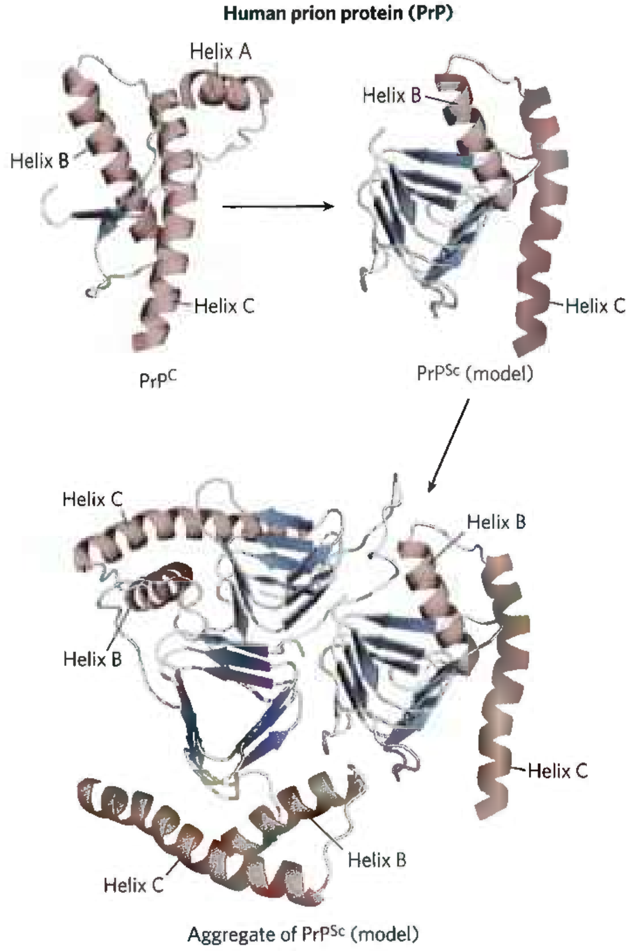


FIGURE 2 Structure of the globular domain of human PrP and models of the misfolded, disease-causing conformation PrP$^{\text{Sc}}$, and an aggregate of PrP$^{\text{Sc}}$. The α helices are labeled to help illustrate the modeled conformational change as the globular protein progresses to the aggregate. Helix A is incorporated into the β-sheet structure of the misfolded conformation. [Data for human PrP from PDB ID 1QLX, R. Zahn et al., *Proc. Natl. Acad. Sci. USA* 97:145, 2000. Information for models from C. Govaerts et al., *Proc. Natl. Acad. Sci. USA* 101:8342, 2004.]

the tau protein do not result in Alzheimer disease, but they cause a frontotemporal dementia and parkinsonism (a condition with symptoms resembling Parkinson disease) that can be equally devastating.

Several other neurodegenerative conditions involve intracellular aggregation of misfolded proteins. In Parkinson disease, the misfolded form of the protein α-synuclein aggregates into spherical filamentous masses called Lewy bodies. Huntington disease involves the protein huntingtin, which has a long polyglutamine repeat. In some individuals, the polyglutamine repeat is longer than normal, and a more subtle type of intracellular aggregation occurs. Notably, when the mutant human proteins involved in Parkinson disease and Huntington disease are expressed in *Drosophila melanogaster*, the flies display neurodegeneration expressed as eye deterioration, tremors, and early death. All of these symptoms are highly suppressed if expression of the Hsp70 chaperone is also increased.

Protein misfolding need not lead to amyloid formation to cause serious disease. For example, cystic fibrosis is caused by defects in a membrane-bound protein called *c*ystic *f*ibrosis *t*ransmembrane conductance *r*egulator (CFTR), which acts as a channel for chloride ions. The most common cystic fibrosis–causing mutation is the deletion of a Phe residue at position 508 in CFTR, which causes improper protein folding. Most of this protein is then degraded and its normal function is lost (see Box 11-2). Drugs that can correct certain CFTR misfolding events have been developed. Such drugs are called "correctors" or "pharmacological chaperones." Many of the disease-related mutations in collagen (p. 118) also cause defective folding. A particularly remarkable type of protein misfolding is seen in the prion diseases (**Box 4-4**).

SUMMARY 4.4 *Protein Denaturation and Folding*

- The maintenance of the steady-state collection of active cellular proteins required under a particular set of conditions—called proteostasis—involves an elaborate set of pathways and processes that fold, refold, and degrade polypeptide chains.
- The three-dimensional structure and the function of most proteins can be destroyed by denaturation, demonstrating a relationship between structure and function. Heat, extremes of pH, organic solvents, solutes, and detergents can all be used to denature proteins.
- Some denatured proteins can renature spontaneously to form biologically active protein, showing that tertiary structure is determined by amino acid sequence.
- Protein folding occurs too fast for it to be a completely random process. Instead, protein folding is generally hierarchical. Initially, regions of secondary structure may form, followed by folding into motifs and domains. Large ensembles of folding intermediates are rapidly brought to a single native conformation.
- For many proteins, folding is facilitated by Hsp70 chaperones and by chaperonins. Disulfide-bond formation and the cis-trans isomerization of Pro peptide bonds can also be catalyzed by specific enzymes during folding.
- Protein misfolding is the molecular basis for many human diseases, including cystic fibrosis and amyloidoses such as Alzheimer disease.

4.5 Determination of Protein and Biomolecular Structures

In this chapter we have presented many types of protein structures. How were these structures determined? **P5** Structural biology is the study of the three-dimensional structures of biomolecules, including proteins, nucleic acids, lipid membranes, and oligosaccharides. Structural biologists combine biochemical approaches with physical tools and computational methods to obtain these structures. Structural biology is extraordinarily powerful for elucidating the relationships between the structure and function of proteins, the molecular basis for enzymatic catalysis and ligand binding, and evolutionary relationships between proteins. Here we focus primarily on three commonly used methods in structural biology: x-ray crystallography, nuclear magnetic resonance (NMR), and cryo-electron microscopy (cryo-EM). Which method a structural biologist uses depends on the system being studied and what information is to be learned. Often structural biologists combine multiple methods to provide a more complete view of function.

Increasingly, computational methods such as molecular dynamics simulations and in silico protein folding are proving to be essential for structural biologists, and these are discussed in **Box 4-5**.

X-ray Diffraction Produces Electron Density Maps from Protein Crystals

The spacing of atoms in a crystal lattice can be determined by measuring the locations and intensities of spots produced on a detector by a beam of x-rays of given wavelength, after the beam has been diffracted by the electrons of the atoms. For example, x-ray analysis of sodium chloride crystals shows that Na^+ and Cl^- ions are arranged in a simple cubic lattice. The spacing of the different kinds of atoms in complex organic molecules, even very large ones such as proteins, can also be analyzed by x-ray diffraction methods. However, the technique for analyzing crystals of complex molecules is far more laborious than the technique for analyzing simple salt crystals. When the repeating pattern of the crystal is a molecule as large as, say, a protein, the numerous atoms in the molecule yield thousands of diffraction spots that must be analyzed by computer.

Consider how images are generated in a light microscope. Light from a point source is focused on an object. The object scatters the light waves, and these scattered waves are recombined by a series of lenses to generate an enlarged image of the object. The smallest object whose structure can be determined by such a system—that is, the resolving power of the microscope—is determined by the wavelength of the light, in this case visible light, with wavelengths in the range of 400 to 700 nm. Objects smaller than half the wavelength of the incident light cannot be resolved. To resolve objects as small as proteins we must use x-rays, with wavelengths in the range of 0.7 to 1.5 Å (0.07 to 0.15 nm). However, there are no lenses that can recombine x-rays to form an image; instead, the pattern of diffracted x-rays is collected directly and an image is reconstructed by mathematical techniques.

The amount of information obtained from **x-ray crystallography** depends on the degree of structural order in the sample. Some important structural parameters were obtained from early studies of the diffraction patterns of the fibrous proteins arranged in regular arrays in hair and wool. However, the orderly bundles formed by fibrous proteins are not crystals—the molecules are aligned side by side, but not all are oriented in the same direction. More-detailed three-dimensional structural information about proteins requires a highly ordered protein crystal. The structures of many proteins are not yet known, simply because they have proved difficult to crystallize. Practitioners have compared making protein crystals to holding together a stack of bowling balls with cellophane tape.

Operationally, there are several steps in x-ray structural analysis (**Fig. 4-30**). A crystal is placed in an x-ray beam between the x-ray source and a detector, and a regular array of spots, called reflections, is generated. The spots are created by the diffracted x-ray beam, and each atom in a molecule makes a contribution to each spot. An electron-density map of the protein is reconstructed

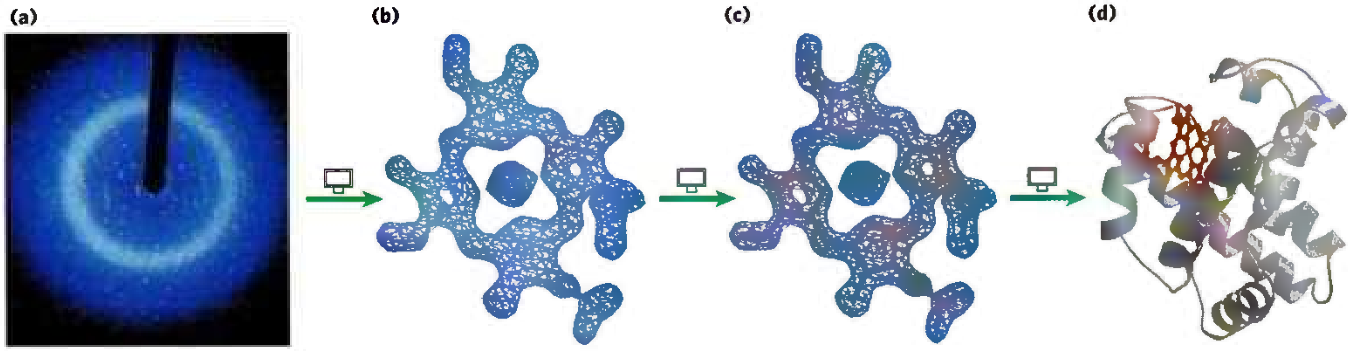


FIGURE 4-30 Steps in determining the structure of sperm whale myoglobin by x-ray crystallography. (a) X-ray diffraction patterns are generated from a crystal of the protein. (b) Data extracted from the diffraction patterns are used to calculate a three-dimensional electron-density map. The electron density of only part of the structure, the heme, is shown here. (c) Regions of greatest electron density reveal the location of atomic nuclei, and this information is used to piece together the final structure. Here, the heme structure is modeled into its electron-density map. (d) The completed structure of sperm whale myoglobin, including the heme. [(a, b, c) Photo and data from George N. Phillips, Jr., University of Wisconsin-Madison, Department of Biochemistry. (d) Data from PDB ID 2MBW, E. A. Brucker et al., *J. Biol. Chem.* 271:25,419, 1996.]

from the overall diffraction pattern of spots by a mathematical technique called a Fourier transform. In effect, the computer acts as a "computational lens." A model for the structure is then built that is consistent with the electron-density map.

John Kendrew found that the x-ray diffraction pattern of crystalline myoglobin (isolated from muscles of the sperm whale) is highly complex, with nearly 25,000 reflections. Computer analysis of these reflections took place in stages. The resolution improved at each stage until, in 1959, the positions of virtually all the nonhydrogen atoms in the protein had been determined. The amino acid sequence of the protein, obtained by chemical analysis, was consistent with the molecular model. Over 100,000 protein structures, many of them much more complex than myoglobin, have since been determined to a similar level of resolution by x-ray crystallography.

The physical environment in a crystal, of course, is not identical to that in solution or in a living cell. A crystal imposes a space and time average on the structure deduced from its analysis, and x-ray diffraction studies provide little information about molecular motion within the protein. The conformation of proteins in a crystal can also be affected by nonphysiological factors such as incidental protein-protein contacts within the crystal. However, when structures derived from the analysis of crystals are compared with structural information obtained by other means (such as NMR, as described below), the crystal-derived structure almost always represents a functional conformation of the protein.

Distances between Protein Atoms Can Be Measured by Nuclear Magnetic Resonance

An advantage of **nuclear magnetic resonance (NMR)** studies is that they are carried out on macromolecules in solution, whereas x-ray crystallography is limited to molecules that can be crystallized. NMR can also illuminate the dynamic side of protein structure, including conformational changes, protein folding, and interactions with other molecules.

NMR is a manifestation of nuclear spin angular momentum, a quantum mechanical property of atomic nuclei. Only certain atoms, including ^{1}H, ^{13}C, ^{15}N, ^{19}F, and ^{31}P, have the kind of nuclear spin that gives rise to an NMR signal. Nuclear spin generates a magnetic dipole. When a strong, static magnetic field is applied to a solution containing a single type of macromolecule, the magnetic dipoles are aligned in the field in one of two orientations: parallel (low energy) or antiparallel (high energy). A short (~10 μs) pulse of electromagnetic energy of suitable frequency (the resonant frequency, which is in the radio frequency range) is applied at right angles to the nuclei aligned in the magnetic field. Some energy is absorbed as nuclei switch to the high-energy state, and the absorption spectrum that results contains information about the identity of the nuclei and their immediate chemical environment. The data from many such experiments on a sample are averaged, increasing the signal-to-noise ratio, and an NMR spectrum such as that in **Figure 4-31** is generated.

^{1}H is particularly important in NMR experiments because of its high sensitivity and natural abundance. For macromolecules, ^{1}H NMR spectra can become quite complicated. Even a small protein has hundreds of ^{1}H atoms, typically resulting in a one-dimensional NMR spectrum too complex for analysis. Structural analysis of proteins became possible with the advent of two-dimensional NMR techniques (Fig. 4-31b, c, d). These methods allow measurement of distance-dependent coupling of nuclear spins in nearby atoms through space (the nuclear Overhauser effect (NOE), in a method dubbed NOESY) or the coupling of nuclear spins in atoms connected by covalent bonds (total correlation spectroscopy, or TOCSY).

BOX 4-5

Video Games and Designer Proteins

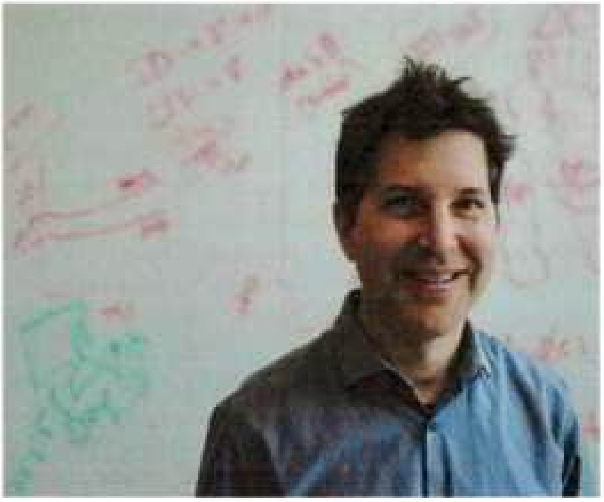
Computational tools are now indispensable to biochemistry. While advances in computers and software have revolutionized how protein structures can be solved by x-ray crystallography, NMR, and cryo-EM, advances in computational chemistry have now allowed a number of studies of protein structure to be carried out entirely in silico using powerful molecular modeling and dynamics software. Key developments in this field were made by Martin Karplus, Michael Levitt, and Arieh Warshel, who received the Nobel Prize in Chemistry in 2013 for their work on the theoretical and computational tools needed to carry out computer simulations of molecules as large as proteins.

Protein folding is a problem particularly well-suited for computational biochemists. Protein-folding landscapes can be complex, and nearly an infinite number of possible peptide conformations are theoretically available for the protein to sample as it folds. However, to understand how folding happens efficiently and by only sampling a small portion of all possible conformations requires computers that can quickly test tens of thousands of possible folding trajectories in a brief time. Sometimes these calculations are carried out on powerful supercomputers, but in other instances research groups have taken to crowdsourcing these problems to thousands of citizen scientists.

David Baker has pioneered crowdsourcing protein-folding problems to engage millions of computer users directly in biochemistry. The Rosetta@home project distributes complex protein-folding problems to citizen scientists who run the software in the background on their home computers. Each home computer is able to complete a portion of a protein-folding experiment, and these results can be combined with those from other users to predict protein structures.

Foldit is a video game in which users compete to solve protein-folding puzzles (Fig. 1). Users are rewarded for making stabilizing contacts in the structure, like hydrogen bonds or favorable van der Waals interactions. Virtual protein designs (sometimes called theozymes) from the video game can then be tested in the real world. DNA genes coding for the virtual proteins can be created in the laboratory and be used to recombinantly produce the proteins in bacteria, using techniques described in Chapter 9. The purified proteins can then be structurally characterized by x-ray crystallography or NMR to see how closely the real-world structures and computationally designed structures match.

One exciting application of computational biochemistry is the creation of "designer proteins." These are proteins with completely novel folds or functions not yet identified in nature. Designer proteins have potential applications in bioengineering, medicine, materials science, and chemistry. In one recent example, the Baker Lab designed a series of Foldit puzzles (such as "Cover the Ligand," in which users had to redesign a ligand-binding site by rebuilding the protein backbone and changing amino acid side chains) to improve the catalytic efficiency of a previously engineered Diels-Alderase (Fig. 2). The Diels-Alder reaction is a common method for organic chemists to create carbon–carbon bonds; however, it is almost never used by naturally occurring enzymes. Diels-Alderase enzymes would



David Baker

[Photo by Ian C. Haydon, courtesy Institute for Protein Design, University of Washington]

Translating a two-dimensional NMR spectrum into a complete three-dimensional structure can be a laborious process. The NOE signals provide some information about the distances between individual atoms, but for these distance constraints to be useful, the atoms giving rise to each signal must be identified. Complementary TOCSY experiments can help identify which NOE signals reflect atoms that are linked by covalent bonds. Certain patterns of NOE signals have been associated with secondary structures such as α helices. Genetic engineering (Chapter 9) can be used to prepare proteins that contain the rare isotopes ^{13}C or ^{15}N. The new NMR signals produced by these atoms, and the coupling with ^{1}H signals resulting from these substitutions, help in the assignment of individual ^{1}H NOE signals. The process is also aided by a knowledge of the amino acid sequence of the polypeptide.

To generate a three-dimensional structure, researchers feed the distance constraints into a computer along with known geometric constraints such as chirality,

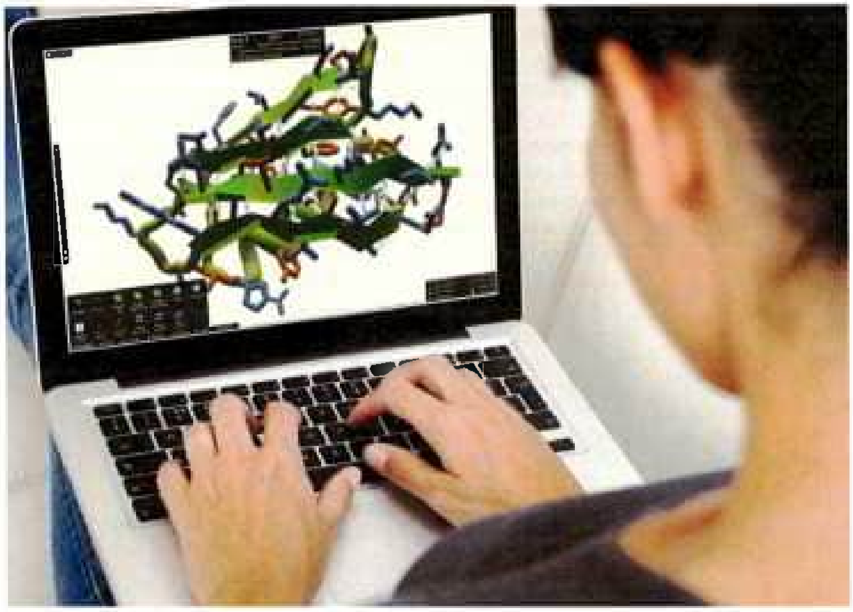


FIGURE 1 Foldit uses a video game interface to crowdsource protein-folding problems. Proteins designed in the video game can be recreated in the laboratory and studied using biochemical and structural methods.

potentially be useful for carrying out environmentally friendly carbon bond forming reactions in water instead of toxic organic solvents. Foldit competitors used the video game to design thousands of virtual enzymes. The players were successful, and once their top virtual enzyme was produced and tested in the real world it was found to be more than 18-fold more active than the original Diels-Alderase. Incredibly, the protein structure created by the Foldit game players was also confirmed by x-ray crystallography. This is definitely a case where we can encourage biochemistry students to spend more time playing video games!

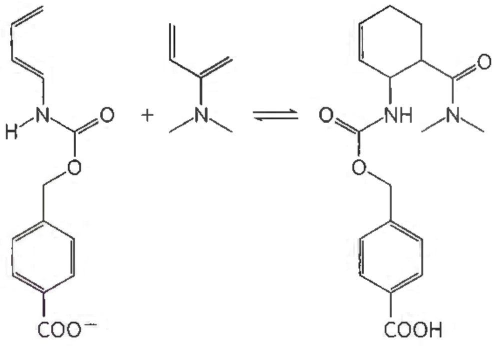


FIGURE 2 The Diels-Alder reaction catalyzed by an enzyme designed by Foldit players. Diels-Alder reactions are commonly used by organic chemists to form C—C bonds; however, there are very few examples of enzymes catalyzing this type of chemistry in nature. [Information from C. B. Eiben et al., *Nature Biotechnol.* 30:190, 2109.]

van der Waals radii, and bond lengths and angles. The computer generates a family of closely related structures that represent the range of conformations consistent with the NOE distance constraints (Fig. 4-31d). The uncertainty in structures generated by NMR is in part a reflection of the molecular vibrations (known as breathing) within a protein structure in solution, discussed in more detail in Chapter 5. Normal experimental uncertainty can also play a role.

Thousands of Individual Molecules Are Used to Determine Structures by Cryo-Electron Microscopy

Our understanding of highly complex processes such as gene expression, mitochondrial respiration, or viral infection is aided immensely by knowing the detailed molecular structures of the proteins that participate in these processes. However, it is often difficult to determine the molecular structure of large, dynamic, macromolecular complexes that contain dozens of individual

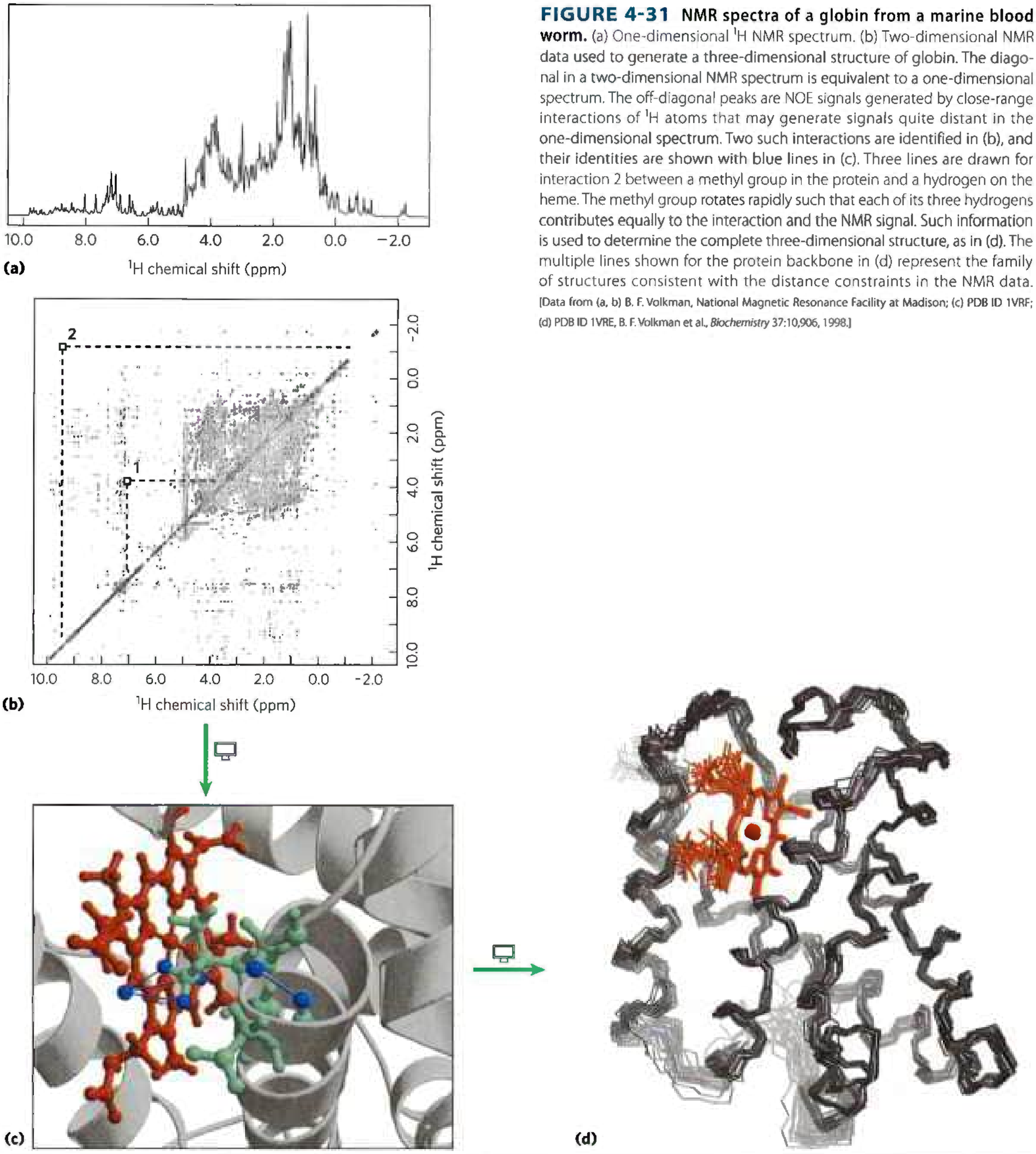


FIGURE 4-31 NMR spectra of a globin from a marine blood worm. (a) One-dimensional ^1H NMR spectrum. (b) Two-dimensional NMR data used to generate a three-dimensional structure of globin. The diagonal in a two-dimensional NMR spectrum is equivalent to a one-dimensional spectrum. The off-diagonal peaks are NOE signals generated by close-range interactions of ^1H atoms that may generate signals quite distant in the one-dimensional spectrum. Two such interactions are identified in (b), and their identities are shown with blue lines in (c). Three lines are drawn for interaction 2 between a methyl group in the protein and a hydrogen on the heme. The methyl group rotates rapidly such that each of its three hydrogens contributes equally to the interaction and the NMR signal. Such information is used to determine the complete three-dimensional structure, as in (d). The multiple lines shown for the protein backbone in (d) represent the family of structures consistent with the distance constraints in the NMR data. [Data from (a, b) B. F. Volkman, National Magnetic Resonance Facility at Madison; (c) PDB ID 1VRF; (d) PDB ID 1VRE, B. F. Volkman et al., *Biochemistry* 37:10,906, 1998.]

protein subunits. Moreover, integral membrane proteins often resist crystallization once they are removed from their lipid environment, making their structures difficult to solve by x-ray diffraction, and many are too large for NMR. In principle, discrete objects in the diameter range 100 to 300 Å can be visualized by electron microscopy (EM). In practice, the high intensity of the EM beam often damages the specimen before a high-resolution image can be obtained. In **cryo-electron microscopy (cryo-EM)**, a sample containing many individual copies of the structure of interest is quick-frozen in vitreous (or noncrystalline) ice and kept frozen while being observed in two dimensions with the electron microscope, greatly reducing damage to the specimen by the electron beam.

Particles such as purified, multisubunit enzymes, arranged randomly on the microscope grid, are visualized

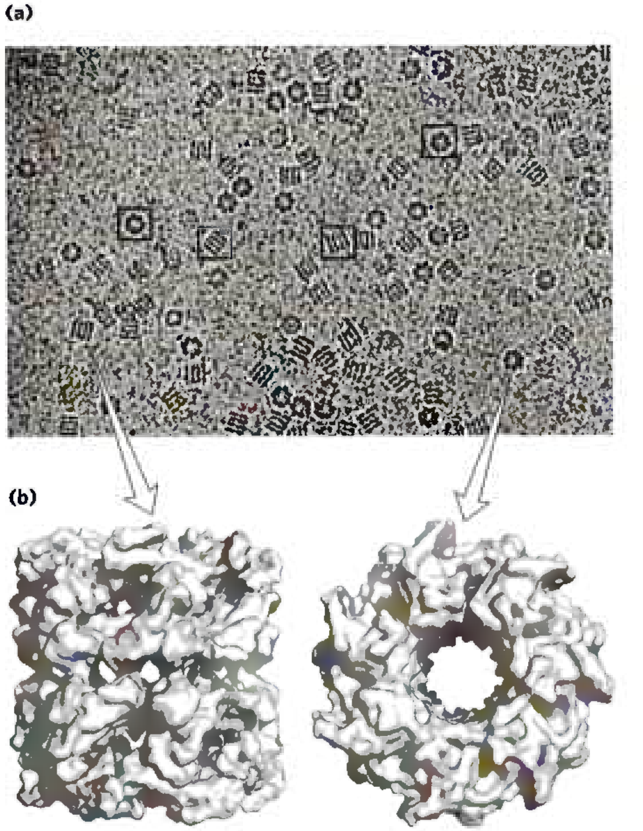


FIGURE 4-32 Structure of the chaperone protein GroEL as determined by single-particle cryo-EM. (a) Cryo-EM images of many individual GroEL particles. (b) Side and top views of the three-dimensional structure derived from analysis of the EM images. [(a) Alberto Bartesaghi and Sriram Subramaniam, National Cancer Institute, National Institutes of Health (b) Data from PDB ID 3E76, P. D. Kaiser et al., *Acta Crystallogr.* 65:967, 2009.]

with the cryo-electron microscope. When cryo-EM is combined with powerful algorithms for transforming the two-dimensional structures of tens of thousands of individual, randomly oriented complexes into a three-dimensional composite, it is sometimes possible to determine molecular structures at a level comparable to that obtained by x-ray crystallography (**Fig. 4-32**). In favorable cases, the repetitive aspects—choice of objects to be included in the analysis, imaging of each object individually, and calculations to produce a three-dimensional structure from the huge number of two-dimensional images—can be automated. The EMDataResource (www.emdataresource.org) is a unified resource for accessing structure maps deposited into data banks and assigned EMDataBank (EMDB) accession codes.

Many novel structures have now been obtained by cryo-EM without models based on prior x-ray or NMR structures. Since cryo-EM relies on imaging of single molecules of a complex, this technique can also be used to computationally sort the imaged particles and simultaneously determine structures of multiple conformational states. Cryo-EM has now been used to solve the structures of some of the most dynamic and largest molecular complexes in the cell, such as the human telomerase enzyme (**Fig. 4-33**). Telomerase is an essential enzyme for

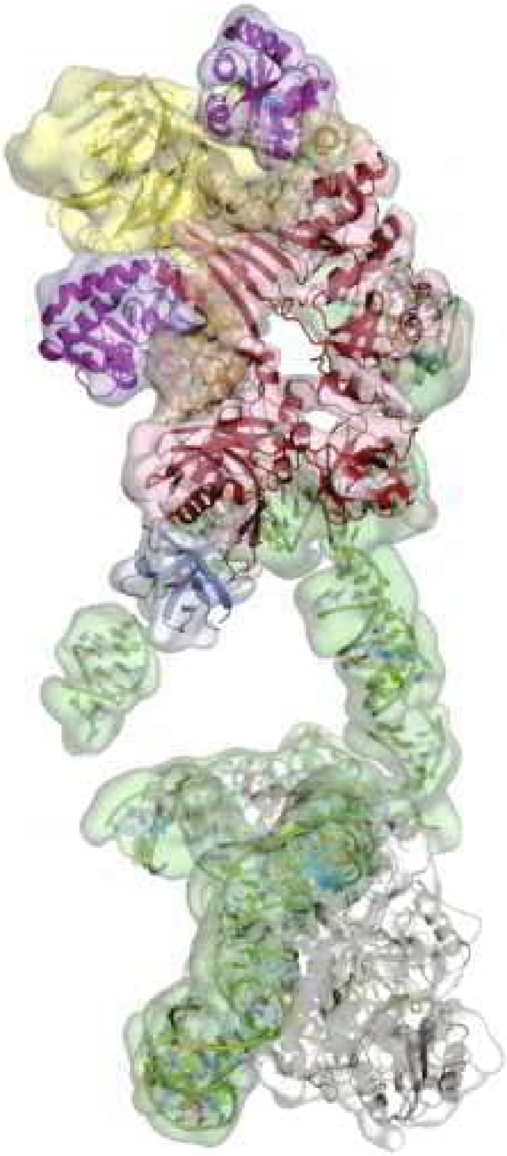


FIGURE 4-33 Cryo-EM structure of human telomerase. The structures of the RNA (green) and protein (ribbon representations) components of human telomerase are shown embedded in the calculated 10.2 Å EM density map. [Data from EMDB ID EMD-7521, T. Nguyen et al., *Nature* 557:190, 2018.]

Eva Nogales
[Courtesy Eva Nogales.]

Kathleen Collins
[Courtesy Kathleen Collins.]

maintaining chromosome integrity in humans (see Chapter 26) and is the target of significant medical research due to its roles in aging and cancer. Cryo-EM was critical for the laboratories of Eva Nogales and Kathleen Collins in determining the architecture of telomerase due to the heterogeneity of the complex and because only minute quantities could be purified from human cells—far too little for crystallization, but enough to observe single molecules by cryo-EM.

SUMMARY 4.5 *Determination of Protein and Biomolecular Structures*

- In x-ray crystallography, protein molecules are crystallized in well-ordered orientations that diffract x-rays. The patterns and intensities of the diffracted x-rays depend on the structure of the protein and its crystalline properties. Mathematical methods can then reconstruct the protein structure that produces a particular diffraction pattern.
- NMR is often carried out on molecules in solution and yields information about atomic nuclei and their chemical environment. Protein structures can be computed from NMR data using hundreds of distance and geometric constraints obtained from multi-dimensional NMR experiments.
- Biomolecules are frozen in vitreous ice for imaging by cryo-EM. The individual molecules are then identified and computationally sorted. The sorted two-dimensional images are then combined using computers to produce a three-dimensional structure.

KEY TERMS

Terms in bold are defined in the glossary.

conformation 106
native conformation 107
hydrophobic effect 108
solvation layer 108
secondary structure 111
α helix 111
β conformation 114
β sheet 114
β turn 114
Ramachandran plot 115
circular dichroism (CD) spectroscopy 116
tertiary structure 116
quaternary structure 116
fibrous proteins 116
globular proteins 116
intrinsically disordered proteins 117
α-keratin 117
collagen 118
fibroin 120
Protein Data Bank (PDB) 122
motif 123
fold 123
domain 123
topology diagram 125
protein family 125
multimer 127
oligomer 127
protomer 127
proteostasis 129
denaturation 129
renaturation 130
chaperone 132
Hsp70 132
chaperonin 133
protein disulfide isomerase (PDI) 133
peptide prolyl cis-trans isomerase (PPI) 133
amyloid 133
amyloidoses 133
prion 134
x-ray crystallography 136
nuclear magnetic resonance (NMR) spectroscopy 137
cryo-electron microscopy (cryo-EM) 140

PROBLEMS

1. **Properties of the Peptide Bond** In x-ray studies of crystalline peptides, Linus Pauling and Robert Corey found that the C—N bond in the peptide link is intermediate in length (1.32 Å) between a typical C—N single bond (1.49 Å) and a C═N double bond (1.27 Å). They also found that the peptide bond is planar (all four atoms attached to the C—N group are located in the same plane) and that the two α-carbon atoms attached to the C—N are always trans to each other (on opposite sides of the peptide bond).

(a) What does the length of the C—N bond in the peptide linkage indicate about its strength and its bond order (i.e., whether it is single, double, or triple)?

(b) What do Pauling and Corey's observations tell us about the ease of rotation about the C—N peptide bond?

2. **Structural and Functional Relationships in Fibrous Proteins** William Astbury discovered that the x-ray diffraction pattern of wool shows a repeating structural unit spaced about 5.2 Å along the length of the wool fiber. When he steamed and stretched the wool, the x-ray pattern showed a new repeating structural unit at a spacing of 7.0 Å. Steaming and stretching the wool and then letting it shrink gave an x-ray pattern consistent with the original spacing of about 5.2 Å. Although these observations provided important clues to the molecular structure of wool, Astbury was unable to interpret them at the time.

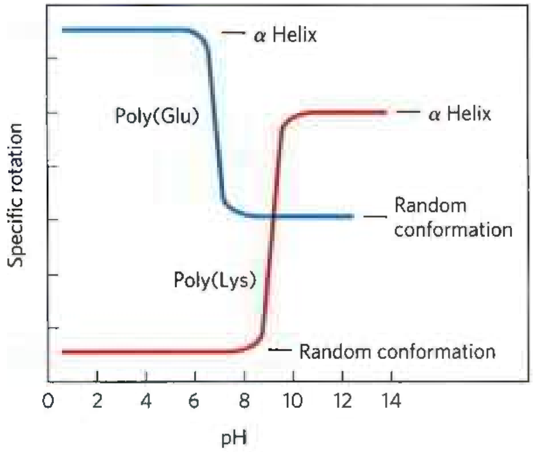
(a) Given our current understanding of the structure of wool, interpret Astbury's observations.

(b) When wool sweaters or socks are washed in hot water or heated in a dryer, they shrink. Silk, on the other hand, does not shrink under the same conditions. Explain.

3. **Rate of Synthesis of Hair α-Keratin** Hair grows at a rate of 15 to 20 cm/yr. All this growth is concentrated at the base of the hair fiber, where α-keratin filaments are synthesized inside living epidermal cells and assembled into ropelike structures (see Fig. 4-10). The fundamental structural element of α-keratin is the α helix, which has 3.6 amino acid residues per turn and a rise of 5.4 Å per turn (see Fig. 4-3a). Assuming that the biosynthesis of α-helical keratin chains is the rate-limiting factor in the growth of hair, calculate the rate at which peptide bonds of α-keratin chains must be synthesized (peptide bonds per second) to account for the observed yearly growth of hair.

4. **Effect of pH on the Conformation of α-Helical Secondary Structures** Specific rotation is a measure of a solution's capacity to rotate circularly polarized light. The unfolding of the α helix of a polypeptide to a randomly coiled conformation is accompanied by a large decrease in a property called specific rotation. Polyglutamate, a polypeptide made up of only L-Glu residues, is an α helix at pH 3. When researchers raise the pH to 7, there is a large decrease in the specific rotation of the solution. Similarly, polylysine (L-Lys residues) is an α helix at pH 10, but when researchers

lower the pH to 7, the specific rotation also decreases, as shown in the graph.



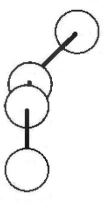
Explain the effect of the pH changes on the conformations of poly(Glu) and poly(Lys). Why does the transition occur over such a narrow range of pH?

5. Disulfide Bonds Determine the Properties of Many Proteins Some natural proteins are rich in disulfide bonds, and their mechanical properties, such as tensile strength, viscosity, and hardness, correlate with the degree of disulfide bonding.

(a) Glutenin, a wheat protein rich in disulfide bonds, imparts the cohesive and elastic character of dough made from wheat flour. Similarly, the hard, tough nature of tortoise shell results from the extensive disulfide bonding in its α-keratin. What is the molecular basis for the correlation between disulfide-bond content and mechanical properties of the protein?

(b) Most globular proteins denature and lose their activity when they are briefly heated to 65 °C. However, the denaturation of globular proteins that contain multiple disulfide bonds often requires longer heat exposure at higher temperatures. One such protein is bovine pancreatic trypsin inhibitor (BPTI), which has 58 amino acid residues in a single peptide chain and contains three disulfide bonds. After a solution of denatured BPTI is cooled, the protein regains its activity. What is the molecular basis for this property of BPTI?

6. Dihedral Angles Consider the series of torsion angles, ϕ and ψ, that might be taken up by the peptide backbone. Which of these closely correspond to ϕ and ψ for an idealized collagen triple helix? Refer to Figure 4-8 as a guide.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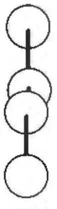
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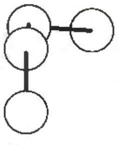
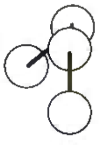

(c)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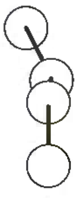
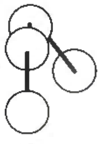

(e)


(f)

7. Amino Acid Sequence and Protein Structure Our growing understanding of how proteins fold allows researchers to make predictions about protein structure based on primary amino acid sequence data. Consider this amino acid sequence.

1 2 3 4 5 6 7 8 9 10
Ile–Ala–His–Thr–Tyr–Gly–Pro–Phe–Glu–Ala–

11 12 13 14 15 16 17 18 19 20
Ala–Met–Cys–Lys–Trp–Glu–Ala–Gln–Pro–Asp–

21 22 23 24 25 26 27 28
Gly–Met–Glu–Cys–Ala–Phe–His–Arg

(a) Where might bends or β turns occur?

(b) Where might intrachain disulfide cross-linkages form?

(c) Suppose that this sequence is part of a larger globular protein. Indicate the probable location (external surface or interior of the protein) of each amino acid residue: Asp, Ile, Thr, Ala, Gln, Lys. Explain your reasoning. (Hint: See the hydropathy index in Table 3-1.)

8. Amino Acid Contributions to Protein Folding Like ribonuclease A, lysozyme from T4 phage is a model enzyme for understanding the energetics and pathways of protein folding. Unlike ribonuclease A, however, T4 lysozyme does not contain any disulfide bonds. A number of studies have quantified the thermodynamic contributions that individual amino acid residues and their interactions make to T4 lysozyme folding.

(a) An ion pair between an Asp and a His residue in T4 lysozyme contributes 13–21 kJ/mol of favorable folding energy at pH 6.0. However, this ion pair contributes much less to lysozyme folding at either pH 2.0 or pH 10.0. How can you explain this observation?

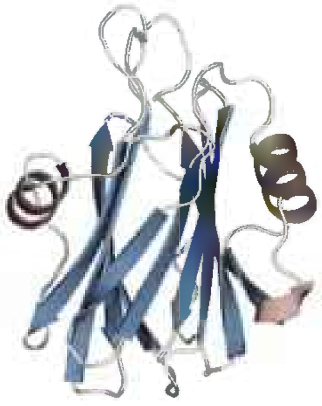
(b) Suppose that a Met residue buried in the folded, hydrophobic core of T4 lysozyme is replaced by mutation with a Lys residue. How would the mutation affect a plot of the thermal denaturation of T4 lysozyme at pH 3.0? (See Fig. 4-24a for an example of a thermal denaturation plot.)

(c) Suppose that the thermal denaturation experiment on the protein with the Met to Lys mutation took place at pH 10.0. Predict whether the mutation would have a greater or a lesser impact on protein stability at pH 10.0 than at pH 3.0. Explain your prediction.

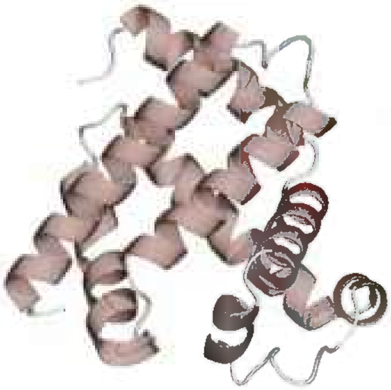
9. Bacteriorhodopsin in Purple Membrane Proteins Under the proper environmental conditions, the salt-loving archaeon *Halobacterium halobium* synthesizes a membrane protein (M_r 26,000) known as bacteriorhodopsin, which is purple because it contains retinal (see Fig. 10-20). Molecules of this protein aggregate into "purple patches" in the cell membrane. Bacteriorhodopsin acts as a light-activated proton pump that provides energy for cell functions. X-ray analysis of this protein reveals that it consists of seven parallel α-helical segments, each of which traverses the bacterial cell membrane (thickness 45 Å). Calculate the minimum number of amino acid residues necessary for one segment of α helix to traverse the membrane completely. Estimate the fraction of the bacteriorhodopsin protein that is involved in membrane-spanning helices. (Use an average amino acid residue weight of 110.)

10. Conservation of Protein Structure Margaret Oakley Dayhoff originated the idea of protein superfamilies after noticing that proteins with diverse amino acid sequences can have similar tertiary structures. Why can protein structure be more highly conserved than individual amino acid sequences?

11. Interpreting Ramachandran Plots Examine the two proteins labeled (a) and (b) below. Which of the two Ramachandran plots, labeled (c) and (d) at right, is more likely to be derived from which protein? Why? [Data from (a) PDB ID 1GWY, J. M. Mancheno et al., *Structure* 11:1319, 2003; (b) PDB ID 1A6M, J. Vojtechovsky et al., *Biophys. J.* 77:2153, 1999.]



(a)



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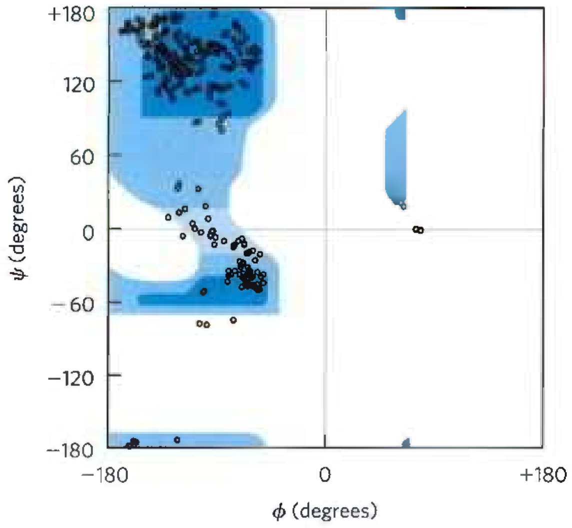

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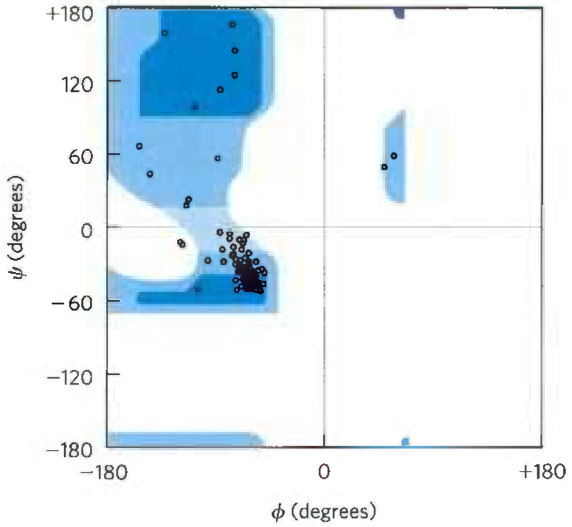
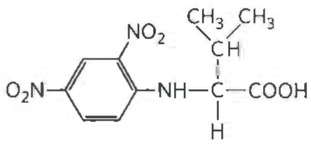


(d)

12. Number of Polypeptide Chains in a Multisubunit Protein A researcher treated a sample (660 mg) of an oligomeric protein of M_r 132,000 with an excess of 1-fluoro-2,4-dinitrobenzene (Sanger's reagent) under slightly alkaline conditions until the chemical reaction was complete. He then completely hydrolyzed the peptide bonds of the protein by heating it with concentrated HCl. The hydrolysate was found to contain 5.5 mg of the compound shown.



2,4-Dinitrophenyl derivatives of the α-amino groups of other amino acids could not be found.

(a) Explain how this information can be used to determine the number of polypeptide chains in an oligomeric protein.

(b) Calculate the number of polypeptide chains in this protein.

(c) What other analytic technique could you employ to determine whether the polypeptide chains in this protein are similar or different?

13. Amyloid Fibers in Disease Several small aromatic molecules, such as phenol red (used as a nontoxic drug model), have been shown to inhibit the formation of amyloid in laboratory model systems. A goal of the research on these small aromatic compounds is to find a drug that efficiently inhibits the formation of amyloid in the brain in people with incipient Alzheimer disease.

(a) Suggest why molecules with aromatic substituents would disrupt the formation of amyloid.

(b) Some researchers suggest that a drug used to treat Alzheimer disease may also be effective in treating type 2 (non-insulin-dependent) diabetes mellitus. Why might a single drug be effective in treating these two different conditions?

14. Protein-Folding Therapies The Food and Drug Administration recently approved the drug lumacaftor for the treatment of cystic fibrosis in patients with the F508ΔCFTR mutation. This mutation is a genetically encoded deletion of amino acid F508 from the protein. About ⅔ of cystic fibrosis patients have this mutation, and lumacaftor is one of the first drugs that functions as a pharmacological chaperone to correct a defect in the protein-folding process. However, lumacaftor is not always effective in treating patients who have other CFTR mutations that result in misfolding. Why is lumacaftor able to correct the misfolding of some mutant CFTR proteins and not others?

15. Structural Biology Methods Which structural biology method (CD, x-ray crystallography, NMR, or cryo-EM) is best suited to each task?
(a) Obtaining an ultra-high resolution (<1.5 Å) structure of a drug bound to its protein target
(b) Obtaining a low-to-medium resolution (5–10 Å) reconstruction of the 11 MDa (11,000,000 Da) bacterial flagellar motor
(c) Identifying the protonation state and pK_a of a His side chain in an enzyme active site
(d) Determining whether a protein is intrinsically disordered or contains secondary structure elements

BIOCHEMISTRY ONLINE

16. Using the PDB The Protein Data Bank (PDB) contains more than 150,000 different three-dimensional biomolecular structures obtained by x-ray crystallography, NMR, and cryo-EM. Each protein structure deposited into the database is given a PDB ID. Several PDB IDs represent proteins whose structures resemble letters from the Roman alphabet. Find each protein structure in the PDB and view the three-dimensional structure using JSmol, PYMOL, or a similar structure viewer.

PDB IDs: 2QYC, 2BNH, 2Q5R, 1XU9, 3H7X, 1OU5, 2WCD

(a) For each protein, identify its quaternary structure and describe the protomer structure as all α, all β, α/β, or $\alpha + \beta$.
(b) What letter does each protein structure most closely resemble?
(c) What word(s) can you spell using these protein structures?

17. Protein Modeling Online A group of patients with Crohn disease (an inflammatory bowel disease) underwent biopsies of their intestinal mucosa in an attempt to identify the causative agent. Researchers identified a protein that was present at higher levels in patients with Crohn disease than in patients with an unrelated inflammatory bowel disease or in unaffected controls. The protein was isolated, and the following *partial* amino acid sequence was obtained (reads left to right):

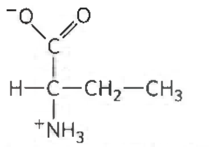
EAELCPDRCI	HSFQNLGIQC	VKKRDLEQAI
SQRIQTNNNP	FQVPIEEQRG	DYDLNAVRLC
FQVTVRDPSG	RPLRLPPVLP	HPIFDNRAPN
TAELKICRVN	RNSGSCLGGD	EIFLLCDKVQ
KEDIEVYFTG	PGWEARGSFS	QADVHRQVAI
VFRTPPYADP	SLQAPVRVSM	QLRRPSDREL
SEPMEFQYLP	DTDDRHRIEE	KRKRTYETFK
SIMKKSPFSG	PTDPRPPPRR	IAVPSRSSAS
VPKPAPQPYP		

(a) You can identify this protein using a protein database such as UniProt (www.uniprot.org). On the home page, click on the link for a "BLAST" search. On the BLAST page, enter about 30 residues from the protein sequence in the appropriate search field and submit it for analysis. What does this analysis tell you about the identity of the protein?
(b) Try using different portions of the amino acid sequence. Do you always get the same result?
(c) A variety of websites provide information about the three-dimensional structure of proteins. Find information about the protein's secondary, tertiary, and quaternary structures using database sites such as the Protein Data Bank (PDB; www.rcsb.org) or Structural Classification of Proteins (SCOP2; http://scop2.mrc-lmb.cam.ac.uk).
(d) In the course of your online searches, what did you learn about the cellular function of the protein?

DATA ANALYSIS PROBLEM

18. Mirror-Image Proteins As noted in Chapter 3, "The amino acid residues in protein molecules are almost all L stereoisomers." It is not clear whether this selectivity is necessary for proper protein function or is an accident of evolution. To explore this question, Milton and colleagues (1992) published a study of an enzyme made entirely of D stereoisomers. The enzyme they chose was HIV protease, a proteolytic enzyme made by HIV that converts inactive viral preproteins to their active forms.

Previously, Wlodawer and coworkers (1989) had reported the complete chemical synthesis of HIV protease from L-amino acids (the L-enzyme), using the process shown in Figure 3-30. Normal HIV protease contains two Cys residues, at positions 67 and 95. Because chemical synthesis of proteins containing Cys is technically difficult, Wlodawer and colleagues substituted the synthetic amino acid L-α-amino-*n*-butyric acid (Aba) for the two Cys residues in the protein. In the authors' words, this was done to "reduce synthetic difficulties associated with Cys deprotection and ease product handling."
(a) The structure of Aba is shown below. Why was this a suitable substitution for a Cys residue? Under what circumstances would it not be suitable?



L-α-Amino-*n*-butyric acid

Wlodawer and coworkers denatured the newly synthesized protein by dissolving it in 6 M guanidine HCl and then allowed it to fold slowly by dialyzing away the guanidine against a neutral buffer (10% glycerol, 25 mM NaH_2PO_4/Na_2HPO_4, pH 7).
(b) There are many reasons to predict that a protein synthesized, denatured, and folded in this manner would not be active. Give three such reasons.
(c) Interestingly, the resulting L-protease was active. What does this finding tell you about the role of disulfide bonds in the native HIV protease molecule?

In a more recent study, Milton and coworkers synthesized HIV protease from D-amino acids, using the same protocol as the earlier study (Wlodawer et al.). Formally, there are three possibilities for the folding of the D-protease: it would be (1) the same shape as the L-protease, (2) the mirror image of the L-protease, or (3) something else, possibly inactive.

(d) For each possibility, decide whether or not it is a likely outcome, and defend your position.

In fact, the D-protease was active: it cleaved a particular synthetic substrate and was inhibited by specific inhibitors. To examine the structure of the D- and L-enzymes, Milton and coworkers tested both forms for activity with D and L forms of a chiral peptide substrate and for inhibition by D and L forms of a chiral peptide-analog inhibitor. Both forms were also tested for inhibition by the achiral inhibitor Evans blue. The findings are given in the table.

			Inhibition		
	Substrate hydrolysis		**Peptide inhibitor**		**Evans blue**
HIV protease	**D-substrate**	**L-substrate**	**D-inhibitor**	**L-inhibitor**	**(achiral)**
L-protease	−	+	−	+	+
D-protease	+	−	+	−	+

(e) Which of the three models proposed is supported by these data? Explain your reasoning.

(f) Why does Evans blue inhibit both forms of the protease?

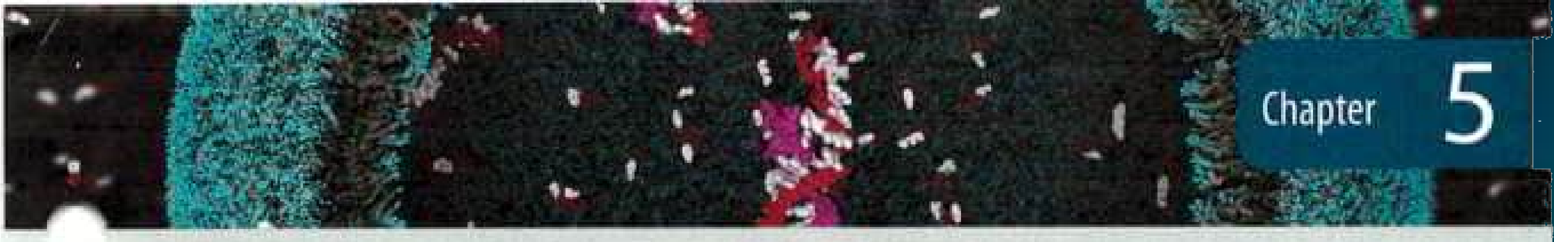
(g) Would you expect chymotrypsin to digest the D-protease? Explain your reasoning.

(h) Would you expect total synthesis from D-amino acids followed by renaturation to yield active enzyme for any enzyme? Explain your reasoning.

References

Milton, R.C., S.C. Milton, and S.B. Kent. 1992. Total chemical synthesis of a D-enzyme: the enantiomers of HIV-1 protease show demonstration of reciprocal chiral substrate specificity. *Science* 256:1445–1448.

Wlodawer, A., M. Miller, M. Jaskólski, B.K. Sathyanarayana, E. Baldwin, I.T. Weber, L.M. Selk, L. Clawson, J. Schneider, and S.B. Kent. 1989. Conserved folding in retroviral proteases: crystal structure of a synthetic HIV-1 protease. *Science* 245:616–621.



Chapter 5

PROTEIN FUNCTION

5.1 Reversible Binding of a Protein to a Ligand: Oxygen-Binding Proteins *148*

5.2 Complementary Interactions between Proteins and Ligands: The Immune System and Immunoglobulins *164*

5.3 Protein Interactions Modulated by Chemical Energy: Actin, Myosin, and Molecular Motors *169*

Knowing the three-dimensional structure of a protein is an important part of understanding protein function, and structural biology often offers insights into molecular interactions. However, the protein structures we have examined so far are deceptively static. Proteins function by interacting dynamically with—binding to—other molecules. We divide these interactions into two types. In some interactions, the result is a reaction that alters the chemical configuration or composition of a bound molecule, with the protein acting as a reaction catalyst, or **enzyme**; we discuss enzymes and their properties in Chapter 6. In other interactions, neither the chemical configuration nor the composition of the bound molecule is changed; such interactions are the subject of this chapter.

It may seem counterintuitive that a protein's interaction with another molecule could be important if it does not alter the associated molecule. Yet, transient interactions of this type are at the heart of many complex physiological processes, such as oxygen transport, transmission of nerve impulses, and immune function. Defining which molecules interact and quantifying such interactions are common and illuminating tasks in every biochemical subdiscipline.

The study of proteins that function through reversible interactions can be organized around six key principles of protein function, some of which will be familiar from Chapter 4:

P1 **The functions of many proteins involve the reversible binding of other molecules.** A molecule bound reversibly by a protein is called a **ligand**. A ligand may be any kind of molecule, including another protein. The transient nature of protein-ligand interactions is critical to life, allowing an organism to respond rapidly and reversibly to changing environmental and metabolic circumstances.

P2 **A ligand binds a protein at a binding site that is complementary to the ligand in size, shape, charge, and hydrophobic or hydrophilic character.** The interaction is specific: the protein can discriminate among the thousands of different molecules in its environment and selectively bind only one or a few types. A given protein may have separate **binding sites** for several different ligands. These specific molecular interactions are crucial in maintaining the high degree of order in a living system.

P3 **Proteins are flexible.** Changes in conformation may be subtle, reflecting molecular vibrations and small movements of amino acid residues throughout the protein. Changes in conformation may also be more dramatic, with major segments of the protein structure moving as much as several nanometers. Specific conformational changes are frequently essential to a protein's function.

P4 **The binding of a protein and a ligand is often coupled to a conformational change in the protein that makes the binding site more complementary to the ligand, permitting tighter binding.** The structural adaptation that occurs between protein and ligand is called **induced fit**.

P5 **In a multisubunit protein, a conformational change in one subunit often affects the conformation of other subunits.**

P6 **Interactions between ligands and proteins may be regulated.**

The themes in our discussion of noncatalytic functions of proteins in this chapter—binding, specificity, and conformational change—are continued in Chapter 6, with the added element of proteins participating in chemical transformations. This discussion excludes the binding of water, which may interact weakly and nonspecifically with many parts of a protein.

5.1 Reversible Binding of a Protein to a Ligand: Oxygen-Binding Proteins

Myoglobin and **hemoglobin** may be the most-studied and best-understood proteins. They were the first proteins for which three-dimensional structures were determined, and these two molecules illustrate almost every aspect of that critical biochemical process: the reversible binding of a ligand to a protein. This classic model of protein function tells us a great deal about how proteins work.

Oxygen Can Bind to a Heme Prosthetic Group

Oxygen is poorly soluble in aqueous solutions (see Table 2-2) and cannot be carried to tissues in sufficient quantity if it is simply dissolved in blood serum. Also, diffusion of oxygen through tissues is ineffective over distances greater than a few millimeters. The evolution of larger, multicellular animals depended on the evolution of proteins that could transport and store oxygen. However, none of the amino acid side chains in proteins are suited for the reversible binding of oxygen molecules. This role is filled by certain transition metals, among them iron and copper, that have a strong tendency to bind oxygen. Multicellular organisms exploit the properties of metals, most commonly iron, for oxygen transport. However, free iron promotes the formation of highly reactive oxygen species such as hydroxyl radicals that can damage DNA and other macromolecules. Iron used in cells is therefore bound in forms that sequester it and/or make it less reactive. In multicellular organisms, iron is often incorporated into a protein-bound prosthetic group called **heme** (or haem). (Recall from Chapter 3 that a prosthetic group is a compound permanently associated with a protein that contributes to the protein's function.) Heme is found in many oxygen-transporting proteins, as well as in some proteins, such as the cytochromes, that participate in oxidation-reduction (electron-transfer) reactions (Chapter 19).

Heme consists of a complex organic ring structure, **protoporphyrin**, to which is bound a single iron atom in its ferrous (Fe^{2+}) state (**Fig. 5-1**). The iron atom has six coordination bonds: four to nitrogen atoms that are part of the flat **porphyrin ring** system, and two perpendicular to the porphyrin.

P1 Iron in the Fe^{2+} state binds oxygen reversibly; in the Fe^{3+} state it does not bind oxygen. The structure of heme and the globins to which it is bound represents an evolutionary adaptation to prevent Fe^{2+} oxidation. Free heme molecules (heme not bound to protein) leave Fe^{2+} with two "open" coordination bonds. Simultaneous reaction of one O_2 molecule with two free heme molecules (or two free Fe^{2+}) can result in irreversible conversion of Fe^{2+} to Fe^{3+}. The coordinated nitrogen atoms (which have an electron-donating character) help prevent conversion of the heme iron to the ferric (Fe^{3+}) state. In heme-containing proteins, this reaction is further prevented by sequestering each heme deep within the protein structure. Thus, access to the two open coordination bonds is restricted. In the globins that will shortly

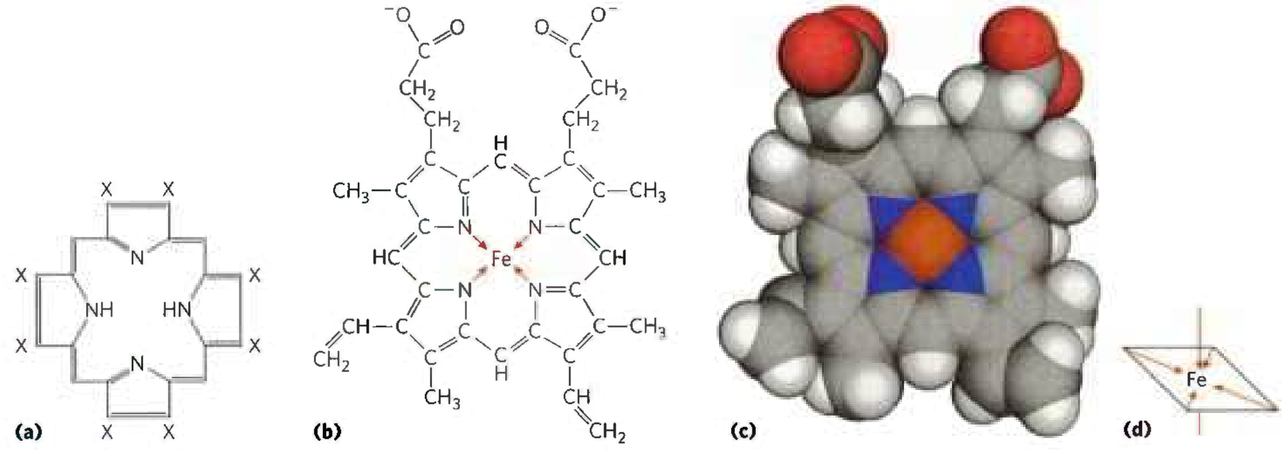


FIGURE 5-1 **Heme.** The heme group is present in myoglobin, hemoglobin, and many other proteins, designated heme proteins. Heme consists of a complex organic ring structure, protoporphyrin IX, with a bound iron atom in its ferrous (Fe^{2+}) state. (a) Porphyrins, of which protoporphyrin IX is just one example, consist of four pyrrole rings linked by methene bridges, with substitutions at one or more of the positions denoted X. (b, c) Two representations of heme. The iron atom of heme has six coordination bonds: four in the plane of, and bonded to, the flat porphyrin ring system, and (d) two perpendicular to it. [(c) Data from PDB ID 1CCR, H. Ochi et al., *J. Mol. Biol.* 166:407, 1983.]

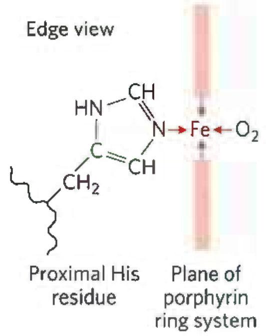


FIGURE 5-2 The heme group viewed from the side. This view shows the two coordination bonds to Fe^{2+} that are perpendicular to the porphyrin ring system. One is occupied by a His residue called the proximal His, His^{93} in myoglobin, also designated His F8 (the eighth residue in α helix F; see Fig. 5-3); the other is the binding site for oxygen. The remaining four coordination bonds are in the plane of, and bonded to, the flat porphyrin ring system.

become the focus of our narrative, one of these two coordination bonds is occupied by a side-chain nitrogen of a highly conserved His residue referred to as the **proximal His**. P2 The other is the binding site for molecular oxygen (O_2) (**Fig. 5-2**). When oxygen binds, the electronic properties of heme iron change; this accounts for the change in color from the dark purple of oxygen-depleted venous blood to the bright red of oxygen-rich arterial blood. Some small molecules, such as carbon monoxide (CO) and nitric oxide (NO), coordinate to heme iron with greater affinity than does O_2. When a molecule of CO is bound to heme, O_2 is excluded, which is why CO is highly toxic to aerobic organisms (a topic explored in Box 5-1). By surrounding and sequestering heme, oxygen-binding proteins regulate the access of small molecules to the heme iron.

Globins Are a Family of Oxygen-Binding Proteins

The **globins** are a widespread family of proteins. Globins are commonly found in eukaryotes of all classes, as well as in archaea and bacteria. All evolved from a common ancestral protein. Their tertiary structure is highly conserved, made up of eight α-helical segments connected by bends. This folding pattern, illustrated by myoglobin (**Fig. 5-3**), constitutes a structural motif known as the **globin fold**. The primary amino acid sequence of globins is less conserved, well reflecting both the ancient origins of this protein family and the evolutionary relatedness of species from which globin comparisons might be made.

Most globins function in oxygen transport or storage, although some play a role in the sensing of oxygen, nitric oxide, or carbon monoxide. The simple nematode worm *Caenorhabditis elegans* has genes encoding 33 different globins. In humans and other mammals, there are at least four kinds of globins. The monomeric myoglobin facilitates oxygen diffusion in muscle tissue. Myoglobin is particularly abundant in the muscles of diving marine mammals such as seals and whales, where it also has an oxygen-storage function for prolonged excursions undersea. The tetrameric hemoglobin is responsible for oxygen transport in the bloodstream. The monomeric neuroglobin is expressed largely in neurons and helps to protect the brain from hypoxia (low oxygen) or ischemia (restricted blood supply). Cytoglobin, another monomeric globin, is found at high concentrations in the walls of blood vessels, where it functions to regulate levels of nitric oxide, a localized signal for muscle relaxation (see Box 12-2).

Myoglobin Has a Single Binding Site for Oxygen

Any detailed discussion of protein function inevitably involves protein structure. Myoglobin (M_r 16,700; abbreviated Mb) is a single polypeptide of 153 amino acid residues with one molecule of heme (Fig. 5-3). About 78% of the amino acid residues in the protein are found in the eight α helices typical of the globin fold, named A through H.

An individual amino acid residue is designated either by its position in the amino acid sequence or by its location in the sequence of a particular α-helical segment. For example, the His residue coordinated to the heme in myoglobin—the proximal His—is His^{93} (the ninety-third residue from the amino-terminal end of the myoglobin polypeptide sequence) and is also called His F8 (the eighth residue in α helix F). The bends in the structure are designated AB, CD, EF, FG, and GH, reflecting the α-helical segments they connect.

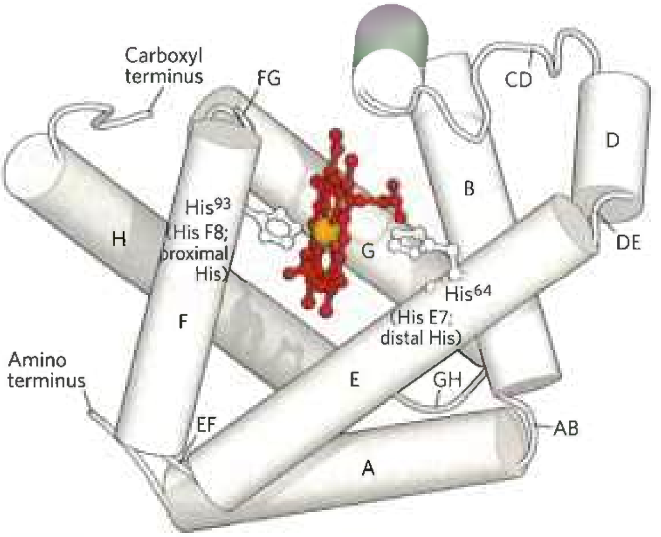

Carboxyl terminus
FG
CD
D
B
His^{93} (His F8; proximal His)
H
G
DE
His^{64} (His E7; distal His)
F
Amino terminus
E
GH
EF
AB
A

FIGURE 5-3 Myoglobin. The eight α-helical segments (shown here as cylinders) are labeled A through H. Nonhelical residues in the bends that connect them are labeled AB, CD, EF, and so forth, indicating the segments they interconnect. A few bends, including BC and DE, are abrupt and do not contain any residues; these are sometimes not labeled. The heme is bound in a pocket made up largely of the E and F helices, although amino acid residues from other segments of the protein also participate. [Data from PDB ID 1MBO, S. E. Phillips, *J. Mol. Biol.* 142:531, 1980.]

Protein-Ligand Interactions Can Be Described Quantitatively

The function of myoglobin depends on the protein's ability not only to bind oxygen but also to release it when and where it is needed. Function in biochemistry often revolves around a reversible protein-ligand interaction of this type. A quantitative description of this interaction is a central part of many biochemical investigations.

In general, the reversible binding of a protein (P) to a ligand (L) can be described by a simple **equilibrium expression**:

$$P + L \rightleftharpoons PL \tag{5-1}$$

The reaction is characterized by an equilibrium constant, K_a, such that

$$K_a = \frac{[PL]}{[P][L]} = \frac{k_a}{k_d} \tag{5-2}$$

where k_a and k_d are rate constants (more on these below). The term $\boldsymbol{K_a}$ is an **association constant** (not to be confused with the K_a that denotes an acid dissociation constant; p. 57) that describes the equilibrium between the complex and the unbound components of the complex. The association constant provides a measure of the affinity of the ligand L for the protein. K_a has units of M^{-1}; a higher value of K_a corresponds to a higher affinity of the ligand for the protein.

The equilibrium term K_a is also equivalent to the ratio of the rates of the forward (association) and reverse (dissociation) reactions that form the PL complex. The association rate is described by the rate constant k_a, and dissociation is described by the rate constant k_d. As discussed further in the next chapter, rate constants are proportionality constants, describing the fraction of a pool of reactant that reacts in a given amount of time. When the reaction involves one molecule, such as the dissociation reaction $PL \rightarrow P + L$, the reaction is *first order* and the rate constant (k_d) has units of reciprocal time (s^{-1}). When the reaction involves two molecules, such as the association reaction $P + L \rightarrow PL$, it is called *second order*, and its rate constant (k_a) has units of $\text{M}^{-1}\,\text{s}^{-1}$.

KEY CONVENTION Equilibrium constants are denoted with a capital *K* and rate constants are denoted with a lowercase *k*. ■

A rearrangement of the first part of Equation 5-2 shows that the ratio of bound to free protein is directly proportional to the concentration of free ligand:

$$K_a[L] = \frac{[PL]}{[P]} \tag{5-3}$$

When the concentration of the ligand is much greater than the concentration of ligand-binding sites, the binding of the ligand by the protein does not appreciably change the concentration of free (unbound) ligand—that is, [L] remains constant. This condition is broadly applicable to most ligands that bind to proteins in cells, and it simplifies our description of the binding equilibrium.

We can now consider the binding equilibrium from the standpoint of the fraction, *Y*, of ligand-binding sites on the protein that are occupied by ligand:

$$Y = \frac{\text{binding sites occupied}}{\text{total binding sites}} = \frac{[PL]}{[PL]+[P]} \tag{5-4}$$

Substituting $K_a[L][P]$ for [PL] (see Eqn 5-3) and rearranging terms gives

$$Y = \frac{K_a[L][P]}{K_a[L][P]+[P]} = \frac{K_a[L]}{K_a[L]+1} = \frac{[L]}{[L]+\dfrac{1}{K_a}} \tag{5-5}$$

The value of K_a can be determined from a plot of *Y* versus the concentration of free ligand, [L] (**Fig. 5-4a**). Any equation of the form $x = y/(y + z)$ describes a hyperbola, and *Y* is thus found to be a hyperbolic function of [L]. The fraction of ligand-binding sites occupied approaches saturation asymptotically as [L] increases. The [L] at which half of the available ligand-binding sites are occupied (that is, $Y = 0.5$) corresponds to $1/K_a$.

It is more common (and intuitively simpler), however, to consider the **dissociation constant, $\boldsymbol{K_d}$**, which

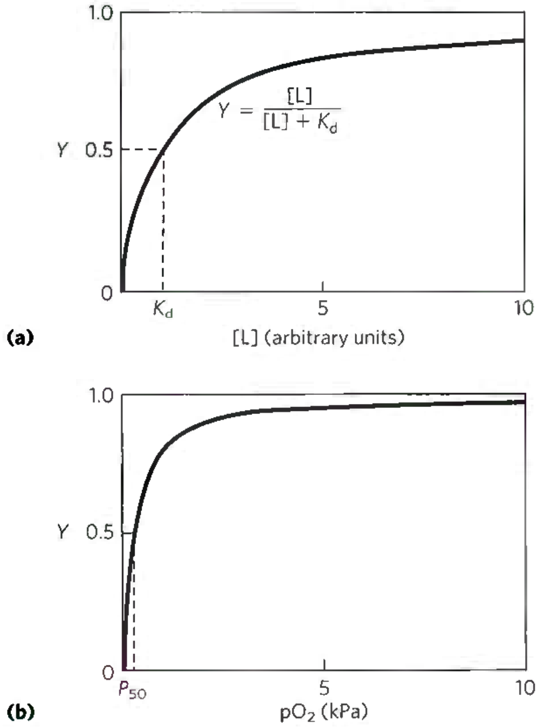
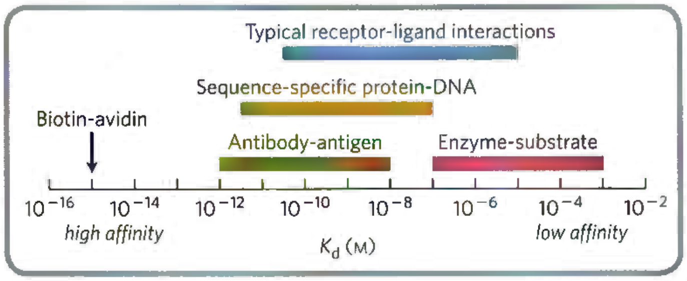


FIGURE 5-4 Graphical representations of ligand binding. The fraction of ligand-binding sites occupied, *Y*, is plotted against the concentration of free ligand. Both curves are rectangular hyperbolas. (a) A hypothetical binding curve for a ligand L. The [L] at which half of the available ligand-binding sites are occupied is equivalent to $1/K_a$, or K_d. The curve has a horizontal asymptote at $Y = 1$ and a vertical asymptote (not shown) at $[L] = -1/K_a$. (b) A curve describing the binding of oxygen to myoglobin. The partial pressure of O_2 in the air above the solution is expressed in kilopascals (kPa). Oxygen binds tightly to myoglobin, with a P_{50} of only 0.26 kPa.

TABLE 5-1 Protein Dissociation Constants: Some Examples and Range

Protein	Ligand	K_d (M)[a]
Avidin (egg white)	Biotin	1×10^{-15}
Insulin receptor (human)	Insulin	1×10^{-10}
Anti-HIV immunoglobulin (human)[b]	gp41 (HIV-1 surface protein)	4×10^{-10}
Nickel-binding protein (*E. coli*)	Ni^{2+}	1×10^{-7}
Calmodulin (rat)[c]	Ca^{2+}	3×10^{-6}
		2×10^{-5}



Color bars indicate the range of dissociation constants typical of various classes of interactions in biological systems. A few interactions, such as that between the protein avidin and the enzyme cofactor biotin, fall outside the normal ranges. The avidin-biotin interaction is so tight it may be considered irreversible. Sequence-specific protein-DNA interactions reflect proteins that bind to a particular sequence of nucleotides in DNA, as opposed to general binding to any DNA site.

[a] A reported dissociation constant is valid only for the particular solution conditions under which it was measured. K_d values for a protein-ligand interaction can be altered, sometimes by several orders of magnitude, by changes in the solution's salt concentration, pH, or other variables.

[b] This immunoglobulin was isolated as part of an effort to develop a vaccine against HIV. Immunoglobulins (described later in the chapter) are highly variable, and the K_d reported here should not be considered characteristic of all immunoglobulins.

[c] Calmodulin has four binding sites for calcium. The values shown reflect the highest- and lowest-affinity binding sites observed in one set of measurements.

is the reciprocal of K_a ($K_d = 1/K_a$) and has units of molar concentration (M). K_d is the equilibrium constant for the release of ligand. The relevant expressions change to

$$K_d = \frac{[P][L]}{[PL]} = \frac{k_d}{k_a} \tag{5-6}$$

$$[PL] = \frac{[P][L]}{K_d} \tag{5-7}$$

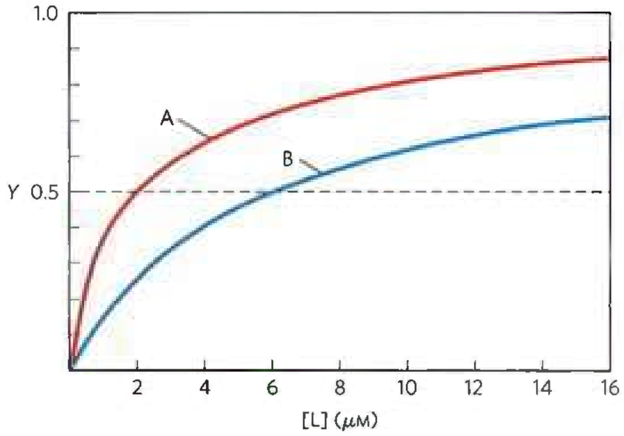
$$Y = \frac{[L]}{[L] + K_d} \tag{5-8}$$

When [L] equals K_d, half of the ligand-binding sites are occupied. As [L] falls below K_d, progressively less of the protein has ligand bound to it. For 90% of the available ligand-binding sites to be occupied, [L] must be nine times greater than K_d.

P1 In practice, K_d is used much more often than K_a to express the affinity of a protein for a ligand. Note that a lower value of K_d corresponds to a higher affinity of ligand for the protein. The mathematics can be reduced to simple statements: K_d is equivalent to the molar concentration of ligand at which half of the available ligand-binding sites are occupied. At this point, the protein is said to have reached half-saturation with respect to ligand binding. The more tightly a protein binds a ligand, the lower the concentration of ligand required for half the binding sites to be occupied, and thus the lower the value of K_d. Some representative dissociation constants are given in **Table 5-1**; the scale shows typical ranges for dissociation constants found in biological systems.

WORKED EXAMPLE 5-1 *Receptor-Ligand Dissociation Constants*

Two proteins, A and B, bind to the same ligand, L, with the binding curves shown below. What is the dissociation constant, K_d, for each protein? Which protein (A or B) has a greater affinity for ligand L?



SOLUTION: We can determine the dissociation constants by inspecting the graph. Because Y represents the fraction of binding sites occupied by ligand, the concentration of ligand at which half the binding sites are occupied — that is, the point where the binding curve crosses the line where $Y = 0.5$ — is the dissociation constant. For A, $K_d = 2\,\mu\text{M}$; for B, $K_d = 6\,\mu\text{M}$. Because A is half-saturated at a lower [L], it has a higher affinity for the ligand.

WORKED EXAMPLE 5-2 *Protein-Ligand Binding*

A protein has a K_d of 2.0 μM. What is the [L] where $Y = 0.6$?

SOLUTION: Solving Equation 5-8 for [L], first we obtain

$$[\text{L}] = Y([\text{L}] + K_d)$$

Distributing Y on the right side gives

$$[\text{L}] = Y[\text{L}] + YK_d$$

Subtracting Y[L] from both sides

$$[\text{L}] - Y[\text{L}] = YK_d$$

then factoring out [L]

$$[\text{L}](1 - Y) = YK_d$$

and dividing both sides by $1 - Y$ gives the final expression

$$[\text{L}] = \frac{YK_d}{(1 - Y)}$$

Substituting the values for Y and K_d given in the problem gives

$$[\text{L}] = 0.6(2.0\,\mu\text{M})/(1 - 0.6) = 3.0\,\mu\text{M}$$

The binding of oxygen to myoglobin follows the patterns discussed above. However, because oxygen is a gas, we must make some minor adjustments to the equations so that laboratory experiments can be carried out more conveniently. We first substitute the concentration of dissolved oxygen for [L] in Equation 5-8 to give

$$Y = \frac{[\text{O}_2]}{[\text{O}_2] + K_d} \qquad (5\text{-}9)$$

As for any ligand, K_d equals the $[\text{O}_2]$ at which half of the available ligand-binding sites are occupied, or $[\text{O}_2]_{0.5}$. Equation 5-9 thus becomes

$$Y = \frac{[\text{O}_2]}{[\text{O}_2] + [\text{O}_2]_{0.5}} \qquad (5\text{-}10)$$

In experiments using oxygen as a ligand, it is the partial pressure of oxygen (pO_2) in the gas phase above the solution that is varied, because this is easier to measure than the concentration of oxygen dissolved in the solution. The concentration of a volatile substance in solution is always proportional to the local partial pressure of the gas. So, if we define the partial pressure of oxygen at $[\text{O}_2]_{0.5}$ as P_{50}, substitution in Equation 5-10 gives

$$Y = \frac{\text{pO}_2}{\text{pO}_2 + P_{50}} \qquad (5\text{-}11)$$

A binding curve for myoglobin that relates Y to pO_2 is shown in Figure 5-4b.

Protein Structure Affects How Ligands Bind

P3 The binding of a ligand to a protein is rarely as simple as the above equations would suggest. The interaction is greatly affected by protein structure and is often accompanied by conformational changes. For example, the specificity with which heme binds its various ligands is altered when the heme is a component of myoglobin. For free heme molecules, carbon monoxide binds more than 20,000 times better than does O_2 (that is, the K_d or P_{50} for CO binding to free heme is more than 20,000 times lower than that for O_2), but it binds only about 40 times better than O_2 when the heme is bound in myoglobin. For free heme, the tighter binding by CO reflects differences in the way the orbital structures of CO and O_2 interact with Fe^{2+}. Those same orbital structures lead to different binding geometries for CO and O_2 when they are bound to heme (**Fig. 5-5a, b**). The change in relative affinity of CO and O_2 for heme when the heme is bound to a globin is mediated by the globin structure.

When heme is bound to myoglobin, its affinity for O_2 is selectively increased by the presence of the **distal His** (His^{64}, or His E7 in myoglobin). The Fe-O_2 complex is much more polar than the Fe-CO complex. A partial negative charge is distributed across the oxygen atoms in the bound O_2 due to partial oxidation of the interacting iron atom. A hydrogen bond between the imidazole side chain of His E7 and the bound O_2 stabilizes this polar complex electrostatically (Fig. 5-5c). The affinity of myoglobin for O_2 is thus selectively increased by a factor of about 500; there is no such effect for Fe-CO binding in myoglobin. Consequently, the 20,000-fold stronger binding affinity of free heme for CO compared with O_2 declines to approximately 40-fold for heme embedded in myoglobin. This favorable electrostatic effect on O_2 binding is even more dramatic in some invertebrate hemoglobins, where two groups in the binding pocket can form strong hydrogen bonds with O_2, causing the heme group to bind O_2 with greater affinity than CO. This selective enhancement of O_2 affinity in globins is physiologically important: it helps prevent poisoning by the CO that is generated in small amounts from metabolism and that is sometimes generated in larger amounts by our industrial-age environment.

P3 The binding of O_2 to the heme in myoglobin also depends on molecular motions, or "breathing," in the protein structure. The heme molecule is deeply buried in the folded polypeptide, with limited direct paths for oxygen to move from the surrounding solution to the ligand-binding site. If the protein were rigid, O_2 could not readily enter or

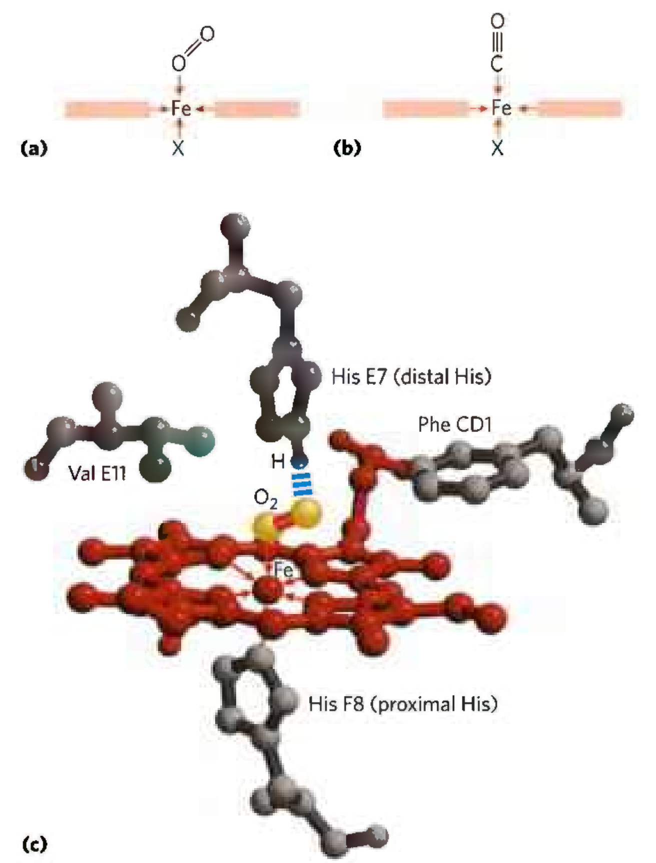


FIGURE 5-5 **Steric effects caused by ligand binding to the heme of myoglobin.** (a) Oxygen binds to heme with the O_2 axis at an angle, a binding conformation readily accommodated by myoglobin. (b) Carbon monoxide binds to free heme with the CO axis perpendicular to the plane of the porphyrin ring. (c) Another view of the heme of myoglobin, showing the arrangement of key amino acid residues around the heme. The bound O_2 is hydrogen-bonded to the distal His, His E7 (His^{64}), facilitating the binding of O_2 compared with its binding to free heme. [(c) Data from PDB ID 1MBO, S. E. Phillips, *J. Mol. Biol.* 142:531, 1980.]

leave the heme pocket. However, rapid molecular flexing of the amino acid side chains produces transient cavities in the protein structure, and O_2 makes its way in and out by moving through these cavities. Computer simulations of rapid structural fluctuations in myoglobin suggest that there are many such pathways. The distal His acts as a gate to control access to one major pocket near the heme iron. Rotation of that His residue to open and close the pocket occurs on a nanosecond (10^{-9} s) time scale.

Thus, subtle conformational changes can be critical for protein activity. Structural changes take on more complexity in the multisubunit hemoglobin, which we consider next.

Hemoglobin Transports Oxygen in Blood

Nearly all the oxygen carried by whole blood in animals is bound and transported by hemoglobin in erythrocytes (red blood cells). Normal human erythrocytes are small (6 to 9 μm in diameter), biconcave disks. They are formed from precursor stem cells called **hemocytoblasts**. In the maturation process, the stem cell produces daughter cells that form large amounts of hemoglobin and then lose their organelles—nucleus, mitochondria, and endoplasmic reticulum. Erythrocytes are thus incomplete, vestigial cells, unable to reproduce and, in humans, destined to survive for only about 120 days. Their main function is to carry hemoglobin, which is dissolved in the cytosol at a very high concentration (~34% by weight).

In arterial blood passing from the lungs through the heart to the peripheral tissues, hemoglobin is about 96% saturated with oxygen. In the venous blood returning to the heart, hemoglobin is only about 64% saturated. Thus, each 100 mL of blood passing through a tissue releases about one-third of the oxygen it carries, or 6.5 mL of O_2 gas at atmospheric pressure and body temperature.

Myoglobin, with its hyperbolic binding curve for oxygen (Fig. 5-4b), is relatively insensitive to small changes in the concentration of dissolved oxygen, so it functions well as an oxygen-storage protein. Hemoglobin, with its multiple subunits and O_2-binding sites, is better suited to oxygen transport. As we shall see, interactions between the subunits of a multimeric protein can permit a highly sensitive response to small changes in ligand concentration. **P5** Interactions among the subunits in hemoglobin cause conformational changes that alter the affinity of the protein for oxygen. The modulation of oxygen binding allows the O_2-transport protein to respond to changes in oxygen demand by tissues.

Hemoglobin Subunits Are Structurally Similar to Myoglobin

Hemoglobin (M_r 64,500; abbreviated Hb) is roughly spherical, with a diameter of nearly 5.5 nm. It is a tetrameric protein containing four heme prosthetic groups, one associated with each polypeptide chain. Adult hemoglobin contains two types of globin: two α chains (141 residues each) and two β chains (146 residues each). The three-dimensional structures of the two types of subunits are very similar to each other and to myoglobin (**Fig. 5-6**), reflecting their evolution within the larger globin superfamily. However, fewer than half of the amino acid residues are identical in the polypeptide sequences of the α and β subunits, and only 27 are identical in the three polypeptides (**Fig. 5-7**).

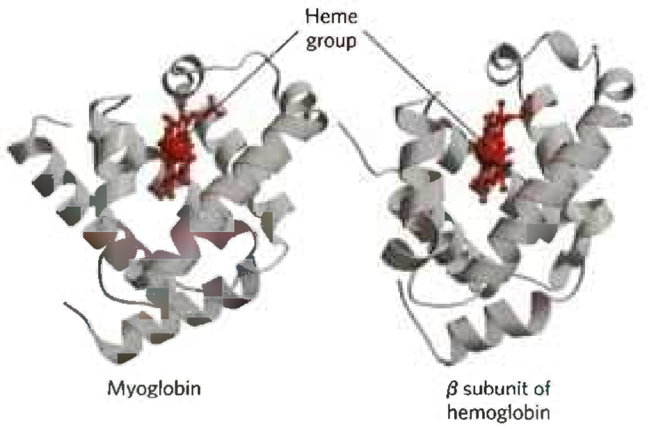
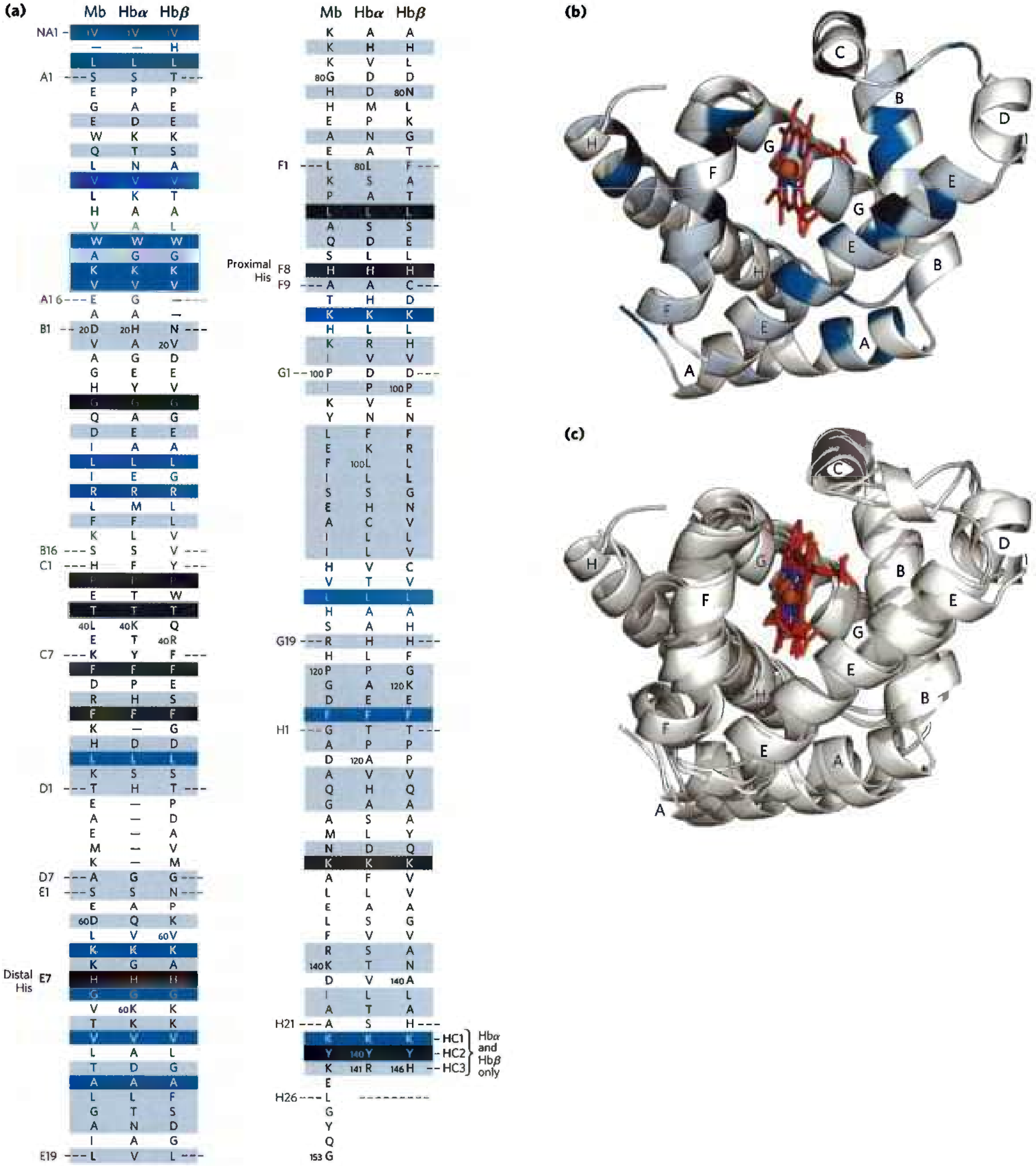


FIGURE 5-6 Comparison of the structures of myoglobin and the β subunit of hemoglobin. [Data from (left) PDB ID 1MBO, S. E. Phillips, *J. Mol. Biol.* 142:531, 1980; (right) PDB ID 1HGA, R. Liddington et al., *J. Mol. Biol.* 228:551, 1992.]



(a)

	Mb	Hbα	Hbβ
NA1	V	V	V
	—	—	H
	L	L	L
A1	S	S	T
	E	P	P
	G	A	E
	E	D	E
	W	K	K
	Q	T	S
	L	N	A
	V	V	V
	L	K	T
	H	A	A
	V	A	L
	W	W	W
	A	G	G
	K	K	K
	V	V	V
A16	E	G	—
	A	A	—
B1	20 D	20 H	N
	V	A	20 V
	A	G	D
	G	E	E
	H	Y	V
	G	G	G
	Q	A	G
	D	E	E
	I	A	A
	L	L	L
	I	E	G
	R	R	R
	L	M	L
	F	F	L
	K	L	V
B16	S	S	V
C1	H	F	Y
	P	P	P
	E	T	W
	T	T	T
	40 L	40 K	Q
	E	T	40 R
C7	K	Y	F
	F	F	F
	D	P	E
	R	H	S
	F	F	F
	K	—	G
	H	D	D
	L	L	L
	K	S	S
D1	T	H	T
	E	—	P
	A	—	D
	E	—	A
	M	—	V
	K	—	M
D7	A	G	G
E1	S	S	N
	E	A	P
	60 D	Q	K
	L	V	60 V
	K	K	K
	K	G	A
Distal His E7	H	H	H
	G	G	G
	V	60 K	K
	T	K	K
	V	V	V
	L	A	L
	T	D	G
	A	A	A
	L	L	F
	G	T	S
	A	N	D
	I	A	G
E19	L	V	L

	Mb	Hbα	Hbβ	
	K	A	A	
	K	H	H	
	K	V	L	
	80 G	D	D	
	H	D	80 N	
	H	M	L	
	E	P	K	
	A	N	G	
	E	A	T	
F1	L	80 L	F	
	K	S	A	
	P	A	T	
	L	L	L	
	A	S	S	
	Q	D	E	
	S	L	L	
Proximal His F8	H	H	H	
F9	A	A	C	
	T	H	D	
	K	K	K	
	H	L	L	
	K	R	H	
	I	V	V	
G1	100 P	D	D	
	I	P	100 P	
	K	V	E	
	Y	N	N	
	L	F	F	
	E	K	R	
	F	100 L	L	
	I	L	L	
	S	S	G	
	E	H	N	
	A	C	V	
	I	L	L	
	I	L	V	
	H	V	C	
	V	T	V	
	L	L	L	
	H	A	A	
	S	A	H	
G19	R	H	H	
	H	L	F	
	120 P	P	G	
	G	A	120 K	
	D	E	E	
	F	F	F	
H1	G	T	T	
	A	P	P	
	D	120 A	P	
	A	V	V	
	Q	H	Q	
	G	A	A	
	A	S	A	
	M	L	Y	
	N	D	Q	
	K	K	K	
	A	F	V	
	L	L	V	
	E	A	A	
	L	S	G	
	F	V	V	
	R	S	A	
	140 K	T	N	
	D	V	140 A	
	I	L	L	
	A	T	A	
H21	A	S	H	
	K	K	K	HC1 Hbα and Hbβ only
	Y	140 Y	Y	HC2 Hbα and Hbβ only
	K	141 R	146 H	HC3 Hbα and Hbβ only
	E			
H26	L			
	G			
	Y			
	Q			
	153 G			

FIGURE 5-7 Comparison of whale myoglobin with the α and β chains of human hemoglobin. (a) Sequence alignment with dashed lines marking helix boundaries. For optimal alignment, short gaps are introduced into both Hb sequences where a few amino acids are present in the other sequences. With the exception of the missing D helix in the Hb α chain (Hbα), this alignment permits the use of the helix lettering convention that emphasizes the common positioning of amino acid residues. Residues conserved in all known globins are shaded black. Residues conserved in the three globins shown here are shaded dark blue. Positions where the amino acid class (hydrophobic, charged, etc.) is conserved are shaded light blue. The common helix-letter-and-number designation for amino acids does not necessarily correspond to a common position in the linear sequence. For example, the distal His residue is His E7 in all three structures, but corresponds to His^{64}, His^{58}, and His^{63} in the linear sequences of Mb, Hbα, and Hbβ, respectively. Nonhelical residues at the amino and carboxyl termini are labeled NA and HC, respectively. (b) The location of shaded residues is shown in a ribbon representation of myoglobin structure. (c) Ribbon representations of the three globin structures are overlaid to show structural conservation. [Data from PDB ID 1MBO, S. E. Phillips, *J. Mol. Biol.* 142:531, 1980; PDB ID 1HGA, R. Liddington et al., *J. Mol. Biol.* 228:551, 1992.]

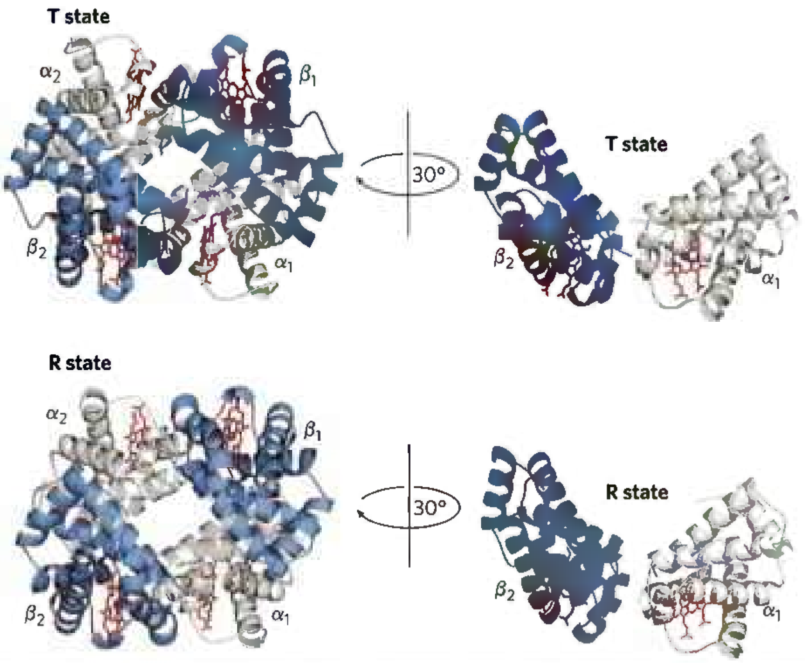


FIGURE 5-8 Dominant interactions between hemoglobin subunits. In this representation, α subunits are light and β subunits are dark. The strongest subunit interactions (highlighted) occur between unlike subunits. When oxygen binds, the $\alpha_1\beta_1$ contact changes little, but there is a large change at the $\alpha_1\beta_2$ contact, with several ion pairs broken. [Data from PDB ID 1HGA, R. Liddington et al., *J. Mol. Biol.* 228:551, 1992.]

The helix-naming convention described for myoglobin is also applied to the hemoglobin polypeptides, except that the α subunit lacks the short D helix. The heme-binding pocket is made up largely of the E and F helices in each of the subunits.

The quaternary structure of hemoglobin features strong interactions between unlike subunits. The $\alpha_1\beta_1$ interface (and its $\alpha_2\beta_2$ counterpart) involves more than 30 residues, and its interaction is sufficiently strong that although mild treatment of hemoglobin with urea tends to disassemble the tetramer into $\alpha\beta$ dimers, these dimers remain intact. The $\alpha_1\beta_2$ (and $\alpha_2\beta_1$) interface involves 19 residues (**Fig. 5-8**). The hydrophobic effect plays the major role in stabilizing these interfaces, but there are also many hydrogen bonds and a few ion pairs (or salt bridges), whose importance is discussed below.

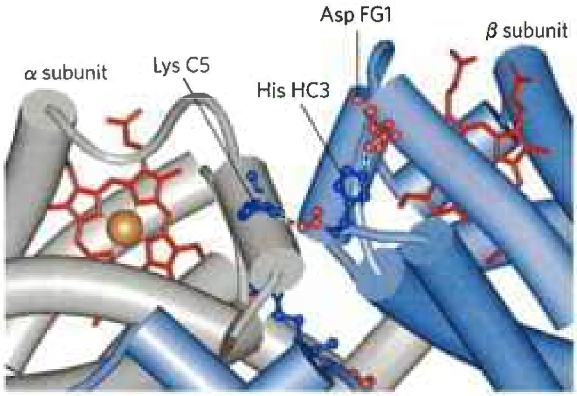
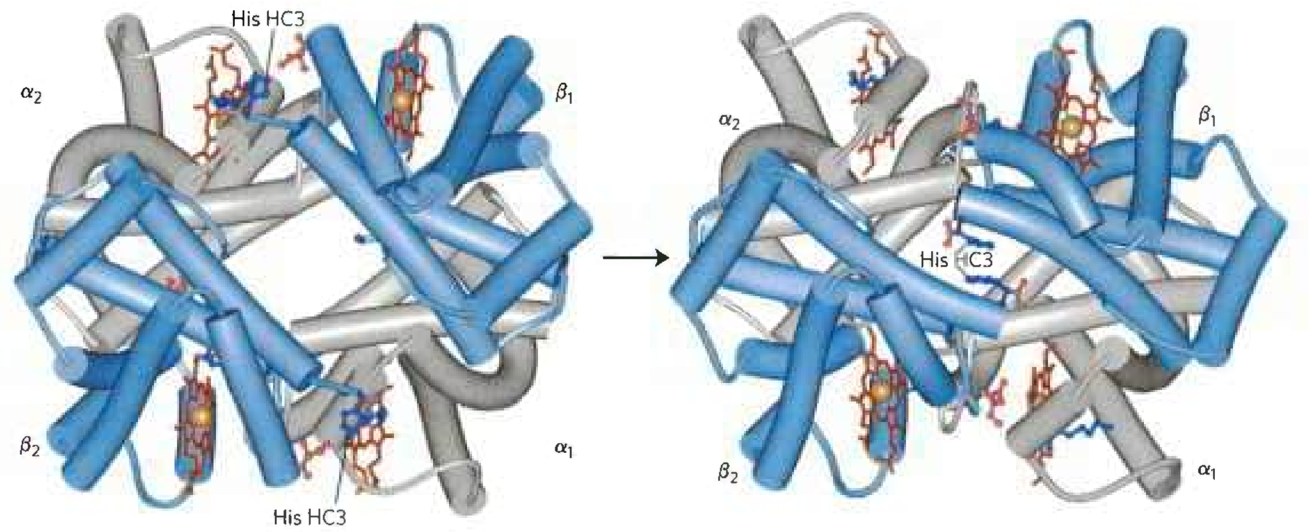


FIGURE 5-9 Some ion pairs that stabilize the T state of deoxyhemoglobin. Many of the ion pairs stabilizing the T state lie at the $\alpha_1\beta_2$ (and $\alpha_2\beta_1$) interface. Close-up view of a portion of a deoxyhemoglobin molecule in the T state. Interactions between the ion pairs His HC3 and Asp FG1 of the β subunit (blue) and between Lys C5 of the α subunit (gray) and His HC3 (its α-carboxyl group) of the β subunit are shown with dashed lines. (Recall that HC3 is the carboxyl-terminal residue of the β subunit.) These examples do not represent the entire network of ion pairs that stabilize the structure. [Data from PDB ID 1HGA, R. Liddington et al., *J. Mol. Biol.* 228:551, 1992.]

Hemoglobin Undergoes a Structural Change on Binding Oxygen

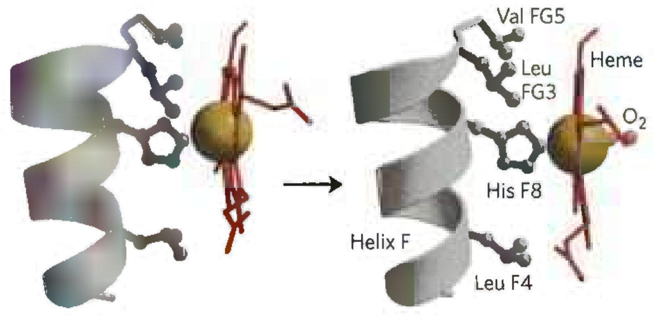
P4 X-ray analysis has revealed two major conformations of hemoglobin: the **R state** and the **T state**. Although oxygen binds to hemoglobin in either state, it has a significantly higher affinity for hemoglobin in the R state. Oxygen binding stabilizes the R state. When oxygen is absent experimentally, the T state is more stable and is thus the predominant conformation of **deoxyhemoglobin**. T and R originally denoted "tense" and "relaxed," respectively, because the T state is stabilized by a greater number of ion pairs, many of which lie at the $\alpha_1\beta_2$ (and $\alpha_2\beta_1$) interface (**Fig. 5-9**). The binding of O_2 to a hemoglobin subunit in the T state triggers a change in conformation to the R state. When the entire protein undergoes this transition, the structures of the individual subunits change little, but the $\alpha\beta$ subunit pairs slide past each other and rotate, narrowing the pocket between the β subunits (**Fig. 5-10**). In this process, some of the ion pairs that stabilize the T state are broken and some new ones are formed.

Max Perutz proposed that the T → R transition is triggered by changes in the positions of key amino acid side chains surrounding the heme. In the T state, the porphyrin is slightly puckered, causing the heme iron to protrude somewhat on the proximal His (His F8) side. The binding of O_2 causes the heme to assume a more



T state **R state**

FIGURE 5-10 The T → R transition. In these depictions of deoxyhemoglobin, as in Figure 5-9, the β subunits are blue and the α subunits are gray. Positively charged side chains and chain termini involved in ion pairs are shown in blue, and their negatively charged partners are shown in red. The Lys C5 of each α subunit and the Asp FG1 of each β subunit are visible but are not labeled (compare Fig. 5-9). Note that the molecule is oriented slightly differently than in Figure 5-9. The transition from the T state to the R state shifts the subunit pairs substantially, affecting certain ion pairs. Most noticeably, the His HC3 residues at the carboxyl termini of the β subunits, which are involved in ion pairs in the T state, rotate in the R state toward the center of the molecule, where they are no longer in ion pairs. Another dramatic result of the T → R transition is a narrowing of the pocket between the β subunits. [T state: Data from PDB ID 1HGA, R. Liddington et al., *J. Mol. Biol.* 228:551, 1992. R state: Data from PDB ID 1BBB, M. M. Silva et al., *J. Biol. Chem.* 267:17,248, 1992.]



T state **R state**

FIGURE 5-11 Changes in conformation near heme on O_2 binding to deoxyhemoglobin. The shift in the position of helix F when heme binds O_2 is thought to be one of the adjustments that triggers the T → R transition. [T state: Data from PDB ID 1HGA, R. Liddington et al., *J. Mol. Biol.* 228:551, 1992. R state: Data from PDB ID 1BBB, M. M. Silva et al., *J. Biol. Chem.* 267:17,248, 1992; R state modified to represent O_2 instead of CO.]

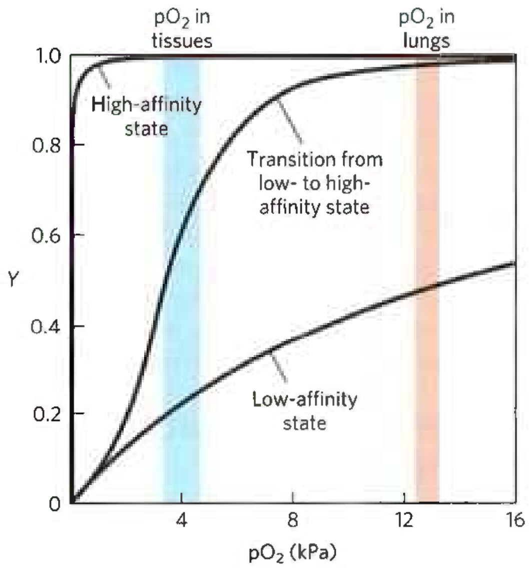


FIGURE 5-12 A sigmoid (cooperative) binding curve. A sigmoid binding curve can be viewed as a hybrid curve reflecting a transition from a low-affinity state to a high-affinity state. Because of its cooperative binding, as manifested by a sigmoid binding curve, hemoglobin is more sensitive to the small differences in O_2 concentration between the tissues and the lungs, allowing it to bind oxygen in the lungs (where pO_2 is high) and release it in the tissues (where pO_2 is low).

planar conformation, shifting the position of the proximal His and the attached F helix (**Fig. 5-11**). These changes lead to adjustments in the ion pairs at the $\alpha_1\beta_2$ interface.

Hemoglobin Binds Oxygen Cooperatively

Hemoglobin must bind oxygen efficiently in the lungs, where the pO_2 is about 13.3 kPa, and it must release oxygen in the tissues, where the pO_2 is about 4 kPa. Myoglobin, or any protein that binds oxygen with a hyperbolic binding curve, would be ill-suited to this function, for the reason illustrated in **Figure 5-12**. A protein that bound O_2 with high affinity would bind it efficiently in the lungs but would not release much of it in the tissues. If the protein bound oxygen with a sufficiently low affinity to release it in the tissues, it would not pick up much oxygen in the lungs.

Hemoglobin solves the problem by undergoing a transition from a low-affinity state (the T state) to a high-affinity state (the R state) as more O_2 molecules are bound. As a result, hemoglobin has a hybrid S-shaped, or sigmoid, binding curve for oxygen (Fig. 5-12). A single-subunit protein with a single ligand-binding site cannot produce a sigmoid binding curve—even if binding elicits a conformational change—because each molecule of ligand binds independently and cannot affect ligand binding to another molecule. P5 In contrast, O_2 binding to individual subunits of hemoglobin can alter the affinity for O_2 in adjacent subunits. The first molecule of O_2 that interacts with deoxyhemoglobin binds weakly, because it binds to a subunit in the T state. Its binding, however, leads to conformational changes that are communicated to adjacent subunits, making it easier for additional molecules of O_2 to bind. In effect, the T → R transition occurs more readily in the second subunit once O_2 is bound to the first subunit. The last (fourth) O_2 molecule binds to a heme in a subunit that is already in the R state, and hence it binds with much higher affinity than the first molecule.

P5 An **allosteric protein** is one in which the binding of a ligand to one site affects the binding properties of another site on the same protein. The term "allosteric" derives from the Greek *allos*, "other," and *stereos*, "solid" or "shape." P6 Allosteric proteins are those having "other shapes," or conformations, induced by the binding of ligands referred to as **modulators**. The conformational changes induced by the modulator(s) interconvert more-active and less-active forms of the protein. The modulators for allosteric proteins may be either inhibitors or activators. When the normal ligand and modulator are identical, the interaction is termed **homotropic**. When the modulator is a molecule other than the normal ligand, the interaction is **heterotropic**. Some proteins have two or more modulators and therefore can have both homotropic and heterotropic interactions.

Cooperative binding of a ligand to a multimeric protein, such as we observe with the binding of O_2 to hemoglobin, is a form of allosteric binding. The binding of one ligand affects the affinities of any remaining unfilled binding sites, and O_2 can be considered as both a ligand and an activating homotropic modulator. There is only one binding site for O_2 on each subunit, so the allosteric effects giving rise to cooperativity are mediated by conformational changes transmitted from one subunit to another by subunit-subunit interactions. A sigmoid binding curve is diagnostic of cooperative binding. It permits a much more sensitive response to ligand concentration and is important to the function of many multisubunit proteins. The principle of allostery extends readily to regulatory enzymes, as we shall see in Chapter 6.

As is the case with myoglobin, ligands other than oxygen can bind to hemoglobin. An important example is carbon monoxide, which binds to hemoglobin about 250 times better than does oxygen (the critical hydrogen bond between O_2 and the distal His is not quite as strong in human hemoglobin as it is in most mammalian myoglobins, so the binding of O_2 relative to CO is not augmented quite as much). Human exposure to CO can have tragic consequences (**Box 5-1**).

Cooperative Ligand Binding Can Be Described Quantitatively

Cooperative binding of oxygen by hemoglobin was first analyzed by Archibald Hill in 1910. From this work came a general approach to the study of cooperative ligand binding to multisubunit proteins.

For a protein with n binding sites, the equilibrium of Equation 5-1 becomes

$$\mathrm{P} + n\mathrm{L} \rightleftharpoons \mathrm{PL}_n \tag{5-12}$$

and the expression for the association constant becomes

$$K_\mathrm{a} = \frac{[\mathrm{PL}_n]}{[\mathrm{P}][\mathrm{L}]^n} \tag{5-13}$$

The expression for Y (see Eqn 5-8) is

$$Y = \frac{[\mathrm{L}]^n}{[\mathrm{L}]^n + K_\mathrm{d}} \tag{5-14}$$

Rearranging, then taking the log of both sides, yields

$$\frac{Y}{1-Y} = \frac{[\mathrm{L}]^n}{K_\mathrm{d}} \tag{5-15}$$

$$\log\left(\frac{Y}{1-Y}\right) = n\log[\mathrm{L}] - \log K_\mathrm{d} \tag{5-16}$$

where $K_\mathrm{d} = [\mathrm{L}]^n_{0.5}$

Equation 5-16 is the **Hill equation**, and a plot of log $[Y/(1-Y)]$ versus log [L] is called a **Hill plot**. Based on the equation, the Hill plot should have a slope of n. However, the experimentally determined slope actually reflects not the number of binding sites but the degree of interaction between them. The slope of a Hill plot is therefore denoted by n_H, the **Hill coefficient**, which is a measure of the degree of cooperativity. If n_H equals 1, ligand binding is not cooperative, a situation that can arise even in a multisubunit protein if the subunits do not communicate. An n_H of greater than 1 indicates positive cooperativity in ligand binding. This is the situation observed in hemoglobin, in which the binding of one molecule of ligand facilitates the binding of others. The theoretical upper limit for n_H is reached when $n_\mathrm{H} = n$. In this case the binding would be completely cooperative: all binding sites on the protein would bind ligand simultaneously, and no protein molecules partially saturated with ligand would be present under any conditions. This limit is never reached in practice, and the measured value of n_H is always less than the actual number of ligand-binding sites in the protein.

An n_H of less than 1 indicates negative cooperativity, in which the binding of one molecule of ligand *impedes*

BOX 5-1 MEDICINE

Carbon Monoxide: A Stealthy Killer

Summer, 2017. A Florida man drove his late-model SUV into his garage, took his wireless key fob with him inside the house, and went to bed. He was found, days later, dead from carbon monoxide poisoning. The man had assumed that removing the fob was enough to shut down the automobile. Instead, the car had idled in the garage until it ran out of gas, ultimately filling the attached house with CO. Since 2006, several dozen deaths and scores of injuries have resulted from similar incidents. Measures to address the safety issues posed by keyless ignitions, now standard in most new cars, have lagged. Meanwhile, the deaths are a vivid reminder that life depends on the reversibility of protein-ligand interactions.

Carbon monoxide (CO), a colorless, odorless gas, is one of the most common known causes of death due to poisoning (in countries where such records are kept). CO has an approximately 250-fold greater affinity for hemoglobin than does oxygen. Consequently, relatively low levels of CO can have substantial and tragic effects. When CO combines with hemoglobin, the complex is referred to as carboxyhemoglobin, or COHb.

Some CO is produced by natural processes, but locally high levels generally result only from human activities. Engine and furnace exhausts are important sources, as CO is a byproduct of the incomplete combustion of fossil fuels. In the United States alone, nearly 4,000 people succumb to CO poisoning each year, both accidentally and intentionally. Many of the accidental deaths involve undetected CO buildup in enclosed spaces. This might occur when a household furnace malfunctions, or a gas generator is operated indoors during a power outage or shutoff, venting CO into a home. CO poisoning can also occur in open spaces, as unsuspecting people at work or play inhale the exhaust from generators, outboard motors, tractor engines, recreational vehicles, or lawn mowers.

Carbon monoxide levels in the atmosphere are rarely dangerous, ranging from less than 0.05 part per million (ppm) in remote and uninhabited areas to 3 to 4 ppm in some cities of the Northern Hemisphere. In the United States, the government-mandated (Occupational Safety and Health Administration, OSHA) limit for CO at worksites is 35 ppm for people working an eight-hour shift. The tight binding of CO to hemoglobin means that COHb can accumulate over time as people are exposed to a constant low-level source of CO.

In an average, healthy individual, 1% or less of the total hemoglobin is complexed as COHb. Since CO is a product of tobacco smoke, many smokers have COHb levels in the range of 3% to 8% of total hemoglobin, and the levels can rise to 15% for chain-smokers. COHb levels equilibrate at 50% in people who breathe air containing 570 ppm of CO for several hours. Reliable methods have been developed that relate CO content in the atmosphere to COHb levels in the blood (Fig. 1). CO levels in the home of the Florida victim exceeded OSHA-mandated limits by more than 30-fold.

How is a human affected by COHb? At levels of less than 10% of total hemoglobin, symptoms are rarely observed. At 15%, the individual experiences mild headaches. At 20% to 30%, the headache is severe and is generally accompanied by

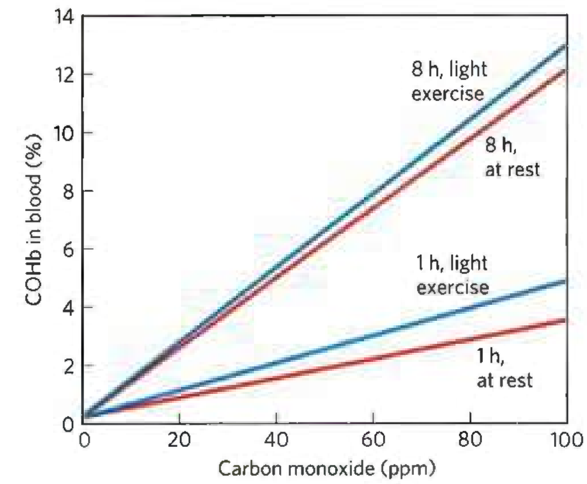


FIGURE 1 Relationship between levels of COHb in blood and concentration of CO in the surrounding air. Four different conditions of exposure are shown, comparing the effects of short exposure versus extended exposure, and exposure at rest versus exposure during light exercise. [Data from R. F. Coburn et al., *J. Clin. Invest.* 44:1899, 1965.]

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the binding of others. Well-documented cases of negative cooperativity are rare.

To adapt the Hill equation to the binding of oxygen to hemoglobin, we must again substitute $\mathrm{pO_2}$ for [L] and P_{50}^n for K_d:

$$\log\left(\frac{Y}{1-Y}\right) = n\log \mathrm{pO_2} - n\log P_{50} \qquad (5\text{-}17)$$

Hill plots for myoglobin and hemoglobin are given in **Figure 5-13**.

Two Models Suggest Mechanisms for Cooperative Binding

In a multisubunit ligand-binding protein like hemoglobin, how does the T → R transition occur? Two models for the cooperative binding of ligands to proteins with multiple binding sites have greatly influenced thinking about this problem, defining the two major possibilities.

The first model was proposed by Jacques Monod, Jeffries Wyman, and Jean-Pierre Changeux in 1965, and is called the **MWC model** or the **concerted model** (**Fig. 5-14a**). The concerted model assumes that the nausea, dizziness, confusion, disorientation, and some visual disturbances; these symptoms are generally reversed if the individual is treated with oxygen. At COHb levels of 30% to 50%, the neurological symptoms become more severe, and at levels near 50%, the individual loses consciousness and can sink into coma. Respiratory failure may follow. With prolonged exposure, some damage becomes permanent. Death normally occurs when COHb levels rise above 60%.

Binding of CO to hemoglobin is affected by many factors, including exercise (Fig. 1) and changes in air pressure related to altitude. Because of their higher base levels of COHb, smokers exposed to a source of CO often develop symptoms faster than nonsmokers. Individuals with heart, lung, or blood diseases that reduce the availability of oxygen to tissues may also experience symptoms at lower levels of CO exposure. Fetuses are at particular risk for CO poisoning. Fetal hemoglobin, with γ subunits replacing β subunits, has a somewhat higher affinity for CO than adult hemoglobin (see p. 148). Cases of CO exposure have been recorded in which the fetus died but the pregnant woman recovered.

It may seem surprising that the loss of half of one's hemoglobin to COHb can prove fatal — we know that people with any of several anemic conditions manage to function reasonably well with half the usual complement of active hemoglobin. However, the binding of CO to hemoglobin does more than remove protein from the pool available to bind oxygen. It also affects the affinity of the remaining hemoglobin subunits for oxygen. As CO binds to one or two subunits of a hemoglobin tetramer, the affinity for O$_2$ is increased substantially in the remaining subunits (Fig. 2). Thus, a hemoglobin tetramer with two bound CO molecules can efficiently bind O$_2$ in the lungs — but it releases very little of it in the tissues. Oxygen deprivation in the tissues rapidly becomes severe. To add to the problem, the effects of CO are not limited to interference with hemoglobin function. CO binds to other heme proteins and a variety of metalloproteins. The effects of these interactions are not yet well understood, but they may be responsible for some of the longer-term effects of acute but nonfatal CO poisoning.

When CO poisoning is suspected, rapid removal of the person from the CO source is essential, but this does not always result in rapid recovery. When an individual is moved from the CO-polluted site to a normal, outdoor atmosphere, O$_2$ begins to replace the CO in hemoglobin — but the COHb level drops only slowly. The half-time is 2 to 6.5 hours, depending on individual and environmental factors. If 100% oxygen is administered with a mask, the rate of exchange can be increased about fourfold; the half-time for O$_2$–CO exchange can be reduced to tens of minutes if 100% oxygen at a pressure of 3 atm (303 kPa) is supplied. Thus, rapid treatment by a properly equipped medical team is critical.

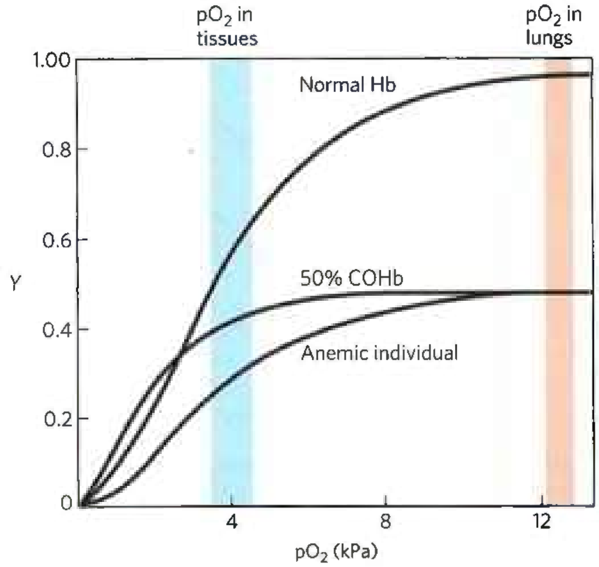


FIGURE 2 Several oxygen-binding curves: for normal hemoglobin, for hemoglobin from an anemic individual with only 50% of her hemoglobin functional, and for hemoglobin from an individual with 50% of his hemoglobin subunits complexed with CO. The pO$_2$ in human lungs and tissues is indicated. [Data from F. J. W. Roughton and R. C. Darling, *Am. J. Physiol.* 141:17, 1944.]

A home carbon monoxide detector is a simple and inexpensive measure to avoid possible tragedy. After completing the research for this box, we immediately purchased several new CO detectors for our homes.

subunits of a cooperatively binding protein are functionally identical, that each subunit can exist in (at least) two conformations, and that all subunits undergo the transition from one conformation to the other simultaneously. In this model, no protein has individual subunits in different conformations. The two conformations are in equilibrium. The ligand can bind to either conformation, but it binds much more tightly to the R state. **P5** Successive binding of ligand molecules to the low-affinity T state (which is more stable in the absence of ligand) gradually shifts the equilibrium to favor the R state.

In the second model, the **sequential model** (Fig. 5-14b), proposed in 1966 by Daniel Koshland and colleagues, ligand binding can induce a change of conformation in an individual subunit. A conformational change in one subunit makes a similar change in an adjacent subunit more likely, and makes the binding of a second ligand molecule more likely as well. There are more potential intermediate states in this model than in the concerted model. The two models are not mutually exclusive; the concerted model may be viewed as the "all-or-none" limiting case of the sequential model.

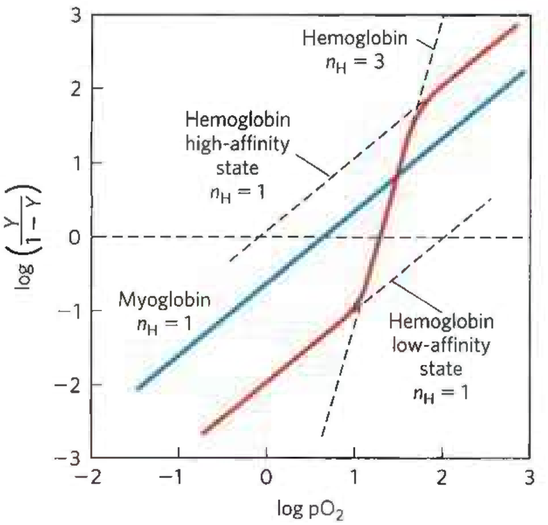


FIGURE 5-13 Hill plots for oxygen binding to myoglobin and hemoglobin. When $n_H = 1$, there is no evident cooperativity. The maximum degree of cooperativity observed for hemoglobin corresponds approximately to $n_H = 3$. Note that while this indicates a high level of cooperativity, n_H is less than n, the number of O_2-binding sites in hemoglobin. This is normal for a protein that exhibits allosteric binding behavior.

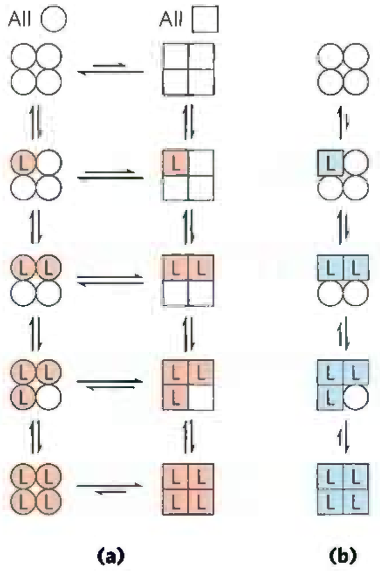


FIGURE 5-14 Two general models for the interconversion of inactive and active forms of a protein during cooperative ligand binding. Although the models may be applied to any protein—including any enzyme (Chapter 6)—that exhibits cooperative binding, here we show four subunits because the model was originally proposed for hemoglobin. (a) In the concerted, or all-or-none, model (MWC model), all subunits are postulated to be in the same conformation, either all ○ (low affinity or inactive) or all □ (high affinity or active). Depending on the equilibrium, K_{eq}, between ○ and □ forms, the binding of one or more ligand molecules (L) will pull the equilibrium toward the □ form. Subunits with bound L are shaded. (b) In the sequential model, each individual subunit can be in either the ○ form or the □ form. The equilibrium is altered as additional ligands are bound, progressively favoring the R state.

Unfortunately, the two models have proven very difficult to distinguish experimentally. In Chapter 6 we use these models to investigate allosteric enzymes.

Hemoglobin Also Transports H^+ and CO_2

In addition to carrying nearly all the oxygen required by cells from the lungs to the tissues, hemoglobin carries two end products of cellular respiration—H^+ and CO_2—from the tissues to the lungs and the kidneys, where they are excreted. The CO_2, produced by oxidation of organic fuels in mitochondria, is hydrated to form bicarbonate:

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

This reaction is catalyzed by **carbonic anhydrase**, an enzyme particularly abundant in erythrocytes. Carbon dioxide is not very soluble in aqueous solution, and bubbles of CO_2 would form in the tissues and blood if it were not converted to bicarbonate. As you can see from the reaction catalyzed by carbonic anhydrase, the hydration of CO_2 results in an increase in the H^+ concentration (a decrease in pH) in the tissues. The binding of oxygen by hemoglobin is profoundly influenced by pH and CO_2 concentration, so the interconversion of CO_2 and bicarbonate is of great importance to the regulation of oxygen binding and release in the blood.

Hemoglobin transports about 40% of the total H^+ and 15% to 20% of the CO_2 formed in the tissues to the lungs and kidneys. (The remainder of the H^+ is absorbed by the plasma's bicarbonate buffer; the remainder of the CO_2 is transported as dissolved HCO_3^- and CO_2.) The structural effects of H^+ and CO_2 binding to hemoglobin favor the T state. P6 Thus, the binding of H^+ and CO_2 is inversely related to the binding of oxygen. At the relatively low pH and high CO_2 concentration of peripheral tissues, the affinity of hemoglobin for oxygen decreases as H^+ and CO_2 are bound, and O_2 is released to the tissues. Conversely, in the capillaries of the lung, as CO_2 is excreted and the blood pH consequently rises, the affinity of hemoglobin for oxygen increases and the protein binds more O_2 for transport to the peripheral tissues. This effect of pH and CO_2 concentration on the binding and release of oxygen by hemoglobin is called the **Bohr effect**, after Christian Bohr, the Danish physiologist (and father of physicist Niels Bohr) who discovered it in 1904.

A complete statement of the binding equilibrium for hemoglobin and one molecule of oxygen can be designated by the reaction

$$HHb^+ + O_2 \rightleftharpoons HbO_2 + H^+$$

where HHb^+ denotes a protonated form of hemoglobin. This equation tells us that the O_2-saturation curve of hemoglobin is influenced by the H^+ concentration

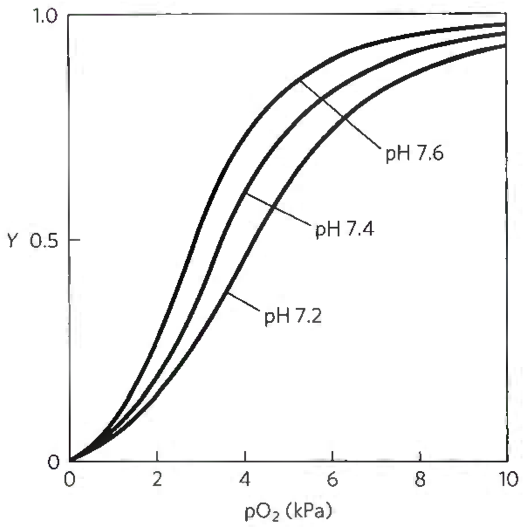
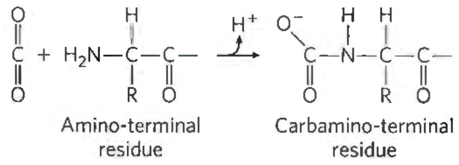


FIGURE 5-15 **Effect of pH on oxygen binding to hemoglobin.** The pH of blood is 7.6 in the lungs and 7.2 in the tissues. Experimental measurements on hemoglobin binding are often performed at pH 7.4.

(**Fig. 5-15**). Both O_2 and H^+ are bound by hemoglobin, but with inverse affinity. When the oxygen concentration is high, as in the lungs, hemoglobin binds O_2 and releases protons. When the oxygen concentration is low, as in the peripheral tissues, H^+ is bound and O_2 is released.

Oxygen and H^+ are not bound at the same sites in hemoglobin. Oxygen binds to the iron atoms of the hemes, whereas H^+ binds to any of several amino acid residues in the protein. A major contribution to the Bohr effect is made by His^{146} (His HC3) of the β subunits. When protonated, this residue forms one of the ion pairs—to Asp^{94} (Asp FG1)—that helps stabilize deoxyhemoglobin in the T state (Fig. 5-9). Protonation of the amino-terminal residues of the α subunits, certain other His residues, and perhaps other groups has a similar effect.

Hemoglobin also binds CO_2, again in a manner inversely related to the binding of oxygen. Carbon dioxide binds as a carbamate group to the α-amino group at the amino-terminal end of each globin chain, forming carbaminohemoglobin:

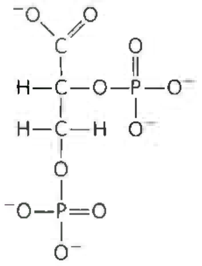


This reaction produces H^+, contributing to the Bohr effect. The bound carbamates also form additional salt bridges (not shown in Fig. 5-9) that help to stabilize the T state and promote the release of oxygen.

When the concentration of carbon dioxide is high, as in peripheral tissues, some CO_2 binds to hemoglobin and the affinity for O_2 decreases, causing its release. Conversely, when hemoglobin reaches the lungs, the high oxygen concentration promotes binding of O_2 and release of CO_2. It is the capacity to communicate ligand-binding information from one polypeptide subunit to the others that makes the hemoglobin molecule so beautifully adapted to integrating the transport of O_2, CO_2, and H^+ by erythrocytes.

Oxygen Binding to Hemoglobin Is Regulated by 2,3-Bisphosphoglycerate

P6 The interaction of **2,3-bisphosphoglycerate (BPG)** with hemoglobin molecules further refines the function of hemoglobin, and provides an example of heterotropic allosteric modulation.



2,3-Bisphosphoglycerate

BPG binds at a site distant from the oxygen-binding site and regulates the O_2-binding affinity of hemoglobin in relation to the pO_2 in the lungs. BPG is present in relatively high concentrations in erythrocytes. When hemoglobin is isolated, it contains substantial amounts of bound BPG, which can be difficult to remove completely. In fact, the O_2-binding curves for hemoglobin that we have examined up to this point were obtained in the presence of bound BPG. 2,3-Bisphosphoglycerate greatly reduces the affinity of hemoglobin for oxygen—there is an inverse relationship between the binding of O_2 and the binding of BPG. We can therefore describe another binding process for hemoglobin:

$$\text{HbBPG} + O_2 \rightleftharpoons \text{HbO}_2 + \text{BPG}$$

BPG is important in the physiological adaptation to the lower pO_2 at high altitudes. For a healthy human at sea level, the binding of O_2 to hemoglobin is regulated such that the amount of O_2 delivered to the tissues is nearly 40% of the maximum that could be carried by the blood (**Fig. 5-16**). Imagine that this person is suddenly transported from sea level to an altitude of 4,500 meters, where the pO_2 is considerably lower. The delivery of O_2 to the tissues is now reduced. However, after just a few hours at the higher altitude, the BPG concentration in the blood has begun to rise, leading to a decrease in the

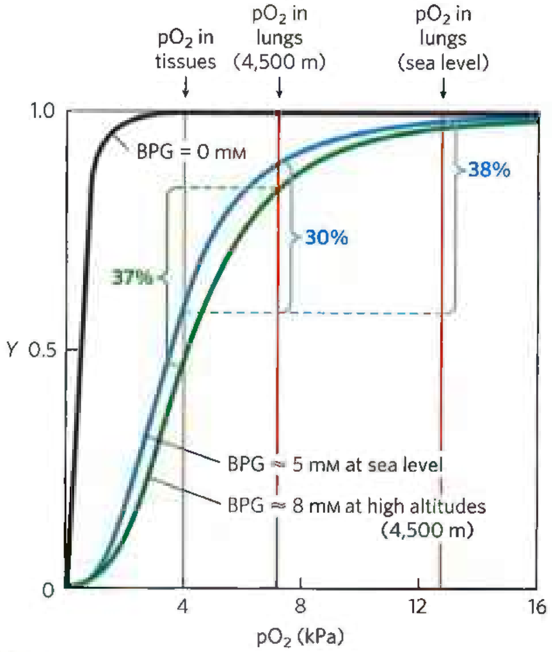
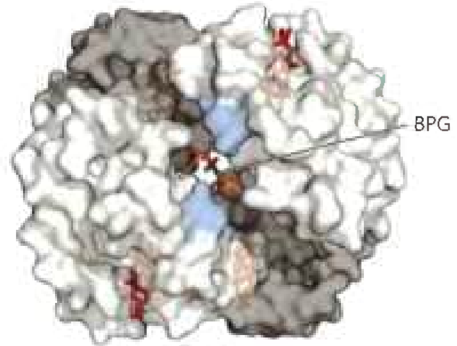
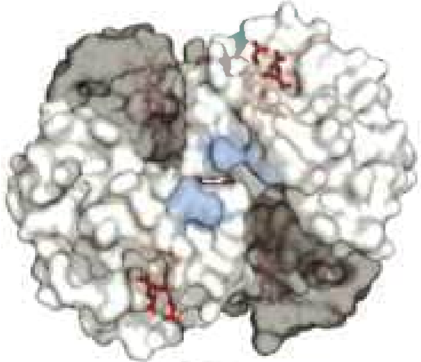


FIGURE 5-16 Effect of 2,3-bisphosphoglycerate on oxygen binding to hemoglobin. The BPG concentration in normal human blood is about 5 mM at sea level and about 8 mM at high altitudes. Note that hemoglobin binds to oxygen quite tightly when BPG is entirely absent, and the binding curve seems to be hyperbolic. In reality, the measured Hill coefficient for O_2-binding cooperativity decreases only slightly (from 3 to about 2.5) when BPG is removed from hemoglobin, but the rising part of the sigmoid binding curve is confined to a very small region close to the origin. At sea level, hemoglobin is nearly saturated with O_2 in the lungs, but is just over 60% saturated in the tissues, so the amount of O_2 released in the tissues is about 38% of the maximum that can be carried in the blood. At high altitudes, O_2 delivery declines by about one-fourth, to 30% of maximum. An increase in BPG concentration, however, decreases the affinity of hemoglobin for O_2, so approximately 37% of what can be carried is again delivered to the tissues.



T state
(a)



R state
(b)

FIGURE 5-17 Binding of 2,3-bisphosphoglycerate to deoxyhemoglobin. (a) BPG binding stabilizes the T state of deoxyhemoglobin. The negative charges of BPG interact with several positively charged groups (shown in blue in this surface contour image) that surround the pocket between the β subunits on the surface of deoxyhemoglobin in the T state. (b) The binding pocket for BPG disappears on oxygenation, following transition to the R state. (Compare with Fig. 5-10.) [Data from (a) PDB ID 1B86, V. Richard et al., *J. Mol. Biol.* 233:270, 1993; (b) PDB ID 1BBB, M. M. Silva et al., *J. Biol. Chem.* 267:17,248, 1992.]

affinity of hemoglobin for oxygen. This adjustment in the BPG level has only a small effect on the binding of O_2 in the lungs, but it has a considerable effect on the release of O_2 in the tissues. As a result, the delivery of oxygen to the tissues is restored to nearly 40% of the O_2 that can be transported by the blood. The situation is reversed when the person returns to sea level. The BPG concentration in erythrocytes also increases in people suffering from **hypoxia**, lowered oxygenation of peripheral tissues due to inadequate functioning of the lungs or circulatory system.

The site of BPG binding to hemoglobin is the cavity between the β subunits in the T state (**Fig. 5-17**). This cavity is lined with positively charged amino acid residues that interact with the negatively charged groups of BPG. Unlike O_2, only one molecule of BPG is bound to each hemoglobin tetramer. BPG lowers hemoglobin's affinity for oxygen by stabilizing the T state. In the absence of BPG, hemoglobin is primarily present in the R state, where it binds O_2 efficiently in the lungs but fails to release it in the tissues.

Regulation of oxygen binding to hemoglobin by BPG has an important role in fetal development. Because a fetus must extract oxygen from its mother's blood, fetal hemoglobin must have greater affinity than the maternal hemoglobin for O_2. The fetus synthesizes γ subunits rather than β subunits, forming $\alpha_2\gamma_2$ hemoglobin. This tetramer has a much lower affinity for BPG than normal adult hemoglobin, and a correspondingly higher affinity for O_2.

Sickle Cell Anemia Is a Molecular Disease of Hemoglobin

The hereditary human disease sickle cell anemia demonstrates strikingly the importance of amino acid sequence in determining the secondary, tertiary, and quaternary structures of globular proteins, and thus their biological functions. Almost 500 genetic variants of hemoglobin are known to occur in the human population; all but a few are quite rare. Most variations consist of differences in a single amino acid residue. The effects on hemoglobin structure and function are often minor but

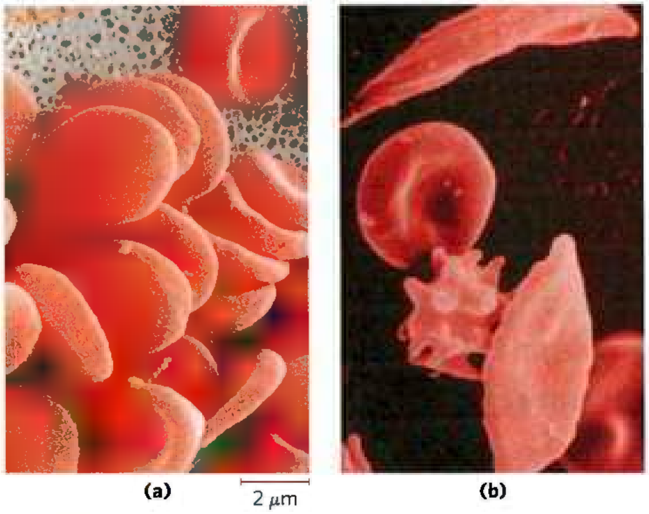


FIGURE 5-18 A comparison of (a) uniform, cup-shaped, normal erythrocytes and (b) the variably shaped erythrocytes seen in sickle cell anemia, which range from normal to spiny or sickle-shaped. [(a) A. Syred/Science; (b) Jackie Lewin, Royal Free Hospital/Science Source.]

can sometimes be extraordinary. Each hemoglobin variation is the product of an altered gene. Variant genes are called alleles. Because humans generally have two copies of each gene, an individual may have two copies of one allele (thus being homozygous for that gene) or one copy of each of two different alleles (thus heterozygous).

Sickle cell anemia occurs in individuals who inherit the allele for sickle cell hemoglobin from both parents. The erythrocytes of these individuals are fewer and also abnormal. In addition to an unusually large number of immature cells, the blood contains many long, thin, sickle-shaped erythrocytes (**Fig. 5-18**). When hemoglobin from sickle cells (called hemoglobin S, or HbS) is deoxygenated, it becomes insoluble and forms polymers that aggregate into tubular fibers (**Fig. 5-19**). Normal hemoglobin (hemoglobin A, or HbA) remains soluble upon deoxygenation. The insoluble fibers of deoxygenated HbS cause the deformed, sickle shape of the erythrocytes, and the proportion of sickled cells increases greatly as blood is deoxygenated.

The altered properties of HbS result from a single amino acid substitution, a Val instead of a Glu residue at position 6 in the two β chains. Replacement of the Glu residue by Val creates a "sticky" hydrophobic contact point at position 6 of the β chain, which is on the outer surface of the molecule. These sticky spots cause deoxyHbS molecules to associate abnormally with each other, forming the long, fibrous aggregates characteristic of this disorder.

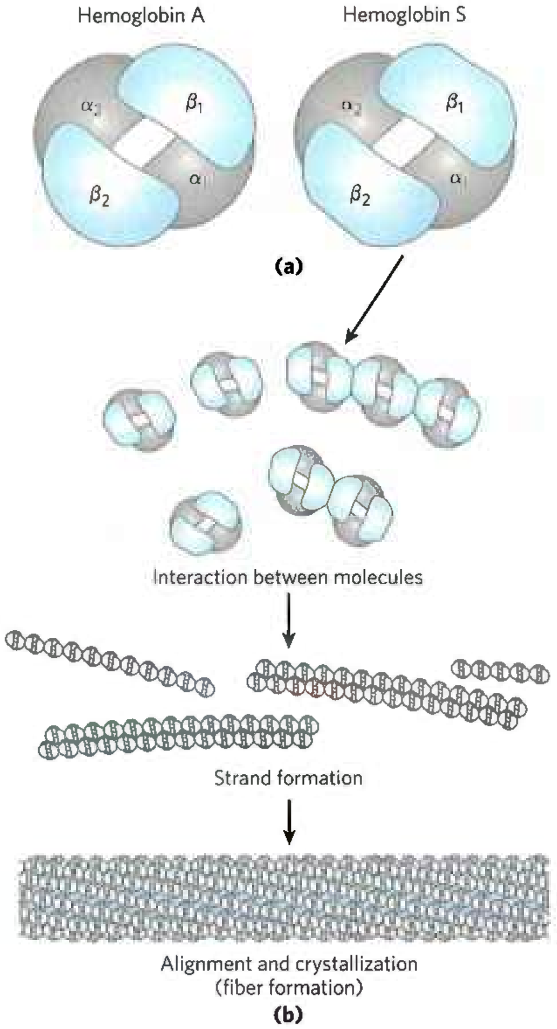


FIGURE 5-19 Normal and sickle cell hemoglobin. (a) Subtle differences between the conformations of HbA and HbS result from a single amino acid change in the β chains. (b) As a result of this change, deoxyHbS has a hydrophobic patch on its surface, which causes the molecules to aggregate into strands that align into insoluble fibers.

Sickle cell anemia is life-threatening and painful. People with this disease suffer repeated crises brought on by physical exertion. They become weak, dizzy, and short of breath, and they also experience heart murmurs and an increased pulse rate. The hemoglobin content of their blood is only about half the normal value of 15 to 16 g/100 mL, because sickled cells are very fragile and rupture easily; this results in anemia ("lack of blood"). An even more serious consequence is that capillaries become blocked by the long, abnormally shaped cells, causing severe pain and interfering with normal organ function—a major factor in the early death of many people with the disease.

Without medical treatment, people with sickle cell anemia usually die in childhood. Curiously, the frequency of the sickle cell allele in populations is unusually high in certain parts of Africa. Investigation into this matter led to the finding that when heterozygous, the allele confers a small but significant resistance to lethal forms of malaria. The heterozygous individuals experience a milder condition called sickle cell trait; only about 1% of their erythrocytes become sickled on deoxygenation. These individuals may live completely normal lives if they avoid vigorous exercise and other stresses on the circulatory system. Natural selection has resulted in an allele population that balances the deleterious effects of the homozygous condition against the resistance to malaria afforded by the heterozygous condition. ■

SUMMARY 5.1 *Reversible Binding of a Protein to a Ligand: Oxygen-Binding Proteins*

- Protein function often entails interactions with other molecules. A protein binds a molecule, known as a ligand, at its binding site.
- Myoglobin contains a heme prosthetic group, which binds oxygen. Heme consists of a single atom of Fe^{2+} coordinated within a porphyrin.
- Globins are a specialized family of transport proteins containing heme; most globins store oxygen.
- Oxygen binds to myoglobin reversibly.
- Reversible binding of a ligand to a protein can be described quantitatively by a dissociation constant K_d. For a monomeric protein such as myoglobin, the fraction of binding sites occupied by a ligand is a hyperbolic function of ligand concentration.
- Proteins may undergo conformational changes when a ligand binds, a process called induced fit. In a multisubunit protein, the binding of a ligand to one subunit may affect ligand binding to other subunits. Ligand binding can be regulated.
- Hemoglobin transports oxygen in blood.
- Normal adult hemoglobin has four heme-containing subunits, two α and two β, similar in structure to each other and to myoglobin.
- Hemoglobin exists in two interchangeable structural states, T and R. The T state is most stable when oxygen is not bound. Oxygen binding promotes transition to the R state.
- Oxygen binding to hemoglobin is both allosteric and cooperative. As O_2 binds to one binding site, the hemoglobin undergoes conformational changes that affect the other binding sites—an example of allosteric behavior.
- Conformational changes between the T and R states, mediated by subunit-subunit interactions, result in cooperative binding; this is described by a sigmoid binding curve and can be analyzed by a Hill plot.
- Two major models have been proposed to explain the cooperative binding of ligands to multisubunit proteins: the concerted model and the sequential model.
- Hemoglobin also binds H^+ and CO_2, resulting in the formation of ion pairs that stabilize the T state and lessen the protein's affinity for O_2 (the Bohr effect).
- Oxygen binding to hemoglobin is also modulated by 2,3-bisphosphoglycerate, which binds to and stabilizes the T state.
- Sickle cell anemia is a genetic disease caused by a single amino acid substitution (Glu^6 to Val^6) in each β chain of hemoglobin. The change produces a hydrophobic patch on the surface of the hemoglobin that causes the molecules to aggregate into bundles of fibers. This homozygous condition results in serious medical complications.

5.2 Complementary Interactions between Proteins and Ligands: The Immune System and Immunoglobulins

We have seen how the conformations of oxygen-binding proteins affect and are affected by the binding of small ligands (O_2 or CO) to the heme group. However, most protein-ligand interactions do not involve a prosthetic group. P2 Instead, the binding site for a ligand is more often like the hemoglobin binding site for BPG—a cleft in the protein lined with amino acid residues, arranged to make the binding interaction highly specific. Effective discrimination between ligands is the norm at binding sites, even when the ligands have only minor structural differences.

Almost all organisms have an immune system of some type that allows them to respond to challenges presented by environmental pathogens. The emergence of vertebrates about 500 million years ago was accompanied by the evolution of an adaptive immune system based on the generation of large numbers of distinct cell clones, each expressing a protein variant that could recognize and bind to a particular type of chemical structure. The system distinguishes molecular "self" from "nonself" and then destroys what is identified as nonself. In this way, the immune system eliminates viruses, bacteria, and other pathogens and molecules that may pose a threat to the organism. On a physiological level, the **immune response** is an intricate and coordinated set of interactions among many classes of proteins, molecules, and cell types. At the level of individual proteins, the immune response demonstrates how an acutely sensitive and specific biochemical system is built upon the reversible binding of ligands to proteins.

The Immune Response Includes a Specialized Array of Cells and Proteins

Immunity is brought about by a variety of **leukocytes** (white blood cells), including **macrophages** and **lymphocytes**, all of which develop from undifferentiated stem cells in the bone marrow. Leukocytes can leave the bloodstream and patrol the tissues, each cell producing one or more proteins capable of recognizing and binding to molecules that might signal an infection.

The immune response consists of two complementary systems, the humoral and cellular immune systems. The **humoral immune system** (Latin *humor*, "fluid") is directed at bacterial infections and extracellular viruses (those found in the body fluids), but it can also respond to individual foreign proteins. The **cellular immune system** destroys host cells infected by viruses and also destroys some parasites and foreign tissues.

At the heart of the humoral immune response are soluble proteins called **antibodies** or **immunoglobulins**, often abbreviated **Ig**. Immunoglobulins bind bacteria, viruses, or large molecules identified as foreign and target them for destruction. Making up 20% of blood protein, the immunoglobulins are produced by **B lymphocytes**, or **B cells**, so named because they complete their development in the *b*one marrow.

The agents at the heart of the cellular immune response are a class of **T lymphocytes**, or **T cells** (so called because the latter stages of their development occur in the *t*hymus), known as **cytotoxic T cells (T_C cells**, also called killer T cells). Recognition of infected cells or parasites involves proteins called **T-cell receptors** on the surface of T_C cells. Receptors are proteins, usually found on the outer surface of cells and extending through the plasma membrane, that recognize and bind extracellular ligands, thus triggering changes inside the cell.

In addition to cytotoxic T cells, there are **helper T cells (T_H cells)**, whose function it is to produce soluble signaling proteins called cytokines, which include the interleukins. T_H cells interact with macrophages. The T_H cells participate only indirectly in the destruction of infected cells and pathogens, stimulating the selective proliferation of those T_C and B cells that can bind to a particular antigen. This process, called **clonal selection**, increases the number of immune system cells that can respond to a particular pathogen. A host organism needs time, often days, to mount an immune response against a new antigen, but **memory cells** permit a rapid response to pathogens previously encountered. A vaccine to protect against a particular viral infection often consists of weakened or killed virus or isolated proteins from a viral or bacterial protein coat. When injected into a person, the vaccine generally does not cause an infection and illness, but it effectively "teaches" the immune system what the viral particles look like, stimulating the production of memory cells. On subsequent infection, these cells can bind to the virus and trigger a rapid immune response.

The importance of T_H cells is dramatically illustrated by the epidemic produced by HIV (human immunodeficiency virus), the virus that causes AIDS (acquired immune deficiency syndrome). T_H cells are the primary targets of HIV infection; elimination of these cells progressively incapacitates the entire immune system.

P1 Each recognition protein of the immune system, either a T-cell receptor or an antibody produced by a B cell, specifically binds some particular chemical structure, distinguishing it from virtually all others. Humans are capable of producing more than 10^8 different antibodies with distinct binding specificities. Given this extraordinary diversity, any chemical structure on the surface of a virus or an invading cell will most likely be recognized and bound by one or more antibodies. Antibody diversity is derived from random reassembly of a set of immunoglobulin gene segments through genetic recombination mechanisms that are discussed in Chapter 25 (see Fig. 25-42).

A specialized lexicon is used to describe the unique interactions between antibodies or T-cell receptors and the molecules they bind. Any molecule or pathogen capable of eliciting an immune response is called an **antigen**. An antigen may be a virus, a bacterial cell wall, or an individual protein or other macromolecule. A complex antigen may be bound by several different antibodies. An individual antibody or T-cell receptor binds only a particular molecular structure within the antigen, called its **antigenic determinant** or **epitope**.

It would be unproductive for the immune system to respond to small molecules that are common intermediates and products of cellular metabolism. Molecules of $M_r < 5{,}000$ are generally not antigenic. However, when small molecules are covalently attached to large proteins in the laboratory, they can be used to elicit an immune response. These small molecules are called **haptens**. The antibodies produced in response to protein-linked haptens will then bind to the same small molecules in their free form. Such antibodies are sometimes used in the development of analytical tests described later in this chapter or as a specific ligand in affinity chromatography (see Fig. 3-17c). We now turn to a more detailed description of antibodies and their binding properties.

Antibodies Have Two Identical Antigen-Binding Sites

Immunoglobulin G (IgG) is the major class of antibody molecule and one of the most abundant proteins in the blood serum. IgG has four polypeptide chains: two large ones, called heavy chains, and two smaller ones, called light chains, linked by noncovalent and disulfide bonds into a complex of M_r 150,000. The heavy chains of an IgG molecule interact at one end, then branch to interact separately with the light chains, forming a Y-shaped molecule (**Fig. 5-20**). At the "hinges" separating the base of an IgG molecule from its branches, the immunoglobulin can be cleaved with proteases. Cleavage with the protease papain liberates the basal fragment, called **Fc** because it usually *c*rystallizes readily, and the two branches, called **Fab**, the *a*ntigen-*b*inding fragments. Each branch has a single antigen-binding site.

The fundamental structure of immunoglobulins was first established by Gerald Edelman and Rodney Porter in the 1960s. Each chain is made up of identifiable domains. Some are constant in sequence and structure from one IgG to the next; others are variable. The constant domains have a characteristic structure known as the **immunoglobulin fold**, a well-conserved structural motif in the all-β class of proteins (Chapter 4). There are three of these constant domains in each heavy chain and one in

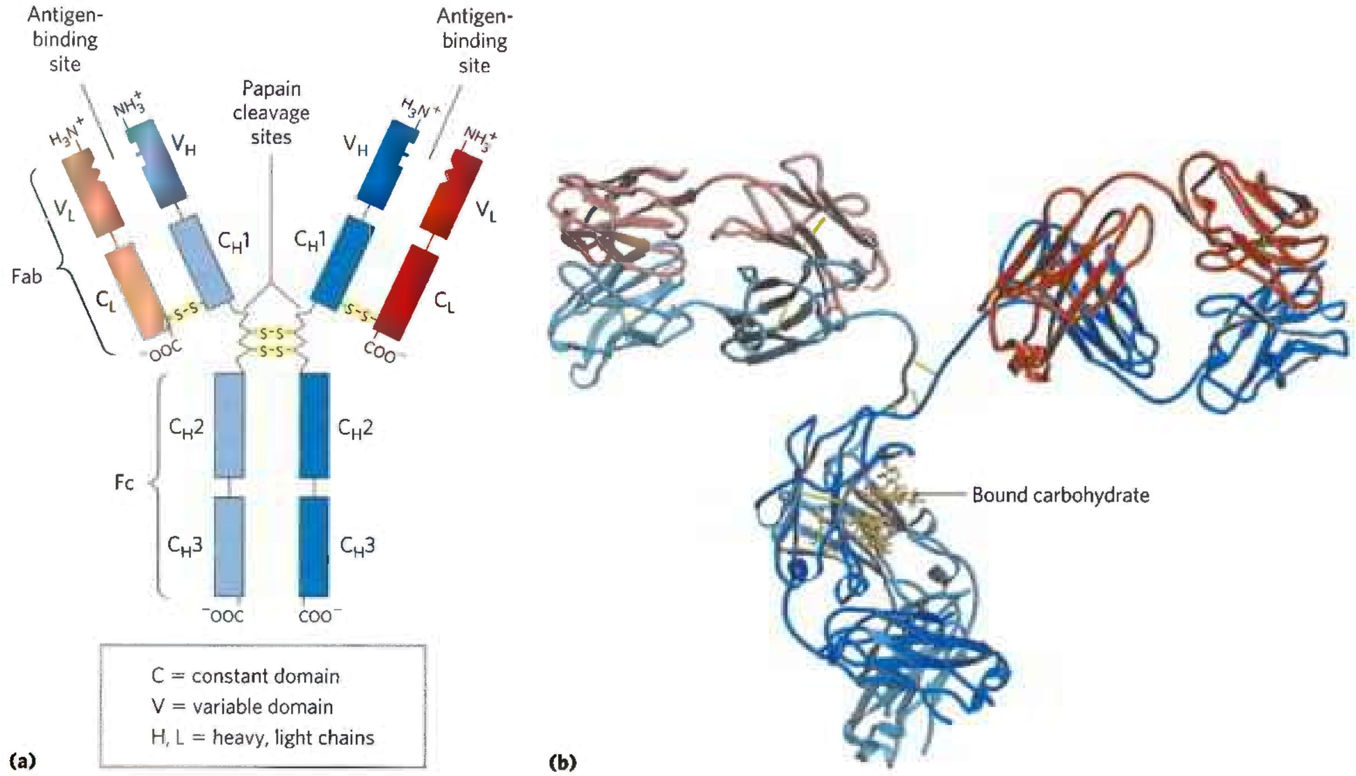


FIGURE 5-20 Immunoglobulin G. (a) Pairs of heavy and light chains combine to form a Y-shaped molecule. Two antigen-binding sites are formed by the combination of variable domains from one light (V_L) chain and one heavy (V_H) chain. Cleavage with papain separates the Fab and Fc portions of the protein in the hinge region. The Fc portion also contains bound carbohydrate (shown in (b)). (b) A ribbon model of an IgG molecule. Although the molecule has two identical heavy chains (two shades of blue) and two identical light chains (two shades of red), it crystallized in the asymmetric conformation shown here. Conformational flexibility is important to the function of immunoglobulins. [(b) Data from PDB ID 1IGT, L. J. Harris et al., *Biochemistry* 36:1581, 1997.]

each light chain. The heavy and light chains also have one variable domain each, in which most of the variability in amino acid sequence is found. The variable domains associate to create the antigen-binding site (Fig. 5-20), allowing formation of an antigen-antibody complex (**Fig. 5-21**).

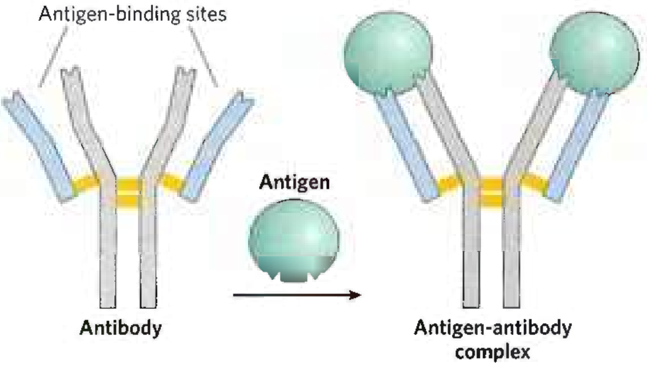


FIGURE 5-21 Binding of IgG to an antigen. To generate an optimal fit for the antigen, the binding sites of IgG often undergo slight conformational changes. Such induced fit is common to many protein-ligand interactions.

In many vertebrates, IgG is but one of five classes of immunoglobulins. Each class has a characteristic type of heavy chain, denoted α, δ, ε, γ, and μ for IgA, IgD, IgE, IgG, and IgM, respectively. Two types of light chain, κ and λ, occur in all classes of immunoglobulins. The overall structures of **IgD** and **IgE** are similar to that of IgG. **IgM** occurs either in a monomeric, membrane-bound form or in a secreted form that is a cross-linked pentamer of this basic structure (**Fig. 5-22**). **IgA**, found principally in secretions such as saliva, tears, and milk, can be a monomer, a dimer, or a trimer. IgM is the first antibody to be made by B lymphocytes and the major antibody in the early stages of a primary immune response. Some B cells soon begin to produce IgD (with the same antigen-binding site as the IgM produced by the same cell), but the particular function of IgD is less clear.

The IgG described above is the major antibody in secondary immune responses, which are initiated by a class of B cells called memory B cells. As part of the organism's ongoing immunity to antigens already encountered and dealt with, IgG is the most abundant immunoglobulin in the blood. When IgG binds to an invading bacterium or virus, it activates certain leukocytes such as macrophages to engulf and destroy the invader, and it also activates some other parts of the immune response. Receptors on the macrophage surface recognize and bind the Fc region of IgG. When these Fc receptors bind an

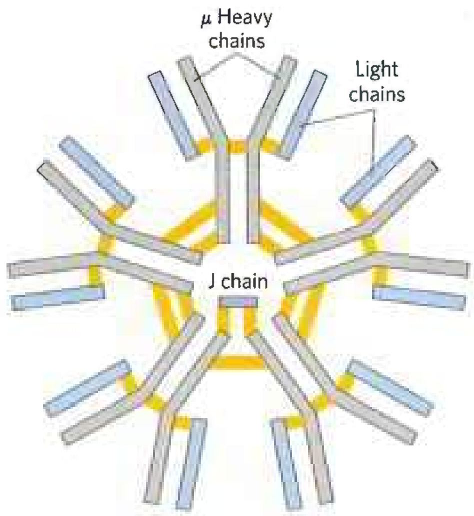


FIGURE 5-22 **IgM pentamer of immunoglobulin units.** The pentamer is cross-linked with disulfide bonds (yellow). The J chain is a polypeptide of M_r 20,000 found in both IgA and IgM.

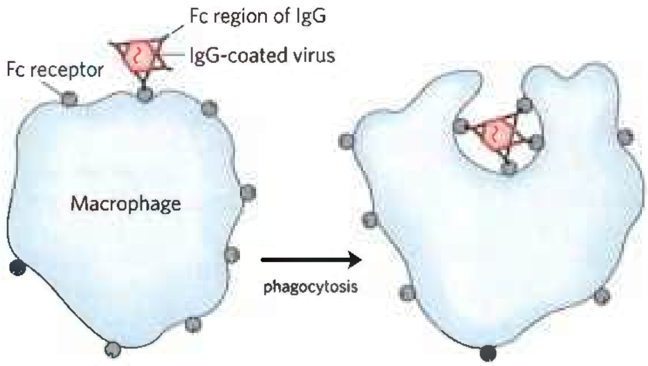


FIGURE 5-23 **Phagocytosis of an antibody-bound virus by a macrophage.** The Fc regions of antibodies bound to the virus now bind to Fc receptors on the surface of a macrophage, triggering the macrophage to engulf and destroy the virus.

antibody-pathogen complex, the macrophage engulfs the complex by phagocytosis (**Fig. 5-23**).

IgE plays an important role in the allergic response, interacting with basophils (phagocytic leukocytes) in the blood and with histamine-secreting cells called mast cells, which are widely distributed in tissues. This immunoglobulin binds, through its Fc region, to special Fc receptors on the basophils or mast cells. In this form, IgE serves as a receptor for antigen. If antigen is bound, the cells are induced to secrete histamine and other biologically active amines that cause the dilation and increased permeability of blood vessels. These effects on the blood vessels are thought to facilitate the movement of immune system cells and proteins to sites of inflammation. They also produce the symptoms normally associated with allergies. Pollen and other allergens are recognized as foreign, triggering an immune response normally reserved for pathogens. ■

Antibodies Bind Tightly and Specifically to Antigen

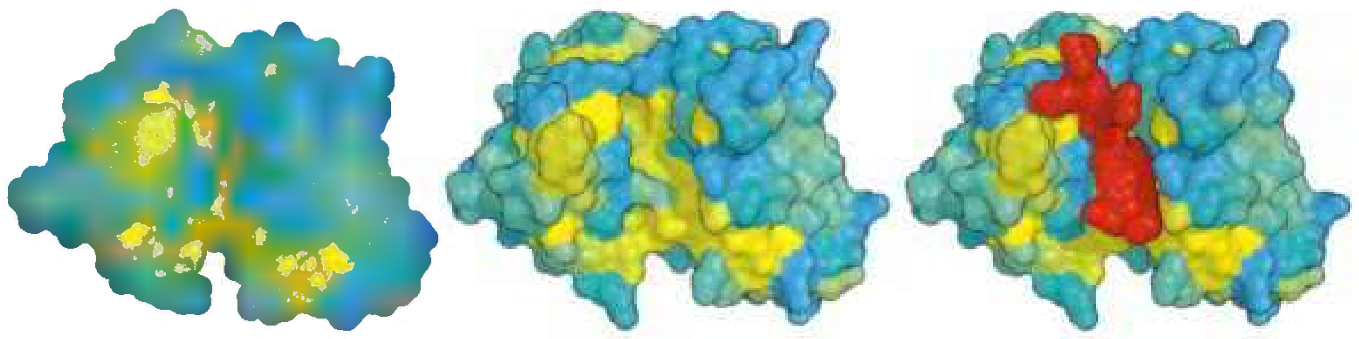
P2 The binding specificity of an antibody is determined by the amino acid residues in the variable domains of its heavy and light chains. Some of those residues, particularly those lining the antigen-binding site, are hypervariable—especially likely to differ. Specificity is conferred by chemical complementarity between the antigen and its specific binding site. For example, a binding site with a negatively charged group may bind an antigen with a positive charge in the complementary position. In many instances, complementarity is achieved interactively as the structures of antigen and binding site influence each other as they come closer together. Conformational changes in the antibody and/or the antigen then allow the complementary groups to interact fully. This is an example of induced fit. The complex of a peptide derived from HIV (a model antigen) and an Fab molecule, shown in **Figure 5-24**, illustrates some of these properties. The changes in structure observed on antigen binding are particularly striking in this example.

A typical antibody-antigen interaction is quite strong, characterized by K_d values as low as 10^{-10} M (recall that a lower K_d corresponds to a stronger binding interaction; see Table 5-1). The K_d reflects the energy derived from the hydrophobic effect and the various ionic, hydrogen-bonding, and van der Waals interactions that stabilize the binding. The binding energy required to produce a K_d of 10^{-10} M is about 65 kJ/mol.

The Antibody-Antigen Interaction Is the Basis for a Variety of Important Analytical Procedures

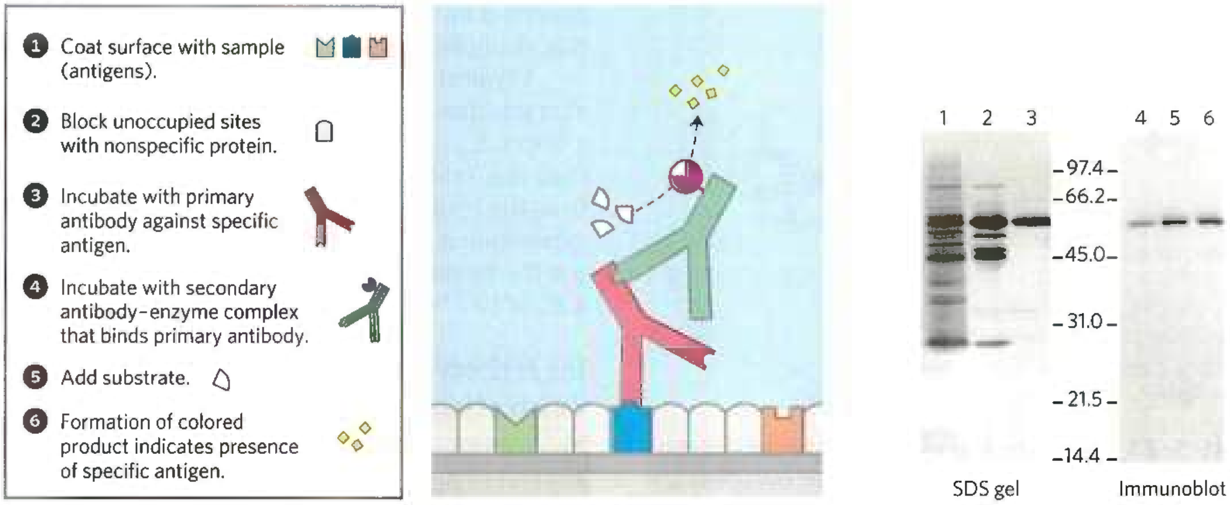
The extraordinary binding affinity and specificity of antibodies make them valuable analytical reagents. Two types of antibody preparations are in use: polyclonal and monoclonal. **Polyclonal antibodies** are those produced by many different B lymphocytes responding to one antigen, such as a protein injected into an animal. Cells in the population of B lymphocytes produce antibodies that bind specific, different epitopes within the antigen. Thus, polyclonal preparations contain a mixture of antibodies that recognize different parts of the protein. **Monoclonal antibodies**, in contrast, are synthesized by a population of identical B cells (a **clone**) grown in cell culture. These antibodies are homogeneous, all recognizing the same epitope.

The specificity of antibodies has practical uses. In a versatile analytical technique, an antibody is attached to a reagent that makes it easy to detect (**Fig. 5-25**). When the antibody binds the target protein, the label reveals the presence of the protein in a solution or its location in a gel, or even in a living cell (Fig. 5-25a). In one application of this technique, an **immunoblot** or **Western blot** assay (Fig. 5-26b), proteins that have been separated by gel electrophoresis are transferred electrophoretically to a nitrocellulose membrane.



(a) Conformation with no antigen bound **(b)** Antigen bound (but not shown) **(c)** Antigen bound (shown)

FIGURE 5-24 Induced fit in the binding of an antigen to IgG. The Fab fragment of an IgG molecule is shown here with the surface contour colored to represent hydrophobicity. Hydrophobic surfaces are yellow and hydrophilic surfaces are blue, with shades of blue to green to yellow in between. (a) View of the Fab fragment in the absence of antigen (a small peptide derived from HIV), looking down on the antigen binding site. (b) The same view, but with the Fab fragment in the "bound" conformation with the antigen omitted to provide an unobstructed view of the altered binding site. Note how the hydrophobic binding cavity has enlarged and several groups have shifted position. (c) The same view as (b) but with the antigen (red) in the binding site. [Data from (a) PDB ID 1GGC, R. L. Stanfield et al., *Structure* 1:83, 1993; (b, c) PDB ID 1GGI, J. M. Rini et al., *Proc. Natl. Acad. Sci. USA* 90:6325, 1993.]


① Coat surface with sample (antigens).
② Block unoccupied sites with nonspecific protein.
③ Incubate with primary antibody against specific antigen.
④ Incubate with secondary antibody–enzyme complex that binds primary antibody.
⑤ Add substrate.
⑥ Formation of colored product indicates presence of specific antigen.
1
2
3
4
5
6
97.4
66.2
45.0
31.0
21.5
14.4
SDS gel
Immunoblot

(a) **(b)**

FIGURE 5-25 Antibodies as analytical reagents. (a) A schematic representation of the use of the specific reaction of an antibody with its antigen to identify and quantify a specific protein in a complex sample. (b) One common application is the immunoblot. Lanes 1 to 3 are from an SDS gel; samples from successive stages in the purification of a protein kinase were separated and stained with Coomassie blue. Lanes 4 to 6 show the same samples, but these were electrophoretically transferred to a nitrocellulose membrane after separation on an SDS gel. The membrane was then "probed" with antibody against the protein kinase. The numbers between the SDS gel and the immunoblot indicate M_r in thousands. [(b) State of Wisconsin Lab of Hygiene, Madison, WI.]

After washing, the membrane is treated successively with primary antibody, secondary antibody linked to enzyme, and substrate. A colored precipitate forms only along the band containing the protein of interest. Immunoblotting allows the detection of a minor component in a sample and provides an approximation of its molecular weight.

We will encounter other aspects of antibodies in later chapters. They are extremely important in medicine and can tell us much about the structure of proteins and the action of genes.

SUMMARY 5.2 *Complementary Interactions between Proteins and Ligands: The Immune System and Immunoglobulins*

- The immune response is mediated by interactions among an array of specialized leukocytes and their associated proteins. T lymphocytes produce T-cell receptors. B lymphocytes produce immunoglobulins.
- Humans have five classes of immunoglobulins, each with different biological functions. The most abundant class is IgG, a Y-shaped protein with two heavy chains

and two light chains. The domains near the upper ends of the Y are hypervariable within the broad population of IgGs and form two antigen-binding sites.

- A given immunoglobulin generally binds to only a part, called the epitope, of a large antigen. Binding often involves a conformational change in the IgG, an induced fit to the antigen.
- The exquisite binding specificity of immunoglobulins is exploited in analytical techniques such as immunoblotting.

5.3 Protein Interactions Modulated by Chemical Energy: Actin, Myosin, and Molecular Motors

Organisms move. Cells move. Organelles and macromolecules within cells move. Most of these movements arise from the activity of a fascinating class of protein-based molecular motors. Fueled by chemical energy, usually derived from ATP, large aggregates of motor proteins undergo cyclic conformational changes that accumulate into a unified, directional force—the tiny force that pulls apart chromosomes in a dividing cell, and the immense force that levers a pouncing, quarter-ton jungle cat into the air.

The interactions among motor proteins, as you might predict, feature complementary arrangements of ionic, hydrogen-bonding, and hydrophobic groups at protein binding sites. In motor proteins, however, the resulting interactions achieve exceptionally high levels of spatial and temporal organization.

Motor proteins underlie the migration of organelles along microtubules, the motion of eukaryotic and bacterial flagella, and the movement of some proteins along DNA. Here, we focus on the well-studied example of the contractile proteins of vertebrate skeletal muscle as a paradigm for how proteins translate chemical energy into motion.

The Major Proteins of Muscle Are Myosin and Actin

The contractile force of muscle is generated by the interaction of two proteins, myosin and actin. These proteins are arranged in filaments that undergo transient interactions and slide past each other to bring about contraction. Together, actin and myosin make up more than 80% of the protein mass of muscle.

Myosin (M_r 520,000) has six subunits: two heavy chains (each of M_r 220,000) and four light chains (each of M_r 20,000). The heavy chains account for much of the overall structure. At their carboxyl termini, they are arranged as extended α helices, wrapped around each other in a fibrous, left-handed coiled coil similar to that of α-keratin (**Fig. 5-26**). At its amino terminus, each heavy chain has a large globular domain containing a site where ATP is hydrolyzed. The light chains are associated with the globular domains.

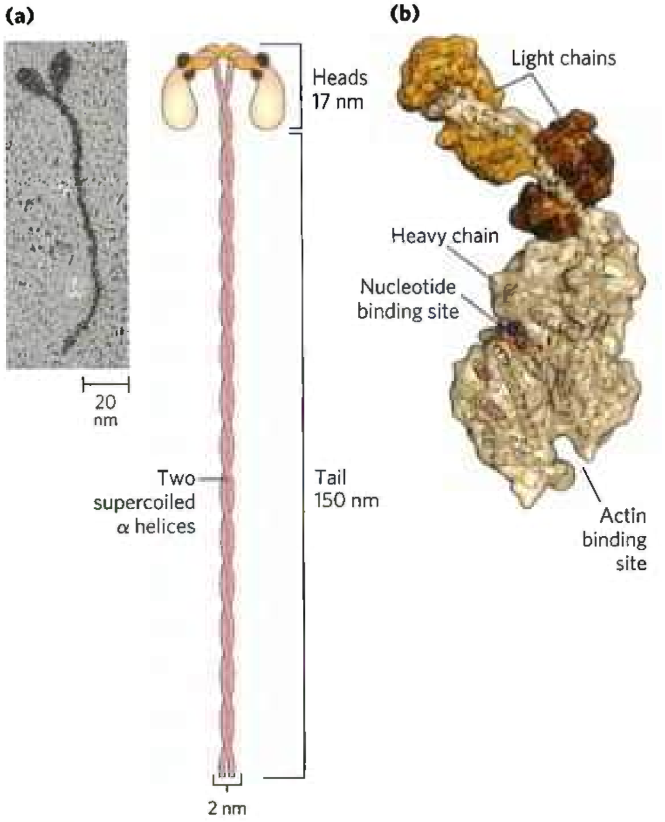


FIGURE 5-26 Myosin. (a) Myosin has two heavy chains: the carboxyl termini forming an extended coiled coil tail and the amino termini having clublike globular head domains. Two myosin light chains are associated with each myosin head. (b) A three-dimensional representation of the myosin head, showing the binding sites for nucleotide (ATP) and actin. [(a) Research from Takeshi Katayama, et al. "Stimulatory effects of arachidonic acid on myosin ATPase activity and contraction of smooth muscle via myosin motor domain," *Am. J. Physiol. Heart Circ. Physiol.* Vol 298, Issue 2, pp. H505–H514, February 2010, Fig. 6b. (b) Data from PDB ID 2MYS, I. Rayment et al., *Science* 261:50, 1993.]

In muscle cells, molecules of myosin aggregate to form structures called **thick filaments** (**Fig. 5-27a**). These rodlike structures are the core of the contractile unit. Within a thick filament, several hundred myosin molecules are arranged with their fibrous "tails" associated to form a long, bipolar structure. The globular domains project from either end of this structure, in regular stacked arrays.

The second major muscle protein, **actin**, is abundant in almost all eukaryotic cells. In muscle, molecules of monomeric actin, called G-actin (*g*lobular actin; M_r 42,000), associate to form a long polymer called F-actin (*f*ilamentous actin). The **thin filament** consists of F-actin (Fig. 5-27b), along with the proteins troponin and tropomyosin (discussed below). The filamentous parts of thin filaments assemble as successive monomeric actin

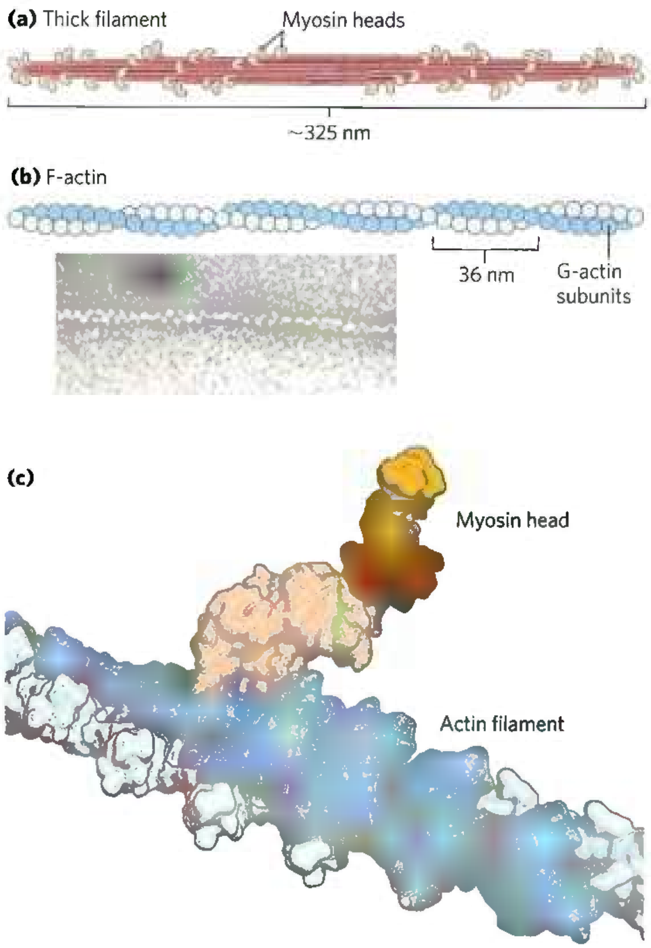


FIGURE 5-27 The major components of muscle. (a) Myosin aggregates to form a bipolar structure called a thick filament. (b) F-actin is a filamentous assemblage of G-actin monomers that polymerize two by two, giving the appearance of two filaments spiraling about one another in a right-handed fashion. (c) Space-filling model of an actin filament with one myosin head bound to an actin monomer within the filament. [(b) Research from Dr. Roger W. Craig PhD, University of Massachusetts Medical School. (c) Data from PDB ID 2MYS, I. Rayment et al., *Science* 261:50, 1993; PDB ID 6BNQ, P. S. Gurel et al., *eLife* 6, 2017, doi 10.7554/eLife.31125]

molecules add to one end. Upon addition, each monomer binds ATP, then hydrolyzes it to ADP, so every actin molecule in the filament is complexed to ADP. This ATP hydrolysis by actin functions only in the assembly of the filaments; it does not contribute directly to the energy expended in muscle contraction. Each actin monomer in the thin filament can bind tightly and specifically to one myosin head group (Fig. 5-27c).

Additional Proteins Organize the Thin and Thick Filaments into Ordered Structures

Skeletal muscle consists of parallel bundles of **muscle fibers**, each fiber a single, very large, multinucleated cell, 20 to 100 μm in diameter, formed from many cells fused together; a single fiber often spans the length of the muscle. Each fiber contains about 1,000 **myofibrils**, 2 μm in diameter, each consisting of a vast number of regularly arrayed thick and thin filaments complexed to other proteins (**Fig. 5-28**). A system of flat membranous vesicles called the **sarcoplasmic reticulum** surrounds each myofibril. Examined under the electron microscope, muscle fibers reveal alternating regions of high electron density and low electron density, called the **A bands** and **I bands** (Fig. 5-28b, c). The A and I bands arise from the arrangement of thick and thin filaments, which are aligned and partially overlapping. The I band is the region of the bundle that in cross section would contain only thin filaments. The darker A band stretches the length of the thick filament and includes the region where parallel thick and thin filaments overlap. Bisecting the I band is a thin structure called the **Z disk**, perpendicular to the thin filaments and serving as an anchor to which the thin filaments are attached. The A band, too, is bisected by a thin line, the **M line** or M disk, a region of high electron density in the middle of the thick filaments. The entire contractile unit, consisting of bundles of thick filaments interleaved at either end with bundles of thin filaments, is called the **sarcomere**. The arrangement of interleaved bundles allows the thick and thin filaments to slide past each other (by a mechanism discussed below), causing a progressive shortening of each sarcomere (Fig. 5-28d).

Myosin Thick Filaments Slide along Actin Thin Filaments

P1 The interaction between actin and myosin, like that between all proteins and ligands, involves weak bonds. When ATP is not bound to myosin, a face on the myosin head group binds tightly to actin (**Fig. 5-29**). When ATP binds to myosin and is hydrolyzed to ADP and phosphate, a coordinated and cyclic series of conformational changes occurs in which myosin releases the F-actin subunit and binds another subunit farther along the thin filament.

The cycle has four major steps (Fig. 5-29). In step ❶, ATP binds to myosin and a cleft in the myosin molecule opens, disrupting the actin-myosin interaction so that the bound actin is released. ATP is then hydrolyzed in step ❷, causing a conformational change in the protein to a "high-energy" state that moves the myosin head and changes its orientation in relation to the actin thin filament. Myosin then binds weakly to an F-actin subunit closer to the Z disk than the one just released. As the phosphate product of ATP hydrolysis is released from myosin in step ❸, another conformational change occurs in which the myosin cleft closes, strengthening the myosin-actin

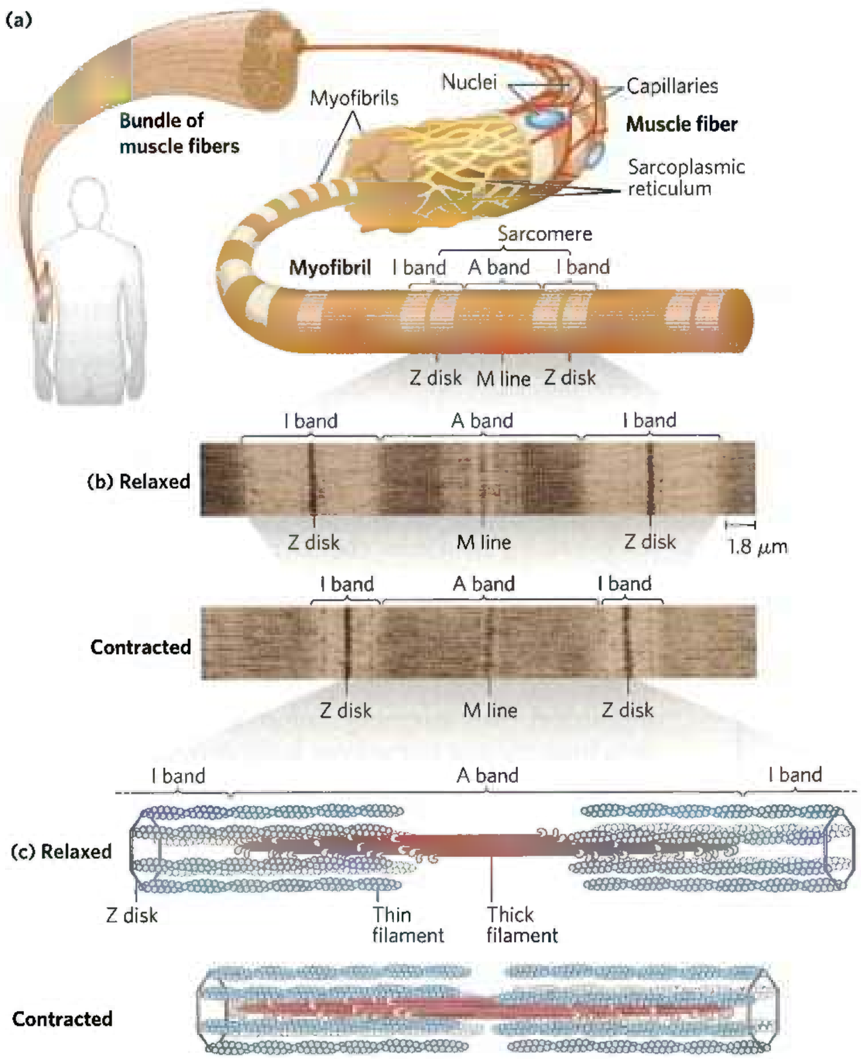

(a)
Bundle of muscle fibers
Myofibrils
Nuclei
Capillaries
Muscle fiber
Sarcoplasmic reticulum
Sarcomere
Myofibril
I band
A band
I band
Z disk
M line
Z disk
I band
A band
I band
(b) Relaxed
Z disk
M line
Z disk
1.8 μm
I band
A band
I band
Contracted
Z disk
M line
Z disk
I band
A band
I band
(c) Relaxed
Z disk
Thin filament
Thick filament
Contracted

FIGURE 5-28 **Skeletal muscle.** (a) Muscle fibers consist of single, elongated, multinucleated cells that arise from the fusion of many precursor cells. The fibers are made up of many myofibrils (only six are shown here for simplicity) surrounded by the membranous sarcoplasmic reticulum. The organization of thick and thin filaments in a myofibril gives it a striated appearance. When muscle contracts, the I bands narrow and the Z disks move closer together, as seen in electron micrographs of (b) relaxed muscle and (c) contracted muscle. (d) Thick filaments are bipolar structures created by the association of many myosin molecules. Muscle contraction occurs by the sliding of the thick and thin filaments past each other so that the Z disks in neighboring I bands draw closer together. The thick and thin filaments are interleaved such that each thick filament is surrounded by six thin filaments. [(b) Medical Images/James E. Dennis.]

binding. This is followed quickly by step ④, a "power stroke" during which the conformation of the myosin head returns to the original resting state, its orientation relative to the bound actin changing so as to pull the tail of the myosin toward the Z disk. ADP is then released to complete the cycle. Each cycle generates about 3 to 4 pN (piconewtons) of force and moves the thick filament 5 to 10 nm relative to the thin filament.

Because there are many myosin heads in a thick filament, at any given moment some (probably 1% to 3%) are bound to thin filaments. This prevents thick filaments from slipping backward when an individual myosin head releases the actin subunit to which it was bound. The thick filament thus actively slides forward past the adjacent thin filaments. This process, coordinated among the many sarcomeres in a muscle fiber, brings about muscle contraction.

The interaction between actin and myosin must be regulated so that contraction occurs only in response to appropriate signals from the nervous system. The regulation is mediated by a complex of two proteins, **tropomyosin** and **troponin** (Fig. 5-30). Tropomyosin binds to the thin filament, blocking the attachment sites for the myosin head groups. Troponin is a Ca^{2+}-binding protein. A nerve impulse causes release of Ca^{2+} ions from the sarcoplasmic reticulum. The released Ca^{2+} binds to troponin (another protein-ligand interaction) and causes a conformational change in the tropomyosin-troponin complexes, exposing the myosin-binding sites on the thin filaments. Contraction follows.

Working skeletal muscle requires two types of molecular functions that are common in proteins: binding and catalysis. The actin-myosin interaction, a protein-ligand interaction like that of immunoglobulins with antigens, is reversible and leaves the participants

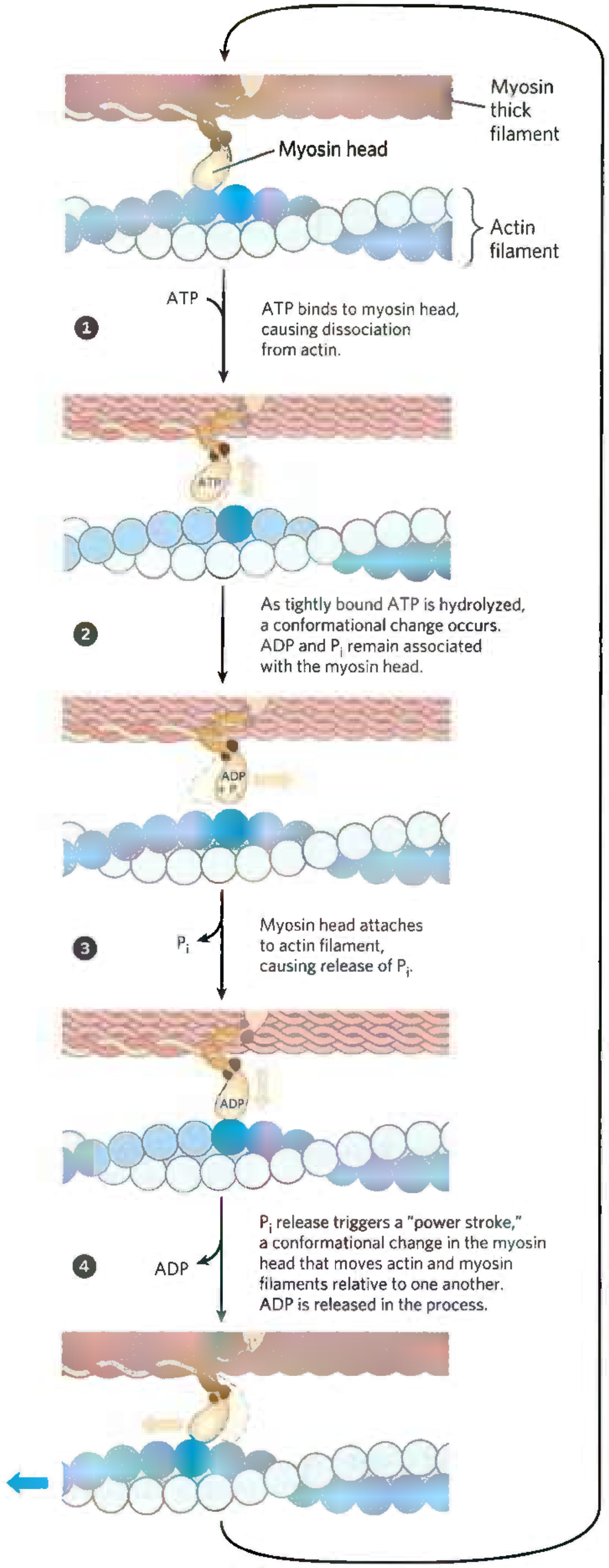

Myosin thick filament
Myosin head
Actin filament
ATP
①
ATP binds to myosin head, causing dissociation from actin.
ATP
②
As tightly bound ATP is hydrolyzed, a conformational change occurs. ADP and P_i remain associated with the myosin head.
ADP + P_i
③
P_i
Myosin head attaches to actin filament, causing release of P_i.
ADP
④
ADP
P_i release triggers a "power stroke," a conformational change in the myosin head that moves actin and myosin filaments relative to one another. ADP is released in the process.

FIGURE 5-29 Molecular mechanism of muscle contraction. Conformational changes in the myosin head that are coupled to stages in the ATP hydrolytic cycle cause myosin to successively dissociate from one actin subunit, then associate with another farther along the actin filament. In this way, the myosin heads slide along the thin filaments, drawing the thick filament array into the thin filament array (see Fig. 5-28).

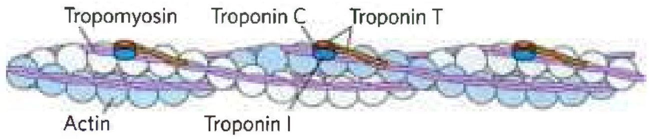

Tropomyosin
Troponin C
Troponin T
Actin
Troponin I

FIGURE 5-30 Regulation of muscle contraction by tropomyosin and troponin. Tropomyosin and troponin are bound to F-actin in the thin filaments. In the relaxed muscle, these two proteins are arranged around the actin filaments so as to block the binding sites for myosin. Tropomyosin is a two-stranded coiled coil of α helices, the same structural motif as in α-keratin (see Fig. 4-11). It forms head-to-tail polymers twisting around the two actin chains. Troponin is attached to the actin-tropomyosin complex at regular intervals of 38.5 nm. Troponin consists of three different subunits: I, C, and T. Troponin I prevents binding of the myosin head to actin; troponin C has a binding site for Ca^{2+}; and troponin T links the entire troponin complex to tropomyosin. When the muscle receives a neural signal to initiate contraction, Ca^{2+} is released from the sarcoplasmic reticulum (see Fig. 5-28a) and binds to troponin C. This causes a conformational change in troponin C, which alters the positions of troponin I and tropomyosin so as to relieve the inhibition by troponin I and allow muscle contraction.

unchanged. This interaction illustrates why reversibility is important; a permanent actin-myosin interaction defines the state of rigor mortis, a state we all want to avoid as long as possible. When ATP binds myosin, however, it is hydrolyzed to ADP and P_i (inorganic phosphate). Myosin is not only an actin-binding protein, but also an ATPase—an enzyme. The function of enzymes in catalyzing chemical transformations is the topic of the next chapter.

SUMMARY 5.3 *Protein Interactions Modulated by Chemical Energy: Actin, Myosin, and Molecular Motors*

- Protein-ligand interactions achieve a special degree of spatial and temporal organization in motor proteins. Muscle contraction results from choreographed interactions between myosin and actin, coupled to the hydrolysis of ATP by myosin.
- Myosin consists of two heavy chains and four light chains, forming a fibrous coiled coil (tail) domain and a globular (head) domain. Myosin molecules are organized into thick filaments, which slide past thin filaments composed largely of actin. ATP hydrolysis in myosin is coupled to a series of conformational changes in the myosin head, leading to dissociation of myosin from one F-actin subunit and its eventual reassociation with another, farther along the thin filament. The myosin thus slides along the actin filaments.
- Muscle contraction is stimulated by the release of Ca^{2+} from the sarcoplasmic reticulum. The Ca^{2+} binds to the protein troponin, leading to a conformational change in a troponin-tropomyosin complex that triggers the cycle of actin-myosin interactions.

KEY TERMS

Terms in bold are defined in the glossary.

ligand	147
binding site	147
induced fit	147
hemoglobin	148
heme	148
porphyrin	148
globins	149
globin fold	149
equilibrium expression	150
association constant, K_a	150
dissociation constant, K_d	150
allosteric protein	157
modulator	157
homotropic	157
heterotropic	157
Hill equation	157
Hill coefficient	157
Bohr effect	160
immune response	164
lymphocytes	164
antibody	165
immunoglobulin (Ig)	165
B lymphocyte (B cell)	165
T lymphocyte (T cell)	165
antigen	165
epitope	165
hapten	165
immunoglobulin fold	165
polyclonal antibodies	167
monoclonal antibodies	167
immunoblotting	167
Western blotting	167
myosin	169
actin	169
myofibril	170
sarcomere	170

PROBLEMS

1. Relationship between Affinity and Dissociation Constant Protein A has a binding site for ligand X with a K_d of 3.0×10^{-7} M. Protein B has a binding site for ligand X with a K_d of 4.0×10^{-8} M. Calculate the K_a for each protein. Which protein has a higher affinity for ligand X? Explain your reasoning.

2. Modeling Apparent Negative Cooperativity Which of these situations would produce a Hill plot with $n_H < 1.0$? Explain your reasoning in each case.

(a) The protein has multiple subunits, each with a single ligand-binding site. Ligand binding to one site decreases the binding affinity of other sites for the ligand.
(b) The protein is a single polypeptide with two ligand-binding sites, each having a different affinity for the ligand.
(c) The protein is a single polypeptide with a single ligand-binding site. As purified, the protein preparation is heterogeneous, containing some protein molecules that are partially denatured and thus have a lower binding affinity for the ligand.
(d) The protein has multiple subunits, each with a single ligand-binding site. Ligands bind independently to each site, do not affect the binding affinity of other sites, and bind with identical affinities.

3. Reversible Ligand Binding I The protein calcineurin binds to the protein calmodulin with an association rate of $8.9 \times 10^3\ \text{M}^{-1}\text{s}^{-1}$ and an overall dissociation constant, K_d, of 10 nM. Calculate the dissociation rate, k_d, including appropriate units.

4. Reversible Ligand Binding II The *E. coli* nickel-binding protein binds to its ligand, Ni^{2+}, with a K_d of 100 nM. Calculate the Ni^{2+} concentration when the fraction of binding sites occupied by the ligand (Y) is **(a)** 0.25, **(b)** 0.6, **(c)** 0.95.

5. Reversible Ligand Binding III You are a technician in a biochemistry lab running receptor binding experiments. The target membrane-bound receptor has been partially purified from mouse, rat, and human cell lines. Using various concentrations of the same radioactive ligand for each receptor in a saturation binding assay, you generate the binding data shown in the table. The dependent variable, Y, is the fraction of binding sites occupied by the ligand.

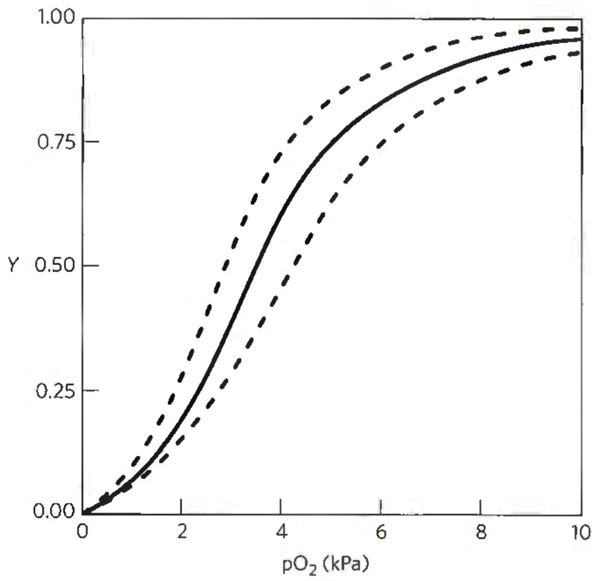
Ligand concentration (nM)	Y		
	Mouse receptor	**Rat receptor**	**Human receptor**
0.2	0.048	0.29	0.17
0.5	0.11	0.50	0.33
1.0	0.20	0.67	0.50
4.0	0.50	0.89	0.80
10	0.71	0.95	0.91
20	0.83	0.97	0.95
50	0.93	0.99	0.98

(a) Determine the mouse receptor K_d in this binding experiment.
(b) Which receptor binds most tightly to this ligand?

6. Reversible Ligand Binding IV Exposure to carbon monoxide can lead to unconsciousness and ultimately death. Suffocation occurs when hemoglobin is half-saturated with CO—that is, when only two of every four oxygen-binding sites are occupied with CO. Explain why death may occur at this point, even though half of the oxygen-binding sites are still available to transport O_2. (Hint: See Box 5-1.)

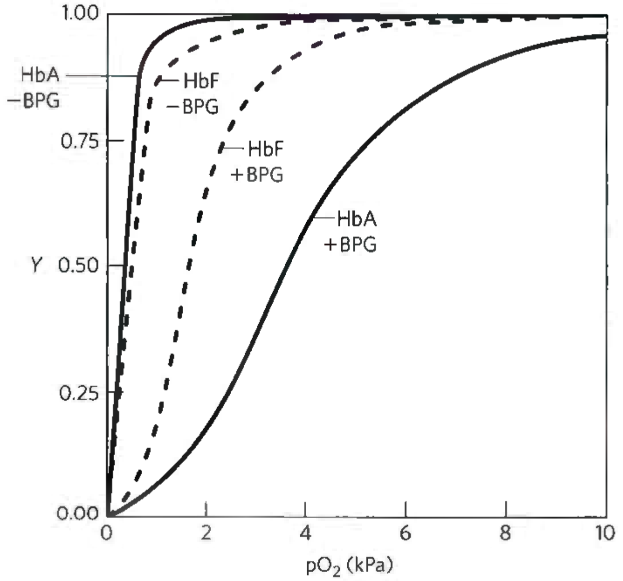
7. Cooperativity in Hemoglobin Under appropriate conditions, hemoglobin dissociates into its four subunits. The isolated α subunit binds oxygen, but the O_2-saturation curve is hyperbolic rather than sigmoid. In addition, the binding of oxygen to the isolated α subunit is not affected by the presence of H^+, CO_2, or BPG. What do these observations indicate about the source of the cooperativity in hemoglobin?

8. Oxygen Binding to Hemoglobin The solid curve in the plot shown is an O_2-binding curve for human hemoglobin. For each condition, indicate whether the stated physiological change would shift the curve to the left (dashed curve), produce no change (black curve), or shift the curve to the right (dashed curve).



(a) an increase in the concentration of CO_2
(b) an increase in the proton concentration (decrease in pH)
(c) an increase in the concentration of 2,3-bisphosphoglycerate (BPG)

9. Comparison of Fetal and Maternal Hemoglobins Studies of oxygen transport in pregnant mammals show that the O_2-saturation curves of fetal and maternal blood are markedly different when measured under the same conditions. Fetal erythrocytes contain a structural variant of hemoglobin, HbF, consisting of two α and two γ subunits ($\alpha_2\gamma_2$), whereas maternal erythrocytes contain HbA ($\alpha_2\beta_2$).
(a) Which hemoglobin has a higher affinity for oxygen under physiologic conditions?
(b) What is the physiological significance of the different O_2 affinities?
When all the BPG is carefully removed from samples of HbA and HbF, the measured O_2-saturation curves (and consequently the O_2 affinities) are displaced to the left. However, HbA now has a greater affinity for oxygen than does HbF. When BPG is reintroduced, the O_2-saturation curves return to normal, as shown in the graph.



(c) What is the effect of BPG on the O_2 affinity of hemoglobin? How can this information be used to explain the different O_2 affinities of fetal and maternal hemoglobin?

10. Hemoglobin Variants There are almost 500 naturally occurring variants of hemoglobin. Most are the result of a single amino acid substitution in a globin polypeptide chain. Some variants produce clinical illness, though not all variants have deleterious effects. A brief sample of hemoglobin variants is shown here.

HbS (sickle cell Hb): substitutes a Val for a Glu on the surface
Hb Cowtown: eliminates an ion pair involved in T-state stabilization
Hb Memphis: substitutes one uncharged polar residue for another of similar size on the surface
Hb Bibba: substitutes a Pro for a Leu involved in an α helix
Hb Milwaukee: substitutes a Glu for a Val
Hb Providence: substitutes an Asn for a Lys that normally projects into the central cavity of the tetramer
Hb Philly: substitutes a Phe for a Tyr, disrupting hydrogen bonding at the $\alpha_1\beta_1$ interface

Select the hemoglobin variants that are described by each statement.
(a) The Hb variant *least* likely to cause pathological symptoms
(b) The variant(s) most likely to show pI values different from that of HbA on an isoelectric focusing gel
(c) The variant(s) most likely to show a decrease in BPG binding and an increase in the overall affinity of the hemoglobin for oxygen

11. Oxygen Binding and Hemoglobin Structure A team of biochemists uses genetic engineering to modify the interface region between hemoglobin subunits. The resulting hemoglobin variants exist in solution primarily as $\alpha\beta$ dimers (few, if any, $\alpha_2\beta_2$ tetramers form). Are these variants likely to bind oxygen more weakly or more tightly? Explain your answer.

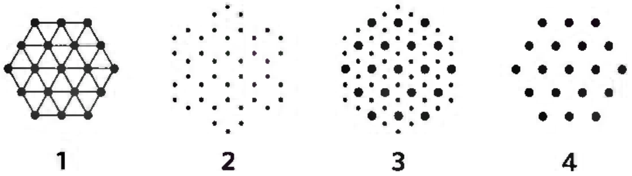
12. Reversible (and Tight) Binding to an Antibody An antibody with high affinity for its antigen has a K_d in the low nanomolar range. Assume an antibody binds an antigen with a K_d of 5×10^{-8} M. Calculate the antigen concentration when Y, the fraction of binding sites occupied by the ligand, is **(a)** 0.4, **(b)** 0.5, **(c)** 0.8, **(d)** 0.9.

13. Using Antibodies to Probe Structure-Function Relationships in Proteins A monoclonal antibody binds to G-actin but not to F-actin. What does this tell you about the epitope recognized by the antibody?

14. The Immune System and Vaccines Some pathogens have developed mechanisms to evade the immune system, making it difficult or impossible to develop effective vaccines against them.
(a) African sleeping sickness is caused by a protozoan called *Trypanosoma brucei*, carried by the tsetse fly. The trypanosome surface is dominated by one coat protein, the variable surface glycoprotein (VSG). The trypanosome genome encodes over 1,000 different versions of VSG. All of the cells in an initial infection feature the same VSG coat on their surfaces, and this is readily recognized as foreign by the immune system. However, an individual trypanosome in the broader population will switch and randomly begin expressing a different variant of the VSG coat. All the descendants of that cell will have the new and different protein on their surface. As the population with the second VSG coat increases, an individual cell will then switch to a third VSG protein coat, and so on.
(b) The human immunodeficiency virus (HIV) has an error-prone system for replicating its genome, effectively introducing mutations at an unusually high rate. Many of the mutations affect the viral protein coat. Describe how each pathogen can survive the immune response of its host.

15. How We Become a "Stiff" When a vertebrate dies, its muscles stiffen as they are deprived of ATP, a state called rigor mortis. Using your knowledge of the catalytic cycle of myosin in muscle contraction, explain the molecular basis of the rigor state.

16. Sarcomeres from Another Point of View The symmetry of thick and thin filaments in a sarcomere is such that six thin filaments ordinarily surround each thick filament in a hexagonal array. Match each cross section (transverse cut) image of a sarcomere with the correct viewpoint.



(a) at the M line
(b) through the I band
(c) through the dense region of the A band
(d) through the less dense region of the A band, adjacent to the M line (see Fig. 5-28b, c)

BIOCHEMISTRY ONLINE

17. IgG and Lysozyme Crystal Structure To fully appreciate how proteins function in a cell, it is helpful to have a three-dimensional view of how proteins interact with other cellular components. Fortunately, this is possible using online protein databases and three-dimensional molecular viewing utilities such as JSmol, a free and user-friendly molecular viewer that is compatible with most browsers and operating systems.

In this exercise, examine the interactions between the enzyme lysozyme and the Fab portion of the antilysozyme antibody. Use the PDB identifier 1FDL to explore the structure of the IgG1 Fab fragment–lysozyme complex (antibody-antigen complex). To answer the questions, use the information on the Structure Summary page at the Protein Data Bank (www.rcsb.org), and view the structure using JSmol or a similar viewer.

(a) Which chains in the three-dimensional model correspond to the antibody fragment, and which correspond to the antigen, lysozyme?
(b) What type of secondary structure predominates in this Fab fragment?
(c) How many amino acid residues are in the heavy and light chains of the Fab fragment? In lysozyme? Estimate the percentage of the lysozyme that interacts with the antigen-binding site of the antibody fragment.
(d) Identify the specific amino acid residues in lysozyme and in the variable regions of the Fab heavy and light chains that are situated at the antigen-antibody interface. Are the residues contiguous in the primary sequence of the polypeptide chains?

18. Exploring Antibodies in the Protein Data Bank The PDB-101 Molecule of the Month article on "Antibodies" (http://pdb101.rcsb.org/motm/21) summarizes what you have read in this chapter regarding antibody structure and function. To paraphrase the article, a variety of antibodies, on the order of one hundred million different types, are always circulating in our bloodstream, searching for foreign invaders to attack. Once an invader is discovered, the antibody binds the invader with its flexible arms, containing the Fab region. Thin, flexible chains connect these flexible arms to the antibody base, called the Fc region. This base determines which class the antibody belongs to, as some antibodies have four or ten binding sites due to their structural formation.

(a) How many specific antigen-binding sites are there on the first immunoglobulin image (derived from PDB ID 1IGT) in the article?
(b) When a virus enters your lungs, how long does it take for you to produce one or more antibodies that bind to it?
(c) Approximately how many types of different antibodies are present in your blood?
(d) Explore the structure of the immunoglobulin molecule (PDB ID 1IGT) by clicking the link in the article or by using a search engine to find the structure summary for PDB ID 1IGT. Use one of the 3D viewers on the PDB site to view a ribbon structure for this immunoglobulin. Identify the two light chains and two heavy chains (use the viewer controls to distinguish them by color).

DATA ANALYSIS PROBLEM

19. Protein Function During the 1980s, the structures of actin and myosin were known only at the resolution shown in Figure 5-26a. Although researchers knew that the globular head portion of myosin bound to actin and hydrolyzed ATP, there was a substantial debate about where in the myosin molecule the contractile force was generated. At the time, two competing models were proposed for the mechanism of force generation in myosin.

In the "hinge" model, the head bound to actin, but the pulling force was generated by contraction of the "hinge region" in the myosin tail. The hinge region is in the heavy meromyosin portion of the myosin molecule; this is roughly the point labeled "Two supercoiled α helices" in Figure 5-26. In the "S1" model (S1 being a name used to describe the head), the pulling force was generated in the S1 "head" itself and the tail was just for structural support.

Many experiments were performed but provided no conclusive evidence. Then, in 1987, James Spudich and his colleagues at Stanford University published a study that, although not conclusive, went a long way toward resolving this controversy.

Recombinant DNA techniques were not sufficiently developed to address this issue in vivo, so Spudich and colleagues used an interesting in vitro motility assay. The alga *Nitella* has extremely long cells, often several centimeters long and about 1 mm in diameter. These cells have actin fibers that run along their long axes, and the cells can be cut open along their length to expose the actin fibers. Spudich and his group had observed that plastic beads coated with myosin would "walk" along these fibers in the presence of ATP, just as myosin would do in contracting muscle.

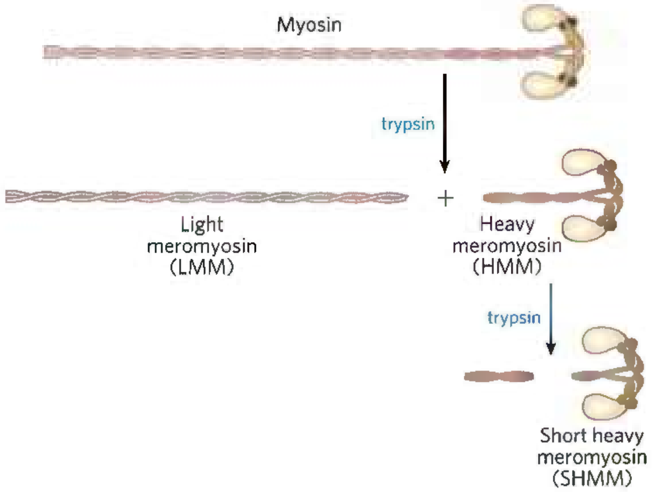
For these experiments, the researchers used a more well-defined method for attaching the myosin to the beads. The "beads" were clumps of killed bacterial (*Staphylococcus aureus*) cells. These cells have a protein on their surface that binds to the Fc region of antibody molecules (Fig. 5-20a). The antibodies, in turn, bind to several (unknown) places along the tail of the myosin molecule. When bead-antibody-myosin complexes were prepared with intact myosin molecules, they would move along *Nitella* actin fibers in the presence of ATP.

(a) Sketch a diagram showing what a bead-antibody-myosin complex might look like at the molecular level.

(b) Why was ATP required for the beads to move along the actin fibers?

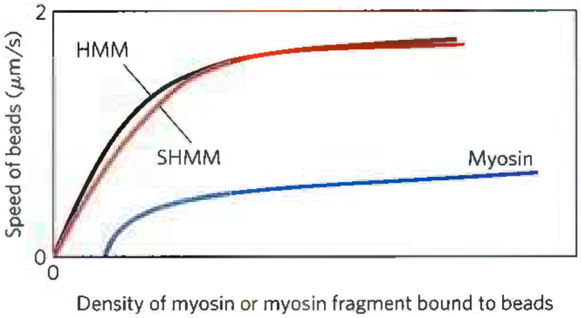
(c) Spudich and coworkers used antibodies that bound to the myosin tail. Why would this experiment have failed if they had used an antibody that bound to the part of S1 that normally bound to actin? Why would this experiment have failed if they had used an antibody that bound to actin?

To help focus on the part of myosin responsible for force production, Spudich and colleagues used trypsin to produce two partial myosin molecules: heavy meromyosin (HMM) and light meromyosin (LMM), by cleavage of a single specific peptide bond in the myosin tail. Additional incubation with trypsin produced an additional cleavage, eliminating more of the tail and the hinge region to generate short heavy meromyosin (SHMM).



(d) Why might trypsin attack this peptide bond first, rather than other peptide bonds in myosin?

Spudich and colleagues prepared bead-antibody-myosin complexes with varying amounts of myosin, HMM, or SHMM and measured their speed of movement along *Nitella* actin fibers in the presence of ATP. The graph below sketches their results.



(e) Which model ("S1" or "hinge") is consistent with these results? Explain your reasoning.

(f) Provide a plausible explanation for the increased speed of the beads with increasing myosin density.

(g) Provide a plausible explanation for the plateauing of the speed of the beads at high myosin density.

Reference

Hynes, T.R., S.M. Block, B.T. White, and J.A. Spudich. 1987. Movement of myosin fragments in vitro: domains involved in force production. *Cell* 48:953–963.

Chapter 6

ENZYMES

6.1 An Introduction to Enzymes *178*
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There are two fundamental conditions for life. First, the organism must be able to self-replicate (a topic to be considered in Part III); second, it must be able to catalyze chemical reactions efficiently and selectively. The central importance of catalysis may seem surprising, but it is easy to demonstrate. As described in Chapter 1, living systems make use of energy from the environment. Many of us, for example, consume substantial amounts of sucrose—common table sugar—as a kind of fuel, usually in the form of sweetened foods and drinks. The conversion of sucrose to CO_2 and H_2O in the presence of oxygen is a highly exergonic process, releasing free energy that we can use to think, move, taste, and see. However, a bag of sugar can remain on the shelf for years without any obvious conversion to CO_2 and H_2O. Although this chemical process is thermodynamically favorable, it is very slow. Yet when sucrose is consumed by a human (or almost any other organism), it releases its chemical energy in seconds. The difference is catalysis. Without catalysis, chemical reactions such as sucrose oxidation could not occur on a useful time scale, and thus could not sustain life. Essentially all reactions that occur in cells are, and must be, catalyzed by enzymes, the most remarkable and highly specialized of the proteins.

Our discussion of enzymes is organized around five principles:

P1 **Enzymes are powerful biological catalysts.** Rate accelerations by enzymes are often far greater than those by synthetic or inorganic catalysts. Like all catalysts, enzymes increase reaction rates, lowering reaction activation barriers. Enzymes do not affect the equilibria of reactions.

P2 **Enzymes exhibit a very high degree of specificity.** Each enzyme catalyzes only one chemical reaction, or sometimes a few closely related reactions. Reaction activation barriers are thus lowered selectively.

P3 **Enzymatic reactions occur in specialized pockets called active sites.** These pockets are similar to ligand binding sites, except that a reaction occurs there—the conversion of a substrate, a molecule that is acted on by an enzyme, to a product.

P4 **Two concepts explain the catalytic power of enzymes.** First, enzymes bind most tightly to the transition state of the catalyzed reaction, using binding energy to lower the activation barrier. Second, enzyme active sites are organized by evolution to facilitate multiple mechanisms of chemical catalysis simultaneously.

P5 **Many enzymes are regulated.** Regulatory mechanisms include reversible covalent modification, binding of allosteric modulators, proteolytic activation, noncovalent binding to regulatory proteins, and elaborate regulatory cascades. Enzymes are often subject to multiple methods of regulation, which allows for exquisite control of every chemical process that occurs in a cell.

The study of enzymes has immense practical importance. Some diseases, especially inheritable genetic disorders, are the result of a deficiency or even a total absence of one or more enzymes. Other disease conditions may be caused by excessive activity of an enzyme. Measurements of the activities of enzymes in blood plasma, erythrocytes, or tissue samples are important in diagnosing certain illnesses. Many drugs act through interactions with enzymes. Enzymes are also important practical tools in chemical engineering, food technology, and agriculture. Virtually every process studied in a biochemical laboratory involves one or often many enzymes. We now turn to a broader description of these remarkable catalysts.

6.1 An Introduction to Enzymes

Much of the history of biochemistry is the history of enzyme research. Biological catalysis was first recognized and described in the late 1700s, in studies on the digestion of meat by secretions of the stomach. The science of biochemistry can be traced to an experiment by Eduard Buchner in 1897, which demonstrated that cell-free yeast extracts could ferment sugar to alcohol. Buchner thus proved that fermentation was promoted by molecules that continued to function when removed from cells. This work marked the end of vitalistic notions advanced by Louis Pasteur that biological catalysis was a process inseparable from living systems. Frederick W. Kühne later gave the name **enzymes** (from the Greek *enzymos*, "leavened") to the molecules detected by Buchner.

Eduard Buchner, 1860–1917 [Science Museum/Science & Society Picture Library.]

Not until the late 1920s did it become clear that enzymes were proteins. In 1926, James Sumner provided the first breakthrough, isolating and crystallizing the enzyme urease. Further work by Northrop, Kunitz, and others led to general acceptance of the enzyme-protein association by the early 1930s. Since the latter part of the twentieth century, thousands of enzymes have been purified, their structures elucidated, and their mechanisms explained.

The power of enzyme catalysts is often astonishing. The enzyme orotidine phosphate decarboxylase, an enzyme involved in the biosynthesis of pyrimidine nucleotides, provides a special example, with a rate enhancement of 10^{17}. **P1** The uncatalyzed reaction has a half-life of 78 million years. On the enzyme, the reaction occurs on a time scale of milliseconds.

Most Enzymes Are Proteins

With the exception of a few classes of catalytic RNA molecules (Chapter 26), enzymes are proteins. Their catalytic activity depends on the integrity of their native protein conformation. If an enzyme is denatured or dissociated into its subunits, catalytic activity is usually lost. The catalytic activity of each enzyme is intimately linked to its primary, secondary, tertiary, and quaternary protein structure.

Enzymes, like other proteins, have molecular weights ranging from about 12,000 to more than 1 million. Some enzymes require no chemical groups for activity other than their amino acid residues. Others require an additional chemical component called a **cofactor**—either one or more inorganic ions, such as Fe^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+} (Table 6-1), or a complex organic or metalloorganic molecule called a **coenzyme**. Coenzymes act as transient carriers of specific functional groups (Table 6-2). Most are derived from vitamins, organic nutrients required in small amounts in the diet. We consider coenzymes in more

TABLE 6-1 Some Inorganic Ions That Serve as Cofactors for Enzymes

Ions	Enzymes
Cu^{2+}	Cytochrome oxidase
Fe^{2+} or Fe^{3+}	Cytochrome oxidase, catalase, peroxidase
K^+	Pyruvate kinase
Mg^{2+}	Hexokinase, glucose 6-phosphatase, pyruvate kinase
Mn^{2+}	Arginase, ribonucleotide reductase
Mo	Dinitrogenase
Ni^{2+}	Urease
Zn^{2+}	Carbonic anhydrase, alcohol dehydrogenase, carboxypeptidases A and B

TABLE 6-2 Some Coenzymes That Serve as Transient Carriers of Specific Atoms or Functional Groups

Coenzyme	Examples of chemical groups transferred	Dietary precursor in mammals
Biocytin	CO_2	Biotin (vitamin B_7)
Coenzyme A	Acyl groups	Pantothenic acid (vitamin B_5) and other compounds
5′-Deoxyadenosylcobalamin (coenzyme B_{12})	H atoms and alkyl groups	Vitamin B_{12}
Flavin adenine dinucleotide	Electrons	Riboflavin (vitamin B_2)
Lipoate	Electrons and acyl groups	Not required in diet
Nicotinamide adenine dinucleotide	Hydride ion ($:H^-$)	Nicotinic acid (niacin, vitamin B_3)
Pyridoxal phosphate	Amino groups	Pyridoxine (vitamin B_6)
Tetrahydrofolate	One-carbon groups	Folate (vitamin B_9)
Thiamine pyrophosphate	Aldehydes	Thiamine (vitamin B_1)

Note: The structures and modes of action of these coenzymes are described in Part II.

TABLE 6-3 International Classification of Enzymes

Class number	Class name	Type of reaction catalyzed
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group transfer
3	Hydrolases	Hydrolysis (transfer of functional groups to water)
4	Lyases	Cleavage of C—C, C—O, C—N, or other bonds by elimination, leaving double bonds or rings, or addition of groups to double bonds
5	Isomerases	Transfer of groups within molecules to yield isomeric forms
6	Ligases	Formation of C—C, C—S, C—O, and C—N bonds by condensation reactions coupled to cleavage of ATP or similar cofactor
7	Translocases	Movement of molecules or ions across membranes or their separation within membranes

detail as we encounter them in the metabolic pathways discussed in Part II. Some enzymes require *both* a coenzyme and one or more metal ions for activity. A coenzyme or metal ion that is very tightly or even covalently bound to the enzyme protein is called a **prosthetic group**. A complete, catalytically active enzyme together with its bound coenzyme and/or metal ions is called a **holoenzyme**. The protein part of such an enzyme is called the **apoenzyme** or **apoprotein**. Finally, some enzyme proteins are modified covalently by phosphorylation, glycosylation, and other processes. Many of these alterations are involved in the regulation of enzyme activity.

Enzymes Are Classified by the Reactions They Catalyze

Many enzymes have been named by adding the suffix "-ase" to the name of their substrate or to a word or phrase describing their activity. Thus, urease catalyzes hydrolysis of urea, and DNA polymerase catalyzes the polymerization of nucleotides to form DNA. Other enzymes were named by their discoverers for a broad function, before the specific reaction catalyzed was known. For example, an enzyme known to act in the digestion of foods was named pepsin, from the Greek *pepsis*, "digestion," and lysozyme was named for its ability to lyse (break down) bacterial cell walls. Still others were named for their source: trypsin, named in part from the Greek *tryein*, "to wear down," was obtained by rubbing pancreatic tissue with glycerin. Sometimes the same enzyme has two or more names, or two different enzymes have the same name. To limit ambiguity, biochemists worldwide have adopted a system for naming and classifying enzymes. This system divides enzymes into seven classes, each with subclasses, based on the type of reaction catalyzed (**Table 6-3**). Each enzyme is assigned a four-part classification number and a systematic name, which identifies the reaction it catalyzes. As an example, the formal systematic name of the enzyme catalyzing the reaction

$$\text{ATP} + \textsc{d}\text{-glucose} \longrightarrow \text{ADP} + \textsc{d}\text{-glucose 6-phosphate}$$

is ATP:D-hexose 6-phosphotransferase, which indicates that it catalyzes the transfer of a phosphoryl group from ATP to glucose. Its Enzyme Commission number (E.C. number) is 2.7.1.1. The first number (2) denotes the class name (transferase); the second number (7), the subclass (phosphotransferase); the third number (1), a phosphotransferase with a hydroxyl group as acceptor; and the fourth number (1), D-glucose as the phosphoryl group acceptor. For many enzymes, a trivial name is more frequently used—in this case, hexokinase. A complete list and description of the thousands of known enzymes is maintained by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (www.qmul.ac.uk/sbcs/iubmb).

SUMMARY 6.1 *An Introduction to Enzymes*

- Life depends on powerful and specific catalysts: enzymes. Almost every biochemical reaction is catalyzed by an enzyme.
- With the exception of a few catalytic RNAs, all known enzymes are proteins. Many require nonprotein coenzymes or cofactors for their catalytic function.
- Enzymes are classified according to the type of reaction they catalyze. All enzymes have formal E.C. numbers and names, and most have trivial names.

6.2 How Enzymes Work

The enzymatic catalysis of reactions is essential to living systems. Under biologically relevant conditions, uncatalyzed reactions tend to be slow—most biological molecules are quite stable in the neutral-pH, mild-temperature, aqueous environment inside cells. Reactions required to digest food, send nerve signals, or contract a muscle simply do not occur at a useful rate without catalysis.

P3 The distinguishing feature of an enzyme-catalyzed reaction is that it takes place within the confines of a pocket on the enzyme called the **active site** (**Fig. 6-1**). The active site provides a specific environment, customized by evolution, in which a given reaction can occur more rapidly. The molecule that is bound in the active site and acted upon

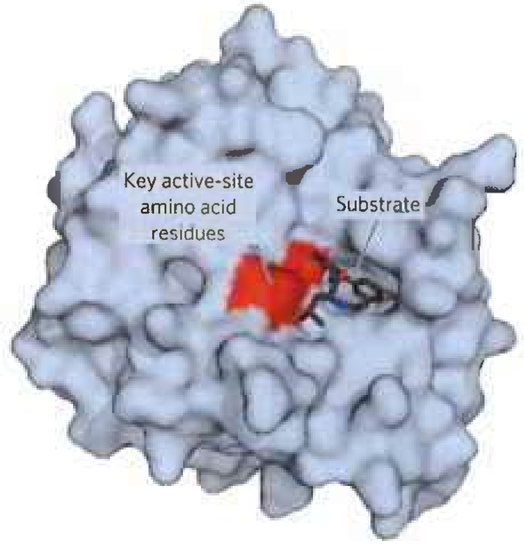

FIGURE 6-1 Binding of a substrate to an enzyme at the active site. The enzyme chymotrypsin with bound substrate. Some key active-site amino acid residues appear as a red splotch on the enzyme surface. [Data from PDB ID 7GCH, K. Brady et al., *Biochemistry* 29:7600, 1990.]

by the enzyme is called the **substrate**. The surface of the active site is lined with amino acid residues with substituent groups that bind the substrate and catalyze its chemical transformation. Often, the active site encloses a substrate, sequestering it completely from solution. The enzyme-substrate complex is central to the action of enzymes. It is also the starting point for mathematical treatments that define the kinetic behavior of enzyme-catalyzed reactions and for theoretical descriptions of enzyme mechanisms.

Enzymes Affect Reaction Rates, Not Equilibria

A simple enzymatic reaction might be written

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P \qquad (6\text{-}1)$$

where E, S, and P represent the enzyme, substrate, and product; ES and EP are transient complexes of the enzyme with the substrate and with the product.

To understand catalysis, we must first appreciate the important distinction between reaction equilibria and reaction rates. P1 The function of a catalyst is to increase the *rate* of a reaction. Catalysts do not affect reaction *equilibria*. (Recall that a reaction is at equilibrium when there is no net change in the concentrations of reactants or products.) Any reaction, such as S $\rightleftharpoons$ P, can be described by a reaction coordinate diagram (**Fig. 6-2**), a picture of the energy changes during the reaction. As discussed in Chapter 1, energy in biological systems is described in terms of free energy, *G*. In the coordinate diagram, the free energy of the system is plotted against the progress of the reaction (the reaction coordinate). The starting point for either the forward reaction or the reverse reaction is called the **ground state**, the contribution to the free energy of the system by an average molecule (S or P) under a given set of conditions.

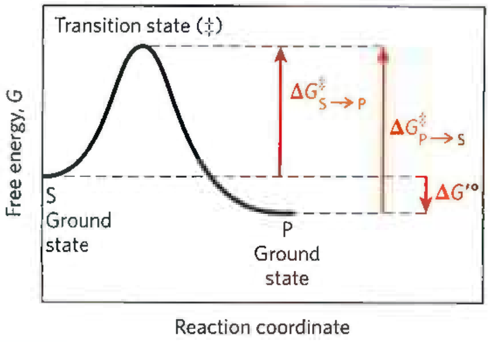

FIGURE 6-2 Reaction coordinate diagram. The free energy of the system is plotted against the progress of the reaction S → P. Such a diagram describes the energy changes during the reaction. The horizontal axis (reaction coordinate) reflects the progressive chemical changes (e.g., bond breakage or formation) as S is converted to P. The activation energies, $\Delta G^{\ddagger}$, for the S → P and P → S reactions are indicated. $\Delta G'^{\circ}$ is the overall standard free-energy change in the direction S → P.

KEY CONVENTION To describe the free-energy changes for reactions, chemists define a standard set of conditions (temperature of 298 K; partial pressure of each gas, 1 atm, or 101.3 kPa; concentration of each solute, 1 M), and express the free-energy change for a reacting system under these conditions as ΔG°, the **standard free-energy change**. Because biochemical systems commonly involve H^+ concentrations far below 1 M, biochemists define a **biochemical standard free-energy change, $\Delta G'^{\circ}$**, the standard free-energy change *at pH 7.0*; we employ this definition throughout the book. A more complete definition of $\Delta G'^{\circ}$ is given in Chapter 13. ■

The equilibrium between S and P reflects the difference in the free energies of their ground states. In the example shown in Figure 6-2, the free energy of the ground state of P is lower than that of S, so $\Delta G'^{\circ}$ for the reaction is negative (the reaction is exergonic) and at equilibrium there is more P than S (the equilibrium favors P).

A favorable equilibrium does not mean that the S → P conversion will occur at a rapid or even detectable rate. The *rate* of a reaction is instead dependent on an entirely different parameter, the energy barrier between S and P. That barrier consists of the energy required for alignment of reacting groups, formation of transient unstable charges, bond rearrangements, and other transformations required for the reaction to proceed in either direction. This is illustrated by the energy "hill" in Figures 6-2 and 6-3. To undergo reaction, the molecules must overcome this barrier and therefore must be raised to a higher energy level. At the top of the energy hill is a point at which decay to the S or P state is equally probable (it is downhill either way). This is called the **transition state**, often symbolized by a double dagger (‡). The transition state is not a chemical species with any significant stability and should not be confused with a reaction intermediate (such as ES or EP). It is simply a fleeting molecular moment in which events such as bond breakage, bond formation, and charge development have proceeded to the precise point at which decay to substrate and decay to product are equally likely.

The difference between the energy level of the ground state and the energy level of the transition state is the **activation energy**, $\Delta G^\ddagger$. The rate of a reaction reflects this activation energy: a higher activation energy corresponds to a slower reaction. Lowering the activation energy increases the rate of reaction. Reaction rates can be increased by raising the temperature and/or pressure, thereby increasing the number of molecules with sufficient energy to overcome the energy barrier. Alternatively, the activation energy can be lowered, and reaction rate increased, by adding a catalyst (**Fig. 6-3**).

Catalysts do not affect reaction equilibria. The bidirectional arrows in Equation 6-1 make this important point: any enzyme that catalyzes the reaction $S \rightarrow P$ also catalyzes the reaction $P \rightarrow S$. The role of enzymes is to *accelerate* the interconversion of S and P. The enzyme is not used up in the process, and the equilibrium point is unaffected. However, the reaction reaches equilibrium much faster when the appropriate enzyme is present, because the rate of the reaction is increased.

This relationship between reaction rate and activation energy is illustrated by the process introduced at the beginning of the chapter: the conversion of sucrose and oxygen to carbon dioxide and water:

$$C_{12}H_{22}O_{11} + 12O_2 \rightleftharpoons 12CO_2 + 11H_2O$$

This conversion, which takes place through a series of separate reactions, has a very large and negative $\Delta G'^\circ$, and at equilibrium the amount of sucrose present is negligible. Yet sucrose is a stable compound, because the activation energy barrier that must be overcome before sucrose reacts with oxygen is quite high. Sucrose can be stored in a container with oxygen almost indefinitely without reacting. In cells, however, sucrose is readily broken down to CO_2 and H_2O in a series of reactions catalyzed by enzymes. These enzymes not only accelerate the reactions, they organize and control them so that much of the energy released is recovered in other chemical forms and made available to the cell for other tasks. The reaction pathway by which sucrose (and other sugars) is broken down is the primary energy-yielding pathway for cells, and the enzymes of this pathway allow the reaction sequence to proceed on a biologically useful (millisecond) time scale.

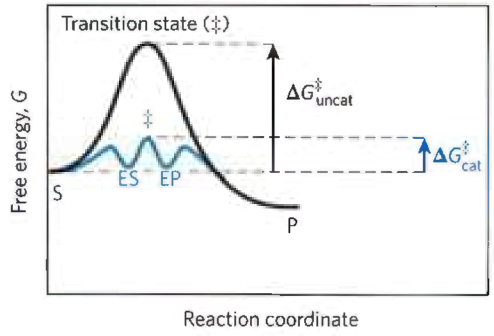

FIGURE 6-3 Reaction coordinate diagram comparing enzyme-catalyzed and uncatalyzed reactions. In the reaction $S \rightarrow P$, the ES and EP intermediates occupy minima in the energy progress curve of the enzyme-catalyzed reaction. The terms $\Delta G^\ddagger_{uncat}$ and $\Delta G^\ddagger_{cat}$ correspond to the activation energy for the uncatalyzed reaction and the overall activation energy for the catalyzed reaction, respectively. The activation energy is lower when the enzyme catalyzes the reaction.

Any reaction may have several steps, involving the formation and decay of transient chemical species called **reaction intermediates**.* A reaction intermediate is any species on the reaction pathway that has a finite chemical lifetime (longer than a molecular vibration, $\sim 10^{-13}$ second). When the $S \rightleftharpoons P$ reaction is catalyzed by an enzyme, the ES and EP complexes can be considered intermediates, even though S and P are stable chemical species (Eqn 6-1); the ES and EP complexes occupy valleys in the reaction coordinate diagram (Fig. 6-3). Additional, less-stable chemical intermediates often exist in the course of an enzyme-catalyzed reaction. The interconversion of two sequential reaction intermediates thus constitutes a reaction step. When several steps occur in a reaction, the overall rate is determined by the step (or steps) with the highest activation energy; this is called the **rate-limiting step**. In a simple case, the rate-limiting step is the highest-energy point in the diagram for interconversion of S and P. In practice, the rate-limiting step can vary with reaction conditions, and for many enzymes several steps may have similar activation energies, which means they are all partially rate-limiting.

Activation energies are energy barriers to chemical reactions. These barriers are crucial to life itself. Without such energy barriers, complex macromolecules would revert spontaneously to much simpler molecular forms, and the complex and highly ordered structures and metabolic processes of cells could not exist. **P2** Over the course of evolution, enzymes have developed to lower activation energies *selectively*, and thus increase rates, for reactions that are needed for cell survival.

Reaction Rates and Equilibria Have Precise Thermodynamic Definitions

P1 Reaction *equilibria* are inextricably linked to the standard free-energy change for the reaction, $\Delta G'^\circ$, and reaction *rates* are linked to the activation energy, $\Delta G^\ddagger$. A basic introduction to these thermodynamic relationships is the next step in understanding how enzymes work.

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* In this chapter, *step* and *intermediate* refer to chemical reactions and chemical species in the reaction pathway of a single enzyme-catalyzed reaction. In the context of metabolic pathways involving many enzymes (discussed in Part II), these terms are used somewhat differently: an entire enzymatic reaction is often referred to as a "step" in a pathway, and the product of one enzymatic reaction (which is the substrate for the next enzyme in the pathway) is referred to as a pathway "intermediate."

An equilibrium such as $S \rightleftharpoons P$ is described by an **equilibrium constant, K_{eq}**, or simply K (p. 23). Under the standard conditions used to compare biochemical processes, an equilibrium constant is denoted K'_{eq} (or K'):

$$K'_{eq} = \frac{[P]}{[S]} \qquad (6\text{-}2)$$

From thermodynamics, the relationship between K'_{eq} and $\Delta G'^\circ$ can be described by the expression

$$\Delta G'^\circ = -RT \ln K'_{eq} \qquad (6\text{-}3)$$

where R is the gas constant, 8.315 J/mol • K, and T is the absolute temperature, 298 K (25 °C). Equation 6-3 is developed and discussed in more detail in Chapter 13. The important point here is that the equilibrium constant is directly related to the overall standard free-energy change for the reaction (**Table 6-4**). A large negative value for $\Delta G'^\circ$ reflects a favorable reaction equilibrium (one in which there is much more product than substrate at equilibrium)—but as already noted, this does not mean the reaction will proceed at a rapid rate.

The rate of any reaction is determined by the concentration of the reactant (or reactants) and by a **rate constant**, usually denoted by k. For the unimolecular reaction $S \rightarrow P$, the rate (or velocity) of the reaction, V—representing the amount of S that reacts per unit of time—is expressed by a **rate equation**:

$$V = k[S] \qquad (6\text{-}4)$$

In this reaction, the rate depends only on the concentration of S. This is called a first-order reaction. The factor k is a proportionality constant that reflects the probability of reaction under a given set of conditions (pH, temperature, and so forth). Here, k is a first-order rate constant and has units of reciprocal time, such as s^{-1}. If a first-order reaction has a rate constant k of 0.03 s^{-1}, this may be interpreted (qualitatively) to mean that 3% of the available S will be converted to P in 1 second. A reaction with a rate constant of 2,000 s^{-1} will be over in a small fraction of a second. If a reaction rate depends on the concentration of two different compounds, or if the reaction is between two molecules of the same compound, the reaction is second order and k is a second-order rate constant, with units of $M^{-1}s^{-1}$. The rate equation then becomes

$$V = k[S_1][S_2] \qquad (6\text{-}5)$$

TABLE 6-4 Relationship between K'_{eq} and $\Delta G'^\circ$

K'_{eq}	$\Delta G'^\circ$ (kJ/mol)
10^{-6}	34.2
10^{-5}	28.5
10^{-4}	22.8
10^{-3}	17.1
10^{-2}	11.4
10^{-1}	5.7
1	0.0
10^{1}	–5.7
10^{2}	–11.4
10^{3}	–17.1

Note: The relationship is calculated from $\Delta G'^\circ = -RT \ln K'_{eq}$ (Eqn 6-3).

From transition-state theory we can derive an expression that relates the magnitude of a rate constant to the activation energy:

$$k = \frac{\mathbf{k}T}{h} e^{-\Delta G^{\ddagger}/RT} \qquad (6\text{-}6)$$

where **k** is the Boltzmann constant and h is Planck's constant. The important point is that the relationship between the rate constant k and the activation energy $\Delta G^{\ddagger}$ is inverse and exponential. In simplified terms, this is the basis for the statement that **P1** a lower activation energy means a faster reaction rate—and lowering activation energies is what enzymes do.

Now we turn from *what* enzymes do to *how* they do it.

A Few Principles Explain the Catalytic Power and Specificity of Enzymes

Enzymes are extraordinary catalysts. The rate enhancements they bring about are in the range of 5 to 17 orders of magnitude (**Table 6-5**). Enzymes are also very specific, readily discriminating between substrates with quite similar structures. How can these enormous and highly selective rate enhancements be explained? What is the source of the energy for the dramatic lowering of the activation energies for specific reactions?

The answer to these questions has two distinct but interwoven parts. The first lies in the *noncovalent* interactions between enzyme and substrate. What

TABLE 6-5 Some Rate Enhancements Produced by Enzymes

Cyclophilin	10^{5}
Carbonic anhydrase	10^{7}
Triose phosphate isomerase	10^{9}
Carboxypeptidase A	10^{11}
Phosphoglucomutase	10^{12}
Succinyl-CoA transferase	10^{13}
Urease	10^{14}
Orotidine monophosphate decarboxylase	10^{17}

really sets enzymes apart from most other catalysts is the formation of a specific ES complex. The interaction between substrate and enzyme in this complex is mediated by the same forces that stabilize protein structure, including hydrogen bonds, ionic interactions, and the hydrophobic effect (Chapter 4). Formation of each weak interaction in the ES complex is accompanied by release of a small amount of free energy that stabilizes the interaction. The energy derived from noncovalent enzyme-substrate interaction is called **binding energy, ΔG_B**. Its significance extends beyond a simple stabilization of the enzyme-substrate interaction. As we will see, **P4** *binding energy is a major source of free energy used by enzymes to lower the activation energies of reactions.*

The second part of the explanation lies in covalent interactions between enzyme and substrate, plus a few additional chemical catalytic mechanisms. Chemical reactions of many types take place between substrates and enzymes' functional groups (specific amino acid side chains, metal ions, and coenzymes). Catalytic functional groups on an enzyme may form a transient covalent bond with a substrate and activate it for reaction, or a group may be transiently transferred from the substrate to the enzyme. In many cases, these reactions occur only in the enzyme active site. Covalent interactions between enzymes and substrates lower the activation energy (and thereby accelerate the reaction) by providing an alternative, lower-energy reaction path. Metal ions facilitate additional mechanisms of catalysis that do not involve covalent interactions.

We now consider noncovalent contributions to catalysis and the additional chemical mechanisms in turn.

Noncovalent Interactions between Enzyme and Substrate Are Optimized in the Transition State

How does an enzyme use noncovalent binding energy to lower the activation energy for a reaction? Formation of the ES complex is not the explanation in itself, although some of the earliest considerations of enzyme mechanisms began with this idea. Studies on enzyme specificity carried out by Emil Fischer led him to propose, in 1894, that enzymes were structurally complementary to their substrates, so that they fit together like a lock and key (**Fig. 6-4**). This elegant idea, that a specific (exclusive) interaction between two biological molecules is mediated by molecular surfaces with complementary shapes, has greatly influenced the development of biochemistry. However, the "lock and key" hypothesis can be misleading when applied to enzymatic catalysis. An enzyme completely complementary to its substrate would be a very poor enzyme, as we can demonstrate.

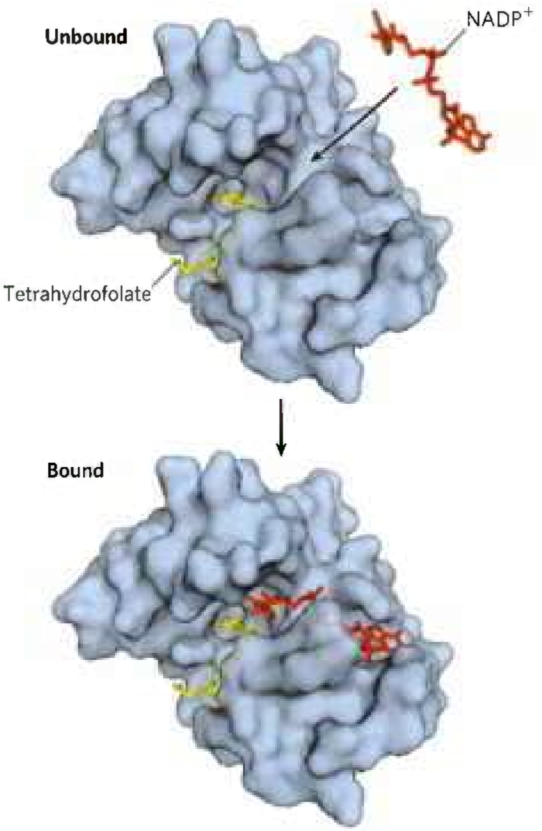
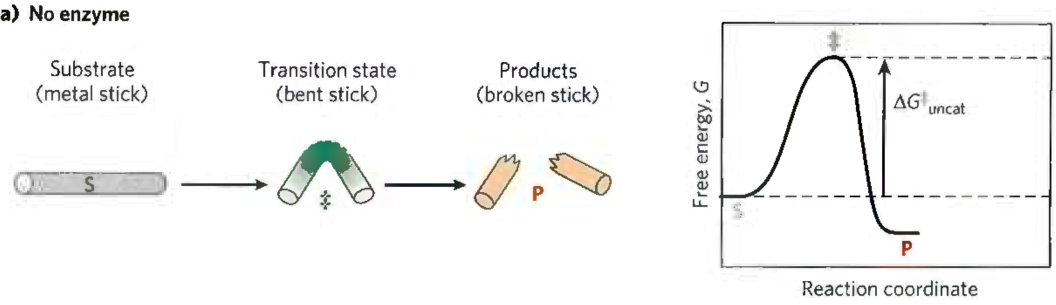


FIGURE 6-4 Complementary shapes of a substrate and its binding site on an enzyme. The enzyme dihydrofolate reductase with its substrate NADP$^+$, unbound and bound; another bound substrate, tetrahydrofolate, is also visible. In this model, the NADP$^+$ binds to a pocket that is complementary to it in shape and ionic properties, an illustration of Emil Fischer's "lock and key" hypothesis of enzyme action. In reality, the complementarity between protein and ligand (in this case, substrate) is rarely perfect, as we saw in Chapter 5. [Data from PDB ID 1RA2, M. R. Sawaya and J. Kraut, *Biochemistry* 36:586, 1997.]

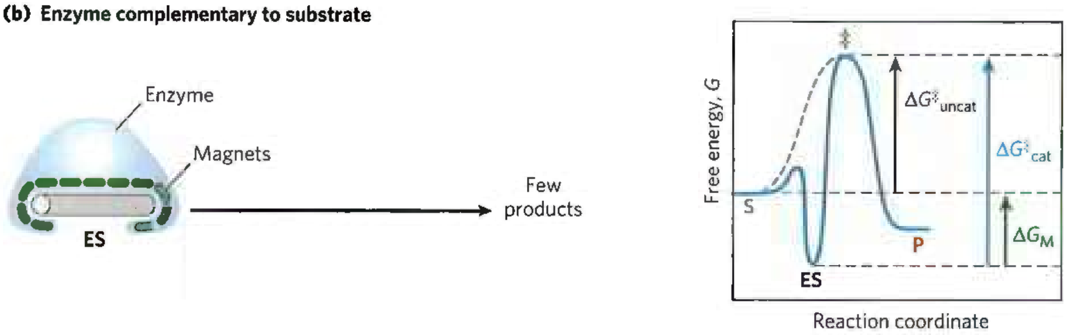
Consider an imaginary reaction, the breaking of a magnetized metal stick. The uncatalyzed reaction is shown in **Figure 6-5a**. Let's examine two imaginary enzymes—two "stickases"—that could catalyze this reaction, both of which employ magnetic forces as a paradigm for the binding energy used by real enzymes. We first design an enzyme perfectly complementary to the substrate (Fig. 6-5b). The active site of this stickase is a pocket lined with magnets. To react (break), the stick must reach the bent transition state of the reaction. However, the snug fit of the stick in this active site means that magnets hinder the required bending. Such an enzyme *impedes* the reaction, stabilizing the substrate instead. In a reaction coordinate diagram (Fig. 6-5b), this kind of ES complex would correspond to an energy trough from which the substrate would have difficulty escaping. Such an enzyme would be useless.

The modern notion of enzymatic catalysis, first proposed by Michael Polanyi (1921) and J. B. S. Haldane (1930), was elaborated by Linus Pauling in 1946 and by William P. Jencks in the 1970s: in order to catalyze reactions, an enzyme must be complementary to the

(a) No enzyme



(b) Enzyme complementary to substrate



(c) Enzyme complementary to transition state

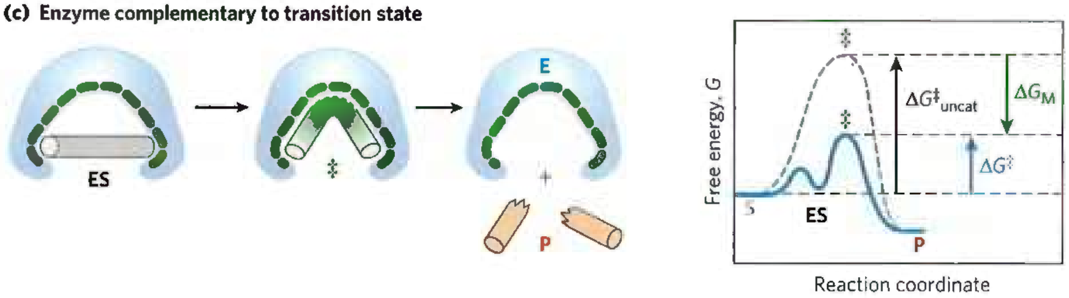


FIGURE 6-5 An imaginary enzyme (stickase) designed to catalyze breakage of a metal stick. (a) Before the stick is broken, it must first be bent (the transition state). In both stickase examples, magnetic interactions take the place of weak bonding interactions between enzyme and substrate. (b) A stickase with a magnet-lined pocket complementary in structure to the stick (the substrate) stabilizes the substrate. Bending is impeded by the magnetic attraction between stick and stickase. (c) An enzyme with a pocket complementary to the reaction transition state helps to destabilize the stick, contributing to catalysis of the reaction. The binding energy of the magnetic interactions compensates for the increase in free energy required to bend the stick. Reaction coordinate diagrams (right) show the energy consequences of complementarity to substrate versus complementarity to transition state (EP complexes are omitted). ΔG_M, the difference between the transition-state energies of the uncatalyzed and catalyzed reactions, is contributed by the magnetic interactions between the stick and stickase. When the enzyme is complementary to the substrate (b), the ES complex is more stable and has less free energy in the ground state than substrate alone. The result is an *increase* in the activation energy.

reaction transition state. This means that optimal interactions between substrate and enzyme occur only in the transition state. Figure 6-5c demonstrates how such an enzyme can work. The metal stick binds to the stickase, but only a few of the possible magnetic interactions are used in forming the ES complex. The bound substrate must still undergo the increase in free energy needed to reach the transition state. Now, however, the increase in free energy required to draw the stick into a bent and partially broken conformation is offset, or "paid for," by the magnetic interactions that form between our imaginary enzyme and substrate (analogous to the binding energy in a real enzyme) in the transition state. Many of these interactions involve parts of the stick that are distant from the point of breakage; thus, interactions between the stickase and nonreacting parts of the stick provide some of the energy needed to catalyze stick breakage. This "energy payment" translates into a lower net activation energy and a faster reaction rate.

Real enzymes work on an analogous principle. **P4** Some weak interactions are formed in the ES complex, but the full complement of such interactions between substrate and enzyme is formed only when the substrate reaches the transition state. The free energy (binding energy) released by the formation of these interactions partially offsets the energy required to reach the top of the energy hill. The summation of the unfavorable (positive) activation energy $\Delta G^{\ddagger}$ and the favorable (negative) binding energy ΔG_B results

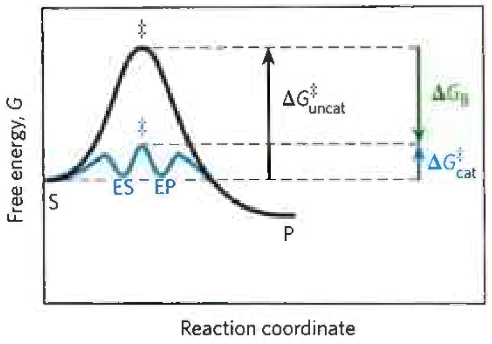


FIGURE 6-6 Role of binding energy in catalysis. To lower the activation energy for a reaction, the system must acquire an amount of energy equivalent to the amount by which $\Delta G^{\ddagger}$ is lowered. Much of this energy comes from binding energy, ΔG_B, contributed by formation of weak noncovalent interactions between substrate and enzyme in the transition state. The role of ΔG_B is analogous to that of ΔG_M in Figure 6-5.

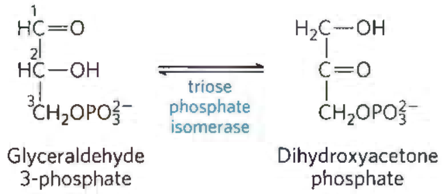
in a lower *net* activation energy (**Fig. 6-6**). Even on the enzyme, the transition state is not a stable species but a brief point in time that the substrate spends atop an energy hill. However, the enzyme-catalyzed reaction is much faster than the uncatalyzed process because the hill is much smaller. **P4** The important principle is that *weak binding interactions between the enzyme and the substrate provide a substantial driving force for enzymatic catalysis*. The groups on the substrate that are involved in these weak interactions can be at some distance from the bonds that are broken or changed. The weak interactions formed only in the transition state are those that make the primary contribution to catalysis.

The requirement for multiple weak interactions to drive catalysis is one reason why enzymes (and some coenzymes) are so large. An enzyme must provide functional groups for ionic, hydrogen-bond, and other interactions, and also must precisely position these groups so that binding energy is optimized in the transition state. **P3** Adequate binding is accomplished most readily by positioning a substrate in a cavity (the active site) where it is effectively removed from water. The size of proteins reflects the need for superstructure to keep interacting groups properly positioned and to keep the cavity from collapsing.

Can we demonstrate quantitatively that binding energy accounts for the huge rate accelerations brought about by enzymes? Yes. As a point of reference, Equation 6-6 allows us to calculate that $\Delta G^{\ddagger}$ must be lowered by about 5.7 kJ/mol to accelerate a first-order reaction by a factor of 10, under conditions commonly found in cells. The energy available from formation of a single weak interaction is generally estimated to be 4 to 30 kJ/mol. The overall energy available from many such interactions is therefore sufficient to lower activation energies by the 60 to 100 kJ/mol required to explain the large rate enhancements observed for many enzymes.

P2 The same binding energy that provides energy for catalysis also gives an enzyme its **specificity**, the ability to discriminate between a substrate and a competing molecule. Conceptually, specificity is easy to distinguish from catalysis, but this distinction is much more difficult to make experimentally, because catalysis and specificity arise from the same phenomenon. If an enzyme active site has functional groups arranged optimally to form a variety of weak interactions with a particular substrate in the transition state, the enzyme will not be able to interact to the same degree with any other molecule. For example, if the substrate has a hydroxyl group that forms a hydrogen bond with a specific Glu residue on the enzyme, any molecule lacking a hydroxyl group at that particular position will be a poorer substrate for the enzyme. **P2** In general, *specificity* is derived from the formation of many weak interactions between the enzyme and its specific substrate molecule.

The importance of binding energy to catalysis can be readily demonstrated. For example, the glycolytic enzyme triose phosphate isomerase catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate:



This reaction rearranges the carbonyl and hydroxyl groups on carbons 1 and 2. However, more than 80% of the enzymatic rate acceleration has been traced to enzyme-substrate interactions involving the phosphate group on carbon 3 of the substrate. This was determined by comparing the enzyme-catalyzed reactions with glyceraldehyde 3-phosphate and with glyceraldehyde (no phosphate group at position 3) as substrate.

The general principles outlined above can be illustrated by a variety of recognized catalytic mechanisms. These mechanisms are not mutually exclusive, and a given enzyme might incorporate several catalytic strategies in its overall mechanism of action.

Consider what needs to occur for a reaction to take place. Prominent physical and thermodynamic factors contributing to $\Delta G^{\ddagger}$, the barrier to reaction, might include (1) the entropy of molecules in solution, which reduces the possibility that they will react together; (2) the solvation shell of hydrogen-bonded water that surrounds and helps to stabilize most biomolecules in aqueous solution; (3) the distortion of substrates that must occur in many reactions; and (4) the need for proper alignment of catalytic functional groups on the enzyme. Binding energy can be used to overcome all these barriers.

First, a large restriction in the relative motions of two substrates that are to react, or **entropy reduction**, is one obvious benefit of binding them to an enzyme. Binding energy constrains the substrates in the proper orientation to react — a substantial contribution to catalysis, because productive collisions between molecules in solution can be exceedingly rare. Substrates can be precisely aligned on the enzyme, with many weak interactions between each substrate and strategically located groups on the enzyme clamping the substrate molecules into the proper positions. Studies have shown that simply constraining the motion of two reactants can produce rate enhancements of many orders of magnitude (**Fig. 6-7**).

Second, in water, many organic molecules are surrounded by a solvation shell of structured water that can hinder reactions (see Fig. 2-8). Formation of weak bonds between substrate and enzyme results in **desolvation** of the substrate. Enzyme-substrate interactions replace most or all of the hydrogen bonds between the substrate and water that would otherwise impede reaction.

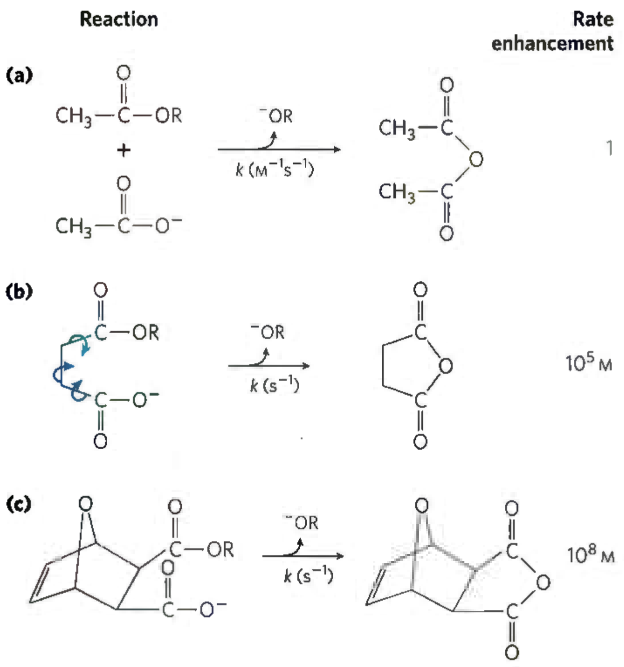


FIGURE 6-7 Rate enhancement by entropy reduction. Shown here are reactions of an ester with a carboxylate group to form an anhydride. The R group is the same in each case. (a) For this bimolecular reaction, the rate constant k is second order, with units of $\text{M}^{-1}\text{s}^{-1}$. (b) When the two reacting groups are in a single molecule, and thus have less freedom of motion, the reaction is much faster. For this unimolecular reaction, k has units of s^{-1}. Dividing the rate constant for (b) by the rate constant for (a) gives a rate enhancement of about 10^5 M. (The enhancement has units of molarity because we are comparing a unimolecular reaction and a bimolecular reaction.) Put another way, if the reactant in (b) were present at a concentration of 1 M, the reacting groups would *behave* as though they were present at a concentration of 10^5 M. Note that the reactant in (b) has freedom of rotation about three bonds (shown with curved arrows), but this still represents a substantial reduction of entropy over (a). If the bonds that rotate in (b) are constrained as in (c), the entropy is reduced further and the reaction exhibits a rate enhancement of 10^8 M relative to (a).

Third, binding energy involving weak interactions that are formed only in the reaction transition state helps to compensate thermodynamically for the unfavorable free-energy change associated with any distortion, primarily electron redistribution, that the substrate must undergo to react.

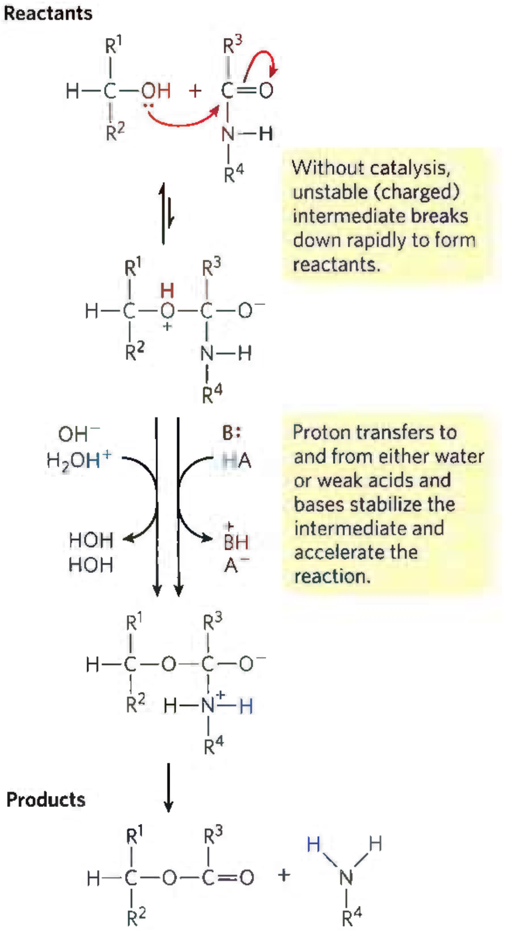
Finally, the enzyme itself usually undergoes a change in conformation when the substrate binds, induced by multiple weak interactions with the substrate. This is referred to as **induced fit**, a mechanism postulated by Daniel Koshland in 1958. The motions can affect a small part of the enzyme near the active site or can involve changes in the positioning of entire domains. Typically, a network of coupled motions occurs throughout the enzyme that ultimately brings about the required changes in the active site. Induced fit serves to bring specific functional groups on the enzyme into the proper position to catalyze the reaction. The conformational change also permits formation of additional weak bonding interactions in the transition state. In either case, the new enzyme conformation has enhanced catalytic properties. As we have seen, induced fit is a common feature of the reversible binding of ligands to proteins (Chapter 5). Induced fit is also important in the interaction of almost every enzyme with its substrate.

Covalent Interactions and Metal Ions Contribute to Catalysis

In most enzymes, noncovalent binding energy is just one of several contributors to the overall catalytic mechanism. P4 Once a substrate is bound to an enzyme, properly positioned catalytic functional groups aid in the cleavage and formation of bonds by a variety of mechanisms, including general acid-base catalysis, covalent catalysis, and metal ion catalysis. The first two of these mechanisms are distinct from those based on binding energy, because they generally involve transient *covalent* interaction with a substrate or group transfer to or from a substrate.

General Acid-Base Catalysis Transfer of a proton from one molecule to another is the single most common reaction in biochemistry. One or, often, many proton transfers occur in the course of most reactions that take place in cells. Many biochemical reactions occur through the formation of unstable charged intermediates that tend to break down rapidly to their constituent reactant species, impeding the forward reaction (**Fig. 6-8**). Charged intermediates can often be stabilized by the transfer of protons to form a species that breaks down more readily to products. These protons are transferred between an enzyme and a substrate or intermediate.

The effects of catalysis by acids and bases are often studied using nonenzymatic model reactions, in which the proton donors or acceptors are either the constituents of water alone or other weak acids and bases.



Without catalysis, unstable (charged) intermediate breaks down rapidly to form reactants.

Proton transfers to and from either water or weak acids and bases stabilize the intermediate and accelerate the reaction.

FIGURE 6-8 How a catalyst circumvents unfavorable charge development during cleavage of an amide. The hydrolysis of an amide bond, shown here, is the same reaction as that catalyzed by chymotrypsin and other proteases. Charge development is unfavorable and can be circumvented by donation of a proton by H_3O^+ (specific acid catalysis) or HA (general acid catalysis), where HA represents any acid. Similarly, charge can be neutralized by proton abstraction by OH^- (specific base catalysis) or B: (general base catalysis), where B: represents any base.

Catalysis of the type that uses only the H^+ (H_3O^+) or OH^- ions present in water is referred to as **specific acid-base catalysis**. However, a further increase in rate can often be afforded by the addition of weak acids and bases to the reaction. Many weak organic acids can supplement water as proton donors, or weak organic bases can supplement water as proton acceptors. The term **general acid-base catalysis** refers to proton transfers mediated by weak acids and bases other than water.

General acid-base catalysis becomes crucial in the active site of an enzyme, where water may not be available as a proton donor or acceptor. Several amino acid side chains can and do take on the role of proton donors and acceptors (**Fig. 6-9**). These groups can be precisely positioned in an enzyme active site to allow proton transfers, providing rate enhancements of the order of 10^2 to 10^5. This type of catalysis occurs on the vast majority of enzymes.

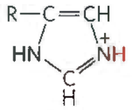
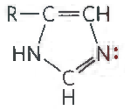
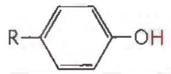
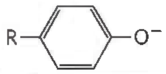
Amino acid residues	General acid form (proton donor)	General base form (proton acceptor)
Glu, Asp	$\mathrm{R{-}COOH}$	$\mathrm{R{-}COO^-}$
Lys, Arg	$\mathrm{R{-}\overset{+}{N}H_3}$	$\mathrm{R{-}\ddot{N}H_2}$
Cys	$\mathrm{R{-}SH}$	$\mathrm{R{-}S^-}$
His		
Ser	$\mathrm{R{-}OH}$	$\mathrm{R{-}O^-}$
Tyr		

FIGURE 6-9 Amino acids in general acid-base catalysis. Many organic reactions that are used to model biochemical processes are promoted by proton donors (general acids) or proton acceptors (general bases). The active sites of some enzymes contain amino acid functional groups, such as those shown here, that can participate in the catalytic process as proton donors or proton acceptors.

Covalent Catalysis In **covalent catalysis**, a transient covalent bond is formed between the enzyme and the substrate. Consider the hydrolysis of a bond between groups A and B:

$$\mathrm{A{-}B} \xrightarrow{\mathrm{H_2O}} \mathrm{A} + \mathrm{B}$$

In the presence of a covalent catalyst (an enzyme with a nucleophilic group X:) the reaction becomes

$$\mathrm{A{-}B} + \mathrm{X{:}} \longrightarrow \mathrm{A{-}X} + \mathrm{B} \xrightarrow{\mathrm{H_2O}} \mathrm{A} + \mathrm{X{:}} + \mathrm{B}$$

Formation and breakdown of a covalent intermediate creates a new pathway for the reaction, but catalysis results *only* when the new pathway has a lower activation energy than the uncatalyzed pathway. Both of the new steps must be faster than the uncatalyzed reaction. Several amino acid side chains, including all those in Figure 6-9, and the functional groups of some enzyme cofactors can serve as nucleophiles in the formation of covalent bonds with substrates. These covalent complexes always undergo further reaction to regenerate the free enzyme. The covalent bond formed between the enzyme and the substrate can activate a substrate for further reaction in a manner that is usually specific to the particular group or coenzyme.

Metal Ion Catalysis Metals, whether tightly bound to the enzyme or taken up from solution along with the substrate, can participate in catalysis in several ways. Ionic interactions between an enzyme-bound metal and a substrate can help orient the substrate for reaction or stabilize charged reaction transition states. This use of weak bonding interactions between metal and substrate is similar to some of the uses of enzyme-substrate binding energy described earlier, and it can contribute to enzyme–transition state complementarity. Metals can also mediate oxidation-reduction reactions by reversible changes in the metal ion's oxidation state. Nearly a third of all known enzymes require one or more metal ions for catalytic activity.

Most enzymes combine several catalytic strategies to bring about a rate enhancement. A good example is the use of covalent catalysis, general acid-base catalysis, and transition-state stabilization in the reaction catalyzed by chymotrypsin, detailed in Section 6.4.

SUMMARY 6.2 *How Enzymes Work*

- Enzymes are highly effective catalysts, commonly enhancing reaction rates by a factor of 10^5 to 10^{17}. Enzyme-catalyzed reactions are characterized by the formation of a complex between substrate and enzyme (an ES complex). Substrate binding occurs in a pocket on the enzyme called the active site.
- The function of enzymes and other catalysts is to lower the activation energy, $\Delta G^{\ddagger}$, for a reaction and thereby enhance the reaction rate. The equilibrium of a reaction is unaffected by the enzyme.
- Reaction equilibria are described by equilibrium constants, K_{eq}, which are related to the biochemical standard free-energy change $\Delta G'^{\circ}$. Reaction rates are described by rate constants, k, which are related to the activation energy $\Delta G^{\ddagger}$.
- The extraordinary rate accelerations provided by enzymes are due to noncovalent binding energy supplemented by covalent interactions or metal ion catalysis.
- Noncovalent binding energy, ΔG_B, is maximized in the transition state of the catalyzed reaction. Enzyme–transition state complementarity is a fundamental principle of enzymatic catalysis. Noncovalent interactions may facilitate the path to the transition state, offsetting the energy required for activation, $\Delta G^{\ddagger}$, by lowering substrate entropy, causing substrate desolvation, or causing a conformational change in the enzyme (induced fit). Binding energy also accounts for the exquisite specificity of enzymes for their substrates.
- General acid-base catalysis and covalent catalysis mechanisms contribute to the catalytic power of enzymes. Covalent interactions between the substrate and the enzyme, group transfers to and from the enzyme, and interactions with metal ions can provide a new, lower-energy reaction path.

6.3 Enzyme Kinetics as an Approach to Understanding Mechanism

Biochemists commonly use several approaches to study the mechanism of action of purified enzymes. The three-dimensional structure of the protein provides important information, which is enhanced by traditional protein chemistry and modern methods of site-directed mutagenesis (changing the amino acid sequence of a protein by genetic engineering; see Fig. 9-10). These technologies permit enzymologists to examine the role of individual amino acids in enzyme structure and action. However, the oldest approach to understanding enzyme mechanisms, and one that remains very important, is to determine the *rate* of a reaction and how it changes in response to changes in experimental parameters, a discipline known as **enzyme kinetics**. We provide here a basic introduction to the kinetics of enzyme-catalyzed reactions.

Substrate Concentration Affects the Rate of Enzyme-Catalyzed Reactions

At any given instant in an enzyme-catalyzed reaction, the enzyme exists in two forms: the free or uncombined form E and the substrate-combined form ES. When the enzyme is first mixed with a large excess of substrate, there is an initial transient period, the **pre–steady state**, during which the concentration of ES builds up. For most enzymatic reactions, this period is very brief. The pre-steady state is frequently too short to be observed easily, lasting only the time (often microseconds) required to convert one molecule of substrate to product (one enzymatic turnover). The reaction quickly achieves a **steady state** in which [ES] (and the concentrations of any other intermediates) remains approximately constant for much of the remainder of the reaction (**Fig. 6–10**). As most of the reaction reflects the steady state, the traditional analysis of reaction rates is referred to as **steady-state kinetics**.

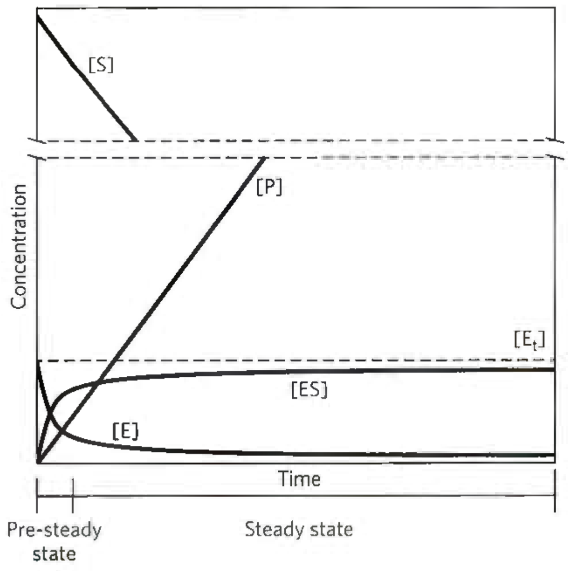


FIGURE 6-10 The course of an enzyme-catalyzed reaction. In a typical reaction, product will increase as substrate declines. The concentration of free enzyme, E, declines rapidly, as the concentration of the ES complex increases and reaches a steady state. The steady-state concentration of ES remains nearly constant for much of the remainder of the reaction.

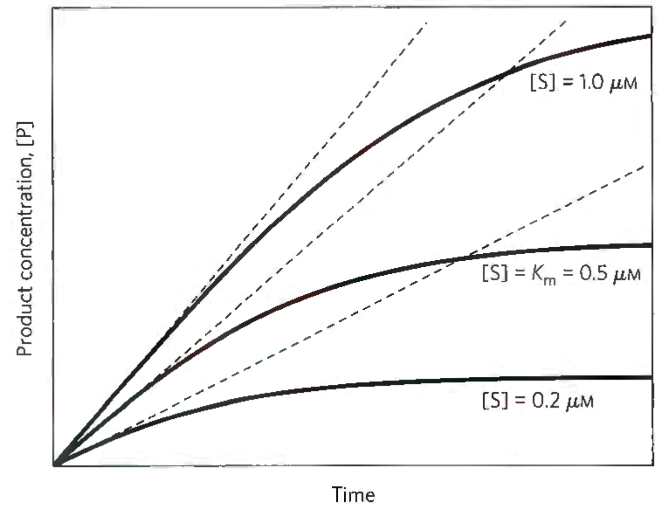


FIGURE 6-11 Initial velocities of enzyme-catalyzed reactions. A theoretical enzyme that catalyzes the reaction S $\rightleftharpoons$ P is present at a concentration of S sufficient to catalyze the reaction at a maximum velocity, defined as V_{max}, of 1 μM/min. The rate observed at a given concentration of S depends on the Michaelis constant, K_m, explained in more detail in the next section. Here, the K_m is 0.5 μM. Progress curves are shown for substrate concentrations below, at, and above the K_m. The rate of an enzyme-catalyzed reaction declines as substrate is converted to product. A tangent to each curve taken at time = 0 (dashed line) defines the initial velocity, V_0, of each reaction.

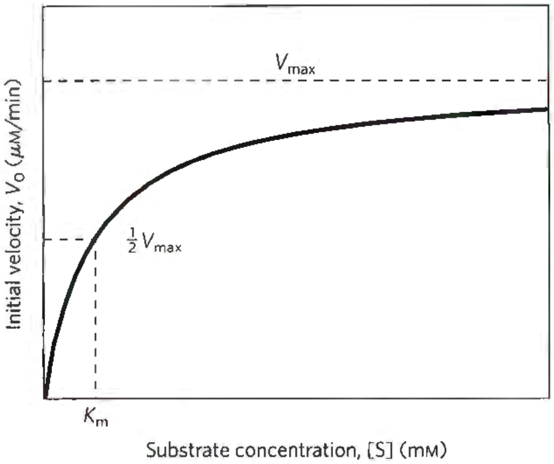


FIGURE 6-12 Effect of substrate concentration on the initial velocity of an enzyme-catalyzed reaction. The maximum velocity, V_{max}, is extrapolated from the plot, because V_0 approaches but never quite reaches V_{max}. The substrate concentration at which V_0 is half-maximal is K_m, the Michaelis constant. The concentration of enzyme in an experiment such as this is generally so low that [S] >> [E] even when [S] is described as low or relatively low. The units shown are typical for enzyme-catalyzed reactions and are given only to help illustrate the meaning of V_0 and [S]. (Note that the curve describes *part* of a rectangular hyperbola, with one asymptote at V_{max}. If the curve were continued below [S] = 0, it would approach a vertical asymptote at [S] = $-K_m$.)

A key factor affecting the rate of a reaction catalyzed by an enzyme is the concentration of substrate, [S]. However, studying the effects of substrate concentration is complicated by the fact that [S] changes during the course of an in vitro reaction as substrate is converted to product. One simplifying approach is to measure the **initial rate** (or **initial velocity**), designated V_0 (**Fig. 6-11**). In a typical reaction, the enzyme may be present in nanomolar quantities, whereas [S] may be five or six orders of magnitude higher. If only the beginning of the reaction is monitored, over a period in which only a small percentage of the available substrate (<2%–3%) is converted to product, [S] can be regarded as constant, to a reasonable approximation. V_0 can then be explored as a function of [S], which is adjusted by the investigator. Note that even the initial rate reflects a steady state.

The effect on V_0 of varying [S] when the enzyme concentration is held constant is shown in **Figure 6-12**. At relatively low concentrations of substrate, V_0 increases almost linearly with an increase in [S]. At higher substrate concentrations, V_0 increases by smaller and smaller amounts in response to increases in [S]. Finally, a point is reached beyond which increases in V_0 are vanishingly small as [S] increases. This plateau-like V_0 region is close to the **maximum velocity, V_{max}**.

The ES complex is the key to understanding this kinetic behavior, just as it was a starting point for our discussion of catalysis. The kinetic pattern in Figure 6-12 led Victor Henri, following a proposal by Adolphe Wurtz a few decades earlier, to propose in 1903 that the combination of an enzyme with its substrate molecule to form an ES complex is a necessary step in enzymatic catalysis. This idea was expanded into a general theory of enzyme action, particularly by Leonor Michaelis and Maud Menten in 1913. They postulated that the enzyme first combines reversibly with its substrate to form an enzyme-substrate complex in a relatively fast reversible step:

$$\mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \tag{6-7}$$

The ES complex then breaks down in a slower second step to yield the free enzyme and the reaction product P:

$$\mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E + P} \tag{6-8}$$

Because the slower second reaction (Eqn 6-8) must limit the rate of the overall reaction, the overall rate must be proportional to the concentration of the species that reacts in the second step—that is, ES.

Leonor Michaelis, 1875–1949
[Rockefeller Archive Center.]

Maud Menten, 1879–1960
[Archives & Special Collections, University of Pittsburgh Library System.]

At low [S], most of the enzyme is in the uncombined form E. Here, the rate is proportional to [S] because the equilibrium of Equation 6-7 is pushed toward formation of more ES as [S] increases. The maximum initial rate of the catalyzed reaction ($V_{\max}$) is observed when virtually all the enzyme is present as the ES complex and [E] is vanishingly small. Under these conditions, the enzyme is "saturated" with its substrate, so that further increases in [S] have no effect on rate. This condition exists when [S] is sufficiently high that essentially all the free enzyme has been converted to the ES form. After the ES complex breaks down to yield the product P, the enzyme is free to catalyze the reaction of another molecule of substrate (and will do so rapidly under saturating conditions). The saturation effect is a distinguishing characteristic of enzymatic catalysts and is responsible for the plateau observed in Figure 6-12, and the pattern seen in the figure is sometimes referred to as saturation kinetics.

The Relationship between Substrate Concentration and Reaction Rate Can Be Expressed with the Michaelis-Menten Equation

The curve expressing the relationship between [S] and V_0 (Fig. 6-12) has the same general shape for most enzymes (it approaches a rectangular hyperbola), which can be expressed algebraically by the Michaelis-Menten equation. Michaelis and Menten derived this equation starting from their basic hypothesis that the rate-limiting step in enzymatic reactions is the breakdown of the ES complex to product and free enzyme. The equation is

$$V_0 = \frac{V_{\max}[\mathrm{S}]}{K_{\mathrm{m}} + [\mathrm{S}]} \tag{6-9}$$

This is the **Michaelis-Menten equation**, the **rate equation** for a one-substrate enzyme-catalyzed reaction. It is a statement of the quantitative relationship between the initial velocity V_0, the maximum velocity $V_{\max}$, and the initial substrate concentration [S], all related through a constant, K_{m}, called the Michaelis constant. All these terms—[S], V_0, $V_{\max}$, and K_{m}—are readily measured experimentally.

Here we develop the basic logic and the algebraic steps in a modern derivation of the Michaelis-Menten equation, which includes the steady-state assumption, a concept introduced by G. E. Briggs and J. B. S. Haldane in 1925. The derivation starts with the two basic steps of the formation and breakdown of ES (Eqns 6-7 and 6-8). Early in the reaction, the concentration of the product, [P], is negligible, and we make the simplifying assumption that the reverse reaction, P → S (described by k_{-2}), can be ignored. This assumption is not critical but it simplifies our task. The overall reaction then reduces to

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E} + \mathrm{P} \tag{6-10}$$

V_0 is determined by the breakdown of ES to form product, which is determined by [ES]:

$$V_0 = k_2[\mathrm{ES}] \tag{6-11}$$

Because [ES] in Equation 6-11 is not easily measured experimentally, we must begin by finding an alternative expression for this term. First, we introduce the term $[\mathrm{E_t}]$, representing the total enzyme concentration (the sum of free and substrate-bound enzyme). Free or unbound enzyme [E] can then be represented by $[\mathrm{E_t}] - [\mathrm{ES}]$. Also, because [S] is ordinarily far greater than $[\mathrm{E_t}]$, the amount of substrate bound by the enzyme at any given time is negligible compared with the total [S]. With these conditions in mind, the following steps lead us to an expression for V_0 in terms of easily measurable parameters.

Step 1 The rates of formation and breakdown of ES are determined by the steps governed by the rate constants k_1 (formation) and $k_{-1} + k_2$ (breakdown to reactants and products, respectively), according to the expressions

$$\text{Rate of ES formation} = k_1([\mathrm{E_t}] - [\mathrm{ES}])[\mathrm{S}] \tag{6-12}$$

$$\text{Rate of ES breakdown} = k_{-1}[\mathrm{ES}] + k_2[\mathrm{ES}] \tag{6-13}$$

Step 2 We now make an important assumption: that the initial rate of reaction reflects a steady state in which [ES] is constant—that is, the rate of formation of ES is equal to the rate of its breakdown. This is called the **steady-state assumption**. The expressions in Equations 6-12 and 6-13 can be equated for the steady state, giving

$$k_1([\mathrm{E_t}] - [\mathrm{ES}])[\mathrm{S}] = k_{-1}[\mathrm{ES}] + k_2[\mathrm{ES}] \tag{6-14}$$

Step 3 In a series of algebraic steps, we now solve Equation 6-14 for [ES]. First, the left side is multiplied out and the right side simplified to give

$$k_1[\mathrm{E_t}][\mathrm{S}] - k_1[\mathrm{ES}][\mathrm{S}] = (k_{-1} + k_2)[\mathrm{ES}] \tag{6-15}$$

Adding the term $k_1[\mathrm{ES}][\mathrm{S}]$ to both sides of the equation and simplifying gives

$$k_1[\mathrm{E_t}][\mathrm{S}] = (k_1[\mathrm{S}] + k_{-1} + k_2)[\mathrm{ES}] \tag{6-16}$$

We then solve this equation for [ES]:

$$[\mathrm{ES}] = \frac{k_1[\mathrm{E_t}][\mathrm{S}]}{k_1[\mathrm{S}] + k_{-1} + k_2} \tag{6-17}$$

This can now be simplified further, combining the rate constants into one expression:

$$[\mathrm{ES}] = \frac{[\mathrm{E_t}][\mathrm{S}]}{[\mathrm{S}] + (k_{-1} + k_2)/k_1} \tag{6-18}$$

The term $(k_{-1} + k_2)/k_1$ is defined as the **Michaelis constant, K_{m}**. Substituting this into Equation 6-18 simplifies the expression to

$$[\mathrm{ES}] = \frac{[\mathrm{E_t}][\mathrm{S}]}{K_{\mathrm{m}} + [\mathrm{S}]} \tag{6-19}$$

Step 4 We can now express V_0 in terms of [ES]. Substituting the right side of Equation 6-19 for [ES] in Equation 6-11 gives

$$V_0 = \frac{k_2[E_t][S]}{K_m + [S]} \tag{6-20}$$

This equation can be simplified further. Because the maximum velocity occurs when the enzyme is saturated (that is, when $[ES] = [E_t]$), V_{max} can be defined as $k_2[E_t]$. Substituting this in Equation 6-20 gives Equation 6-9:

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

Note that K_m has units of molar concentration. Does the equation fit experimental observations? Yes; we can confirm this by considering the limiting situations where [S] is very low or very high, as shown in **Figure 6-13**.

An important numerical relationship emerges from the Michaelis-Menten equation in the special case when V_0 is exactly one-half V_{max} (Fig. 6-13). Then

$$\frac{V_{max}}{2} = \frac{V_{max}[S]}{K_m + [S]} \tag{6-21}$$

On dividing by V_{max}, we obtain

$$\frac{1}{2} = \frac{[S]}{K_m + [S]} \tag{6-22}$$

Solving for K_m, we get $K_m + [S] = 2[S]$, or

$$K_m = [S], \quad \text{when} \quad V_0 = \tfrac{1}{2}V_{max} \tag{6-23}$$

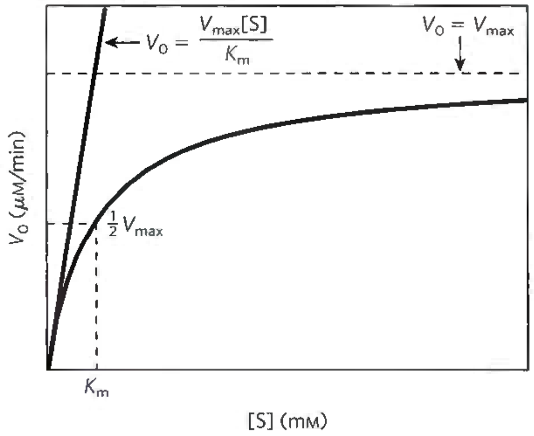


FIGURE 6-13 Dependence of initial velocity on substrate concentration. This graph shows the kinetic parameters that define the limits of the curve at low [S] and high [S]. At low [S], $K_m >> [S]$, and the [S] term in the denominator of the Michaelis-Menten equation (Eqn 6-9) becomes insignificant. The equation simplifies to $V_0 = V_{max}[S]/K_m$, and V_0 exhibits a linear dependence on [S], as observed here. At high [S], where $[S] >> K_m$, the K_m term in the denominator of the Michaelis-Menten equation becomes insignificant and the equation simplifies to $V_0 = V_{max}$; this is consistent with the plateau observed at high [S]. The Michaelis-Menten equation is therefore consistent with the observed dependence of V_0 on [S], and the shape of the curve is defined by the terms V_{max}/K_m at low [S] and V_{max} at high [S].

This is a very useful, practical definition of K_m: K_m is equivalent to the substrate concentration at which V_0 is one-half V_{max}.

Michaelis-Menten Kinetics Can Be Analyzed Quantitatively

There are several ways to determine V_{max} and K_m from a plot relating V_0 to [S]. A traditional approach is to algebraically transform the Michaelis-Menten equation into equations that convert the hyperbolic curve in the V_0 versus [S] plot into a linear form from which values of V_{max} and K_m may be obtained by extrapolation. The most common of these approaches takes the reciprocal of both sides of the Michaelis-Menten equation (Eqn 6-9):

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

$$\frac{1}{V_0} = \frac{K_m + [S]}{V_{max}[S]} \tag{6-24}$$

Separating the components of the numerator on the right side of the equation gives

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{[S]}{V_{max}[S]} \tag{6-25}$$

which simplifies to

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \tag{6-26}$$

This form of the Michaelis-Menten equation is called the **Lineweaver-Burk equation**. For enzymes obeying the Michaelis-Menten relationship, a plot of $1/V_0$ versus $1/[S]$ (the "double reciprocal" of the V_0 versus [S] plot we have been using up to this point) yields a straight line (**Fig. 6-14**). This line has a slope of K_m/V_{max}, an intercept of $1/V_{max}$ on the $1/V_0$ axis, and an intercept of $-1/K_m$ on the $1/[S]$ axis. The Lineweaver-Burk plot is a useful way to

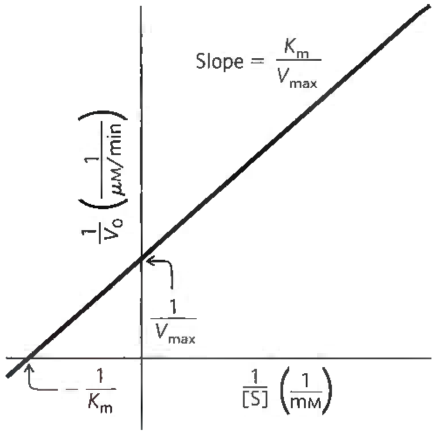


FIGURE 6-14 A double-reciprocal, or Lineweaver-Burk, plot. Plotting $1/V_0$ versus $1/[S]$ puts the data into a linear form. Intercepts on the $1/V_0$ and $1/[S]$ axes are $1/V_{max}$ and $-1/K_m$, respectively.

display data and can provide some mechanistic information, as we will see. However, the double-reciprocal transformation tends to give undue weight to data obtained at low substrate concentration, and can distort errors in the extrapolated values of V_{max} and K_m.

More commonly, the parameters V_{max} and K_m are derived directly from the V_0 versus [S] plot via nonlinear regression, using one of a multitude of curve-fitting programs available online. These are generally easy to use and offer accuracy superior to Lineweaver-Burk or related approaches.

TABLE 6-6 K_m **for Some Enzymes and Substrates**

Enzyme	Substrate	K_m (mM)
Hexokinase (brain)	ATP	0.4
	D-Glucose	0.05
	D-Fructose	1.5
Carbonic anhydrase	HCO_3^-	26
Chymotrypsin	Glycyltyrosinylglycine	108
	N-Benzoyltyrosinamide	2.5
β-Galactosidase	D-Lactose	4.0
Threonine dehydratase	L-Threonine	5.0

Kinetic Parameters Are Used to Compare Enzyme Activities

It is important to distinguish between the Michaelis-Menten equation and the specific kinetic mechanism on which the equation was originally based. The Michaelis-Menten equation does not depend on the relatively simple two-step reaction mechanism proposed by Michaelis and Menten (Eqn 6-10). All enzymes that exhibit a hyperbolic dependence of V_0 on [S] are said to follow **Michaelis-Menten kinetics**. Many enzymes that follow Michaelis-Menten kinetics have quite different reaction mechanisms, and enzymes that catalyze reactions with six or eight identifiable steps often exhibit the same steady-state kinetic behavior. The practical rule that $K_m = [S]$ when $V_0 = \frac{1}{2}V_{max}$ (Eqn 6-23) holds for all enzymes that follow Michaelis-Menten kinetics. The most important exceptions to Michaelis-Menten kinetics are the regulatory enzymes, discussed in Section 6.5.

The parameters K_m and V_{max} can be obtained experimentally for any given enzyme, and their measurement is often a key first step in enzyme characterization. However, the mechanistic insight they offer is limited. Obtaining information about the number, rates, or chemical nature of discrete steps in the reaction usually requires additional complementary approaches. Steady-state kinetics nevertheless is the standard language through which biochemists compare and characterize the catalytic efficiencies of enzymes.

Interpreting K_m and V_{max} Both the magnitude and the meaning of K_m and V_{max} can vary greatly from enzyme to enzyme.

K_m can vary even for different substrates of the same enzyme (Table 6-6). The term is sometimes used (often inappropriately) as an indicator of the affinity of an enzyme for its substrate. The actual meaning of K_m depends on specific aspects of the reaction mechanism, such as the number and relative rates of the individual steps. For reactions with two steps,

$$K_m = \frac{k_2 + k_{-1}}{k_1} \tag{6-27}$$

When k_2 is rate-limiting, $k_2 \ll k_{-1}$, and K_m reduces to k_{-1}/k_1, which is defined as the **dissociation constant, K_d**, of the ES complex. Where these conditions hold, K_m does represent a measure of the affinity of the enzyme for its substrate in the ES complex. However, this scenario does not apply for most enzymes. Sometimes $k_2 \gg k_{-1}$, and then $K_m = k_2/k_1$. In other cases, k_2 and k_{-1} are comparable, and K_m remains a more complex function of all three rate constants (Eqn 6-27). The Michaelis-Menten equation and the characteristic saturation behavior of the enzyme still apply, but K_m cannot be considered a simple measure of substrate affinity. Even more common are cases in which the reaction goes through several steps after formation of ES; K_m can then become a very complex function of many rate constants.

The quantity V_{max} depends upon the rate-limiting step of the enzyme-catalyzed reaction. If an enzyme reacts by the two-step Michaelis-Menten mechanism, $V_{max} = k_2[E_t]$, where k_2 is rate-limiting. However, the number of reaction steps and the identity of the rate-limiting step(s) can vary from enzyme to enzyme. For example, consider the common situation where product release, EP → E + P, is rate-limiting. Early in the reaction (when [P] is low), the overall reaction can be adequately described by the scheme

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \xrightarrow{k_3} E + P \tag{6-28}$$

In this case, most of the enzyme is in the EP form at saturation, and $V_{max} = k_3[E_t]$.

It is useful to define a more general rate constant, $\boldsymbol{k_{cat}}$, to describe the limiting rate of any enzyme-catalyzed reaction at saturation. If the reaction has several steps, one of which is clearly rate-limiting, k_{cat} is equivalent to the rate constant for that limiting step. For the simple reaction of Equation 6-10, $k_{cat} = k_2$. For the reaction of Equation 6-28, when product release is clearly rate-limiting, $k_{cat} = k_3$. When several steps are partially rate-limiting, k_{cat} can become a complex function of several of the rate constants that define each individual reaction step. In the Michaelis-Menten equation, $k_{cat} = V_{max}/[E_t]$. Rearranged, $V_{max} = k_{cat}[E_t]$, and Equation 6-9 becomes

$$V_0 = \frac{k_{cat}[E_t][S]}{K_m + [S]} \tag{6-29}$$

TABLE 6-7 Turnover Number, k_{cat}, of Some Enzymes

Enzyme	Substrate	k_{cat} (s^{-1})
Catalase	H_2O_2	40,000,000
Carbonic anhydrase	HCO_3^-	400,000
Acetylcholinesterase	Acetylcholine	14,000
β-Lactamase	Benzylpenicillin	2,000
Fumarase	Fumarate	800
RecA protein (an ATPase)	ATP	0.5

The constant k_{cat} is a first-order rate constant and hence has units of reciprocal time. It is also called the **turnover number**. It is equivalent to the number of substrate molecules converted to product in a given unit of time on a single enzyme molecule when the enzyme is saturated with substrate. The turnover numbers of several enzymes are given in **Table 6-7**.

Comparing Catalytic Mechanisms and Efficiencies The kinetic parameters k_{cat} and K_m are useful for the study and comparison of different enzymes, whether their reaction mechanisms are simple or complex. Each enzyme has values of k_{cat} and K_m that reflect the cellular environment, the concentration of substrate normally encountered in vivo by the enzyme, and the chemistry of the reaction being catalyzed.

The parameters k_{cat} and K_m also allow us to evaluate the kinetic efficiency of enzymes, but either parameter alone is insufficient for this task. Two enzymes catalyzing different reactions may have the same k_{cat} (turnover number), yet the rates of the uncatalyzed reactions may be different and thus the rate enhancements brought about by the enzymes may differ greatly. Experimentally, the K_m for an enzyme tends to be similar to the cellular concentration of its substrate. An enzyme that acts on a substrate present at a very low concentration in the cell usually has a lower K_m than an enzyme that acts on a substrate that is more abundant.

The best way to compare the catalytic efficiencies of different enzymes or the turnover of different substrates by the same enzyme is to compare the ratio k_{cat}/K_m for the two reactions. This parameter, sometimes called the **specificity constant**, is the rate constant for the conversion of E + S to E + P. When $[S] \ll K_m$, Equation 6-29 reduces to the form

$$V_0 = \frac{k_{cat}}{K_m}[E_t][S] \qquad (6\text{-}30)$$

V_0 in this case depends on the concentration of two reactants, $[E_t]$ and [S], so this is a second-order rate equation, and the constant k_{cat}/K_m is a second-order rate constant with units of $\text{M}^{-1}\text{s}^{-1}$. There is an upper limit to k_{cat}/K_m, imposed by the rate at which E and S can diffuse together in an aqueous solution. This diffusion-controlled limit is 10^8 to 10^9 $\text{M}^{-1}\text{s}^{-1}$, and many enzymes have a k_{cat}/K_m near this range (**Table 6-8**). Such enzymes are said to have achieved catalytic perfection. Note that different values of k_{cat} and K_m can produce the maximum ratio.

WORKED EXAMPLE 6-1 ***Determination of K_m***

An enzyme is discovered that catalyzes the chemical reaction

$$\text{SAD} \rightleftharpoons \text{HAPPY}$$

A team of motivated researchers sets out to study the enzyme, which they call happyase. They find that the k_{cat} for happyase is 600 s^{-1} and they carry out several additional experiments.

When $[E_t] = 20$ nM and [SAD] = 40 μM, the reaction velocity, V_0, is 9.6 $\mu\text{M s}^{-1}$. Calculate K_m for the substrate SAD.

SOLUTION: We know k_{cat}, $[E_t]$, [S], and V_0. We want to solve for K_m. We will first derive an expression for K_m, beginning with the Michaelis-Menten equation (Eqn 6-9). We will calculate V_{max} by equating it to $k_{cat}[E_t]$. We can then substitute the known values to calculate K_m.

$$V_0 = \frac{V_{max}[S]}{(K_m + [S])}$$

First, multiply both sides by $K_m + [S]$:

$$V_0(K_m + [S]) = V_{max}[S]$$

$$V_0K_m + V_0[S] = V_{max}[S]$$

TABLE 6-8 Enzymes for Which k_{cat}/K_m Is Close to the Diffusion-Controlled Limit (10^8 to 10^9 $\text{M}^{-1}\text{s}^{-1}$)

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
Acetylcholinesterase	Acetylcholine	1.4×10^4	9×10^{-5}	1.6×10^8
Carbonic anhydrase	CO_2 HCO_3^-	1×10^6 4×10^5	1.2×10^{-2} 2.6×10^{-2}	8.3×10^7 1.5×10^7
Catalase	H_2O_2	4×10^7	1.1×10^0	4×10^7
Crotonase	Crotonyl-CoA	5.7×10^3	2×10^{-5}	2.8×10^8
Fumarase	Fumarate Malate	8×10^2 9×10^2	5×10^{-6} 2.5×10^{-5}	1.6×10^8 3.6×10^7
β-Lactamase	Benzylpenicillin	2.0×10^3	2×10^{-5}	1×10^8

Information from A. Fersht, *Structure and Mechanism in Protein Science*, p. 166, W. H. Freeman and Company, 1999.

Subtract $V_0[\mathrm{S}]$ from both sides to give

$$V_0K_m = V_{max}[\mathrm{S}] - V_0[\mathrm{S}]$$

Factor out [S] and divide both sides by V_0.

$$K_m = \frac{(V_{max} - V_0)[\mathrm{S}]}{V_0}$$

Now, equate V_{max} to $k_{cat}[\mathrm{E_t}]$, and substitute the given values to obtain K_m.

$$V_{max} = k_{cat}[\mathrm{E_t}] = (600\ \mathrm{s}^{-1})0.02\ \mu\mathrm{M} = 12\ \mu\mathrm{M\ s}^{-1}$$

$$K_m = (12\ \mu\mathrm{M\ s}^{-1} - 9.6\ \mu\mathrm{M\ s}^{-1})40\ \mu\mathrm{M}/9.6\ \mu\mathrm{M\ s}^{-1}$$

$$= 96\ \mu\mathrm{M}^2\ \mathrm{s}^{-1}/9.6\ \mu\mathrm{M\ s}^{-1} = 10\ \mu\mathrm{M}$$

Once you have worked with this equation, you will recognize shortcuts to solve problems like this. For example, rearranging Equation 6-9 by simply dividing both sides by V_{max} gives

$$\frac{V_0}{V_{max}} = \frac{[\mathrm{S}]}{K_m + [\mathrm{S}]}$$

Thus, the ratio $V_0/V_{max} = 9.6\ \mu\mathrm{M\ s}^{-1}/12\ \mu\mathrm{M\ s}^{-1} = [\mathrm{S}]/(K_m + [\mathrm{S}])$. This sometimes simplifies the process of solving for K_m, in this case, giving 0.25[S], or 10 μM.

WORKED EXAMPLE 6-2 *Determination of [S]*

In a separate happyase experiment using $[\mathrm{E_t}] = 10$ mM, the reaction velocity, V_0, is measured as 3 $\mu\mathrm{M\ s}^{-1}$. What is the [S] used in this experiment?

SOLUTION: Using the same logic as in Worked Example 6-1—equating V_{max} to $k_{cat}[\mathrm{E_t}]$—we see that V_{max} for this enzyme concentration is 6 $\mu\mathrm{M\ s}^{-1}$. Note that V_0 is exactly half of V_{max}. Recall that K_m is by definition equal to the [S] at which $V_0 = \frac{1}{2}V_{max}$. Thus, in this example, the [S] must be the same as K_m, or 10 μM. If V_0 were anything other than $\frac{1}{2}V_{max}$, it would be simplest to use the expression $V_0/V_{max} = [\mathrm{S}]/(K_m + [\mathrm{S}])$ to solve for [S].

Many Enzymes Catalyze Reactions with Two or More Substrates

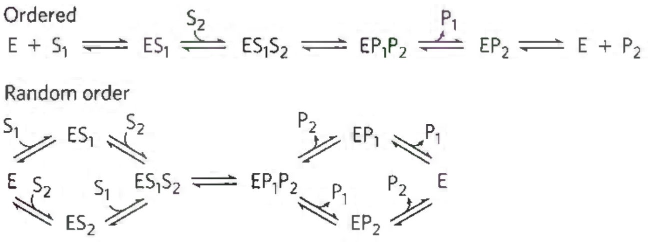
We have seen how [S] affects the rate of a simple enzymatic reaction with only one substrate molecule ($\mathrm{S} \rightarrow \mathrm{P}$). In most enzymatic reactions, however, two (and sometimes more) different substrate molecules bind to the enzyme and participate in the reaction. Nearly two-thirds of all enzymatic reactions have two substrates and two products. These are generally reactions in which a group is transferred from one substrate to the other, or one substrate is oxidized while the other is reduced. For example, in the reaction catalyzed by hexokinase, ATP and glucose are the substrate molecules, and ADP and glucose 6-phosphate are the products:

$$\text{ATP} + \text{glucose} \longrightarrow \text{ADP} + \text{glucose 6-phosphate}$$

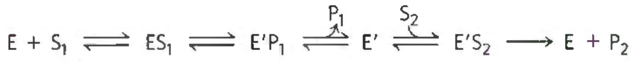
A phosphoryl group is transferred from ATP to glucose. The rates of such bisubstrate reactions can also be analyzed by the Michaelis-Menten approach. Hexokinase has a characteristic K_m for each of its substrates (Table 6-6).

Enzymatic reactions with two substrates proceed by one of several different types of pathways. In some cases, both substrates are bound to the enzyme concurrently at some point in the course of the reaction, forming a noncovalent ternary complex (**Fig. 6-15a**); the substrates bind in a random sequence or in a specific order. Ordered binding occurs when binding of the first substrate creates

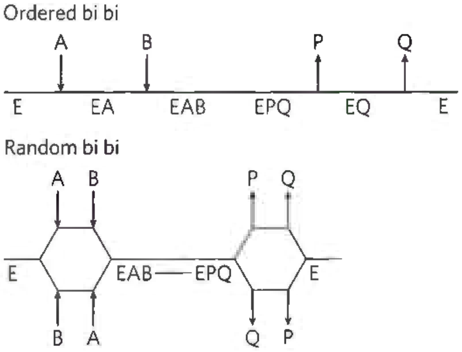
(a) Enzyme reaction involving a ternary complex


Ordered
$E + S_1 \rightleftharpoons ES_1 \rightleftharpoons ES_1S_2 \rightleftharpoons EP_1P_2 \rightleftharpoons EP_2 \rightleftharpoons E + P_2$
S_2
P_1
Random order
S_1
ES_1
S_2
E
S_2
S_1
ES_2
$ES_1S_2 \rightleftharpoons EP_1P_2$
P_2
EP_1
P_1
P_1
P_2
E
EP_2

(b) Enzyme reaction in which no ternary complex is formed


$E + S_1 \rightleftharpoons ES_1 \rightleftharpoons E'P_1 \rightleftharpoons E' \rightleftharpoons E'S_2 \longrightarrow E + P_2$
P_1
S_2

(c) Cleland nomenclature



(d) Ping-Pong in Cleland nomenclature

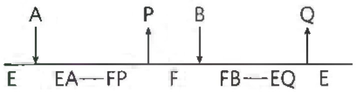


FIGURE 6-15 Common mechanisms for enzyme-catalyzed bisubstrate reactions. (a) The enzyme and both substrates come together to form a ternary complex. In ordered binding, substrate 1 must bind before substrate 2 can bind productively. In random binding, the substrates can bind in either order. Product dissociation can also be ordered or random. (b) An enzyme-substrate complex forms, a product leaves the complex, the altered enzyme forms a second complex with another substrate molecule, and the second product leaves, regenerating the enzyme. Substrate 1 may transfer a functional group to the enzyme (to form the covalently modified E′), which is subsequently transferred to substrate 2. This is called a Ping-Pong or double-displacement mechanism. (c) Ternary complex formation depicted using Cleland nomenclature. In the ordered bi bi and random bi bi reactions shown here, the release of product follows the same pattern as the binding of substrate—both ordered or both random. (d) The Ping-Pong or double-displacement reaction described with Cleland nomenclature.

a condition, often a conformation change, required for the second substrate to bind. In other cases, the first substrate is converted to product and dissociates before the second substrate binds, so no ternary complex is formed. An example of this is the Ping-Pong, or double-displacement, mechanism (Fig. 6-15b).

A shorthand notation developed by W. W. Cleland can be helpful in describing reactions with multiple substrates and products. In this system, referred to as **Cleland nomenclature**, substrates are denoted A, B, C, and D, in the order in which they bind to the enzyme, and products are denoted P, Q, S, and T, in the order in which they dissociate. Enzymatic reactions with one, two, three, or four substrates are referred to as uni, bi, ter, and quad, respectively. The enzyme is, as usual, denoted E, but if it is modified in the course of the reaction, successive forms are denoted F, G, and so on. The progress of the reaction is indicated with a horizontal line, with successive chemical species indicated below it. If there is an alternative in the reaction path, the horizontal line is bifurcated. Steps involving binding and dissociating substrates and products are indicated with vertical lines.

Common reactions with two substrates and two products (bi bi) are described with the shorthand forms illustrated in Figure 6-15c for an ordered bi bi reaction and a random bi bi reaction. In the latter example, the release of product is also random, as indicated by the two sets of bifurcations. Rarely, the binding of substrates is ordered and the release of products is random, or vice versa, eliminating the bifurcation at one end or the other of the progress line. In a Ping-Pong reaction, lacking a ternary complex, the pathway has a transient second form of the enzyme, F (Fig. 6-15d). This is the form in which a group has been transferred from the first substrate, A, to create a transient covalent attachment to the enzyme. As noted above, such reactions are often called double-displacement reactions, as a group is transferred first from substrate A to the enzyme and then from the enzyme to substrate B. Substrates A and B do not encounter each other on the enzyme.

Michaelis-Menten steady-state kinetics can provide only limited information about the number of steps and intermediates in an enzymatic reaction, but the approach can be used to distinguish between pathways that have a ternary intermediate and pathways—including Ping-Pong pathways—that do not (**Fig. 6-16**). As we will see when we consider enzyme inhibition, steady-state kinetics can also distinguish between ordered and random binding of substrates and products in reactions with ternary intermediates.

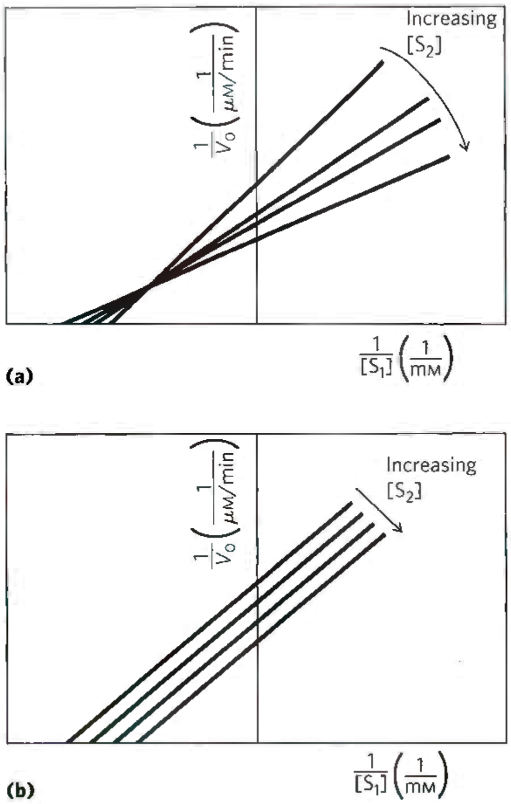


FIGURE 6-16 Steady-state kinetic analysis of bisubstrate reactions. In these double-reciprocal plots, the concentration of substrate 1 is varied while the concentration of substrate 2 is held constant. This is repeated for several values of $[S_2]$, generating several separate lines. (a) Intersecting lines indicate that a ternary complex is formed in the reaction; (b) parallel lines indicate a Ping-Pong (double-displacement) pathway.

Enzyme Activity Depends on pH

In general, steady-state kinetics provides information required to characterize an enzyme and assess its catalytic efficiency. Additional information can be gained by examination of how the key experimental parameters k_{cat} and k_{cat}/K_m change when reaction conditions change, particularly pH. Enzymes have an optimum pH (or pH range) at which their activity is maximal (**Fig. 6-17**); at higher or lower pH, activity decreases. This is not surprising. Amino acid side chains in the active site may act as weak acids and bases only if they maintain a certain state of ionization. Elsewhere in the protein, removing a proton from a His residue, for example, might eliminate an ionic interaction that is essential for stabilizing the active conformation of the enzyme. A less common cause of pH sensitivity is titration of a group on the substrate.

The pH range over which an enzyme undergoes changes in activity can provide a clue to the type of amino acid residue involved (see Table 3-1). A change in activity near pH 7.0, for example, often reflects titration of a His residue. The effects of pH must be interpreted with some caution, however. In the closely packed environment of a protein, the pK_a of amino acid side chains can be significantly altered. For example, a nearby positive charge can lower the pK_a of a Lys residue, and a nearby negative charge can increase it. Such effects sometimes result in a pK_a that is shifted by several pH units from its value in the free amino acid. In the enzyme acetoacetate decarboxylase, for example, one Lys residue has a pK_a of 6.6 (compared with 10.5 in free lysine) due to electrostatic effects of nearby positive charges.

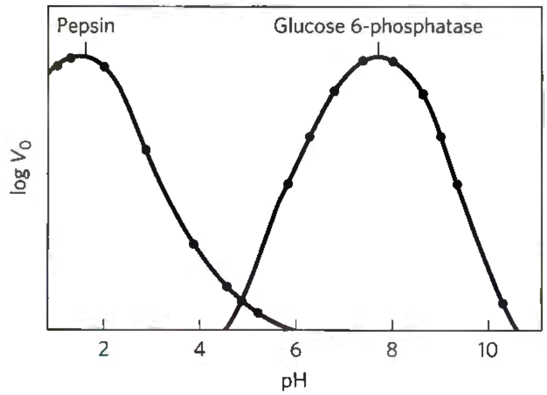


FIGURE 6-17 The pH-activity profiles of two enzymes. These curves are constructed from measurements of initial velocities when the reaction is carried out in buffers of different pH. Because pH is a logarithmic scale reflecting 10-fold changes in [H$^+$], the changes in V_0 are also plotted on a logarithmic scale. The pH optimum for the activity of an enzyme is generally close to the pH of the environment in which the enzyme is normally found. Pepsin, a peptidase found in the stomach, has a pH optimum of about 1.6. The pH of gastric juice is between 1 and 2. Glucose 6-phosphatase of hepatocytes (liver cells), with a pH optimum of about 7.8, is responsible for releasing glucose into the blood. The normal pH of the cytosol of hepatocytes is about 7.2.

Pre–Steady State Kinetics Can Provide Evidence for Specific Reaction Steps

The mechanistic insight provided by steady-state kinetics can be augmented, sometimes dramatically, by an examination of the pre–steady state. Consider an enzyme with a reaction mechanism that conforms to the scheme in Equation 6-28, featuring three steps:

$$\mathrm{E + S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{EP} \xrightarrow{k_3} \mathrm{E + P}$$

Overall catalytic efficiency for this reaction can be assessed with steady-state kinetics, but the rates of the individual steps cannot be determined in this way, and the slow (rate-limiting) step can rarely be identified. For the rate constants of individual steps to be measured, the reaction must be studied during its pre–steady state. The first turnover of an enzyme-catalyzed reaction often occurs in seconds or milliseconds, so researchers use special equipment that allows mixing and sampling on this time scale (**Fig. 6-18a**). Reactions are stopped and protein-bound products are quantified, after the timed addition and rapid mixing of an acid that denatures the protein and releases all bound molecules. A detailed description of pre–steady state kinetics is beyond the scope of this text, but we can illustrate the power of this approach by a simple example of an enzyme that uses the pathway

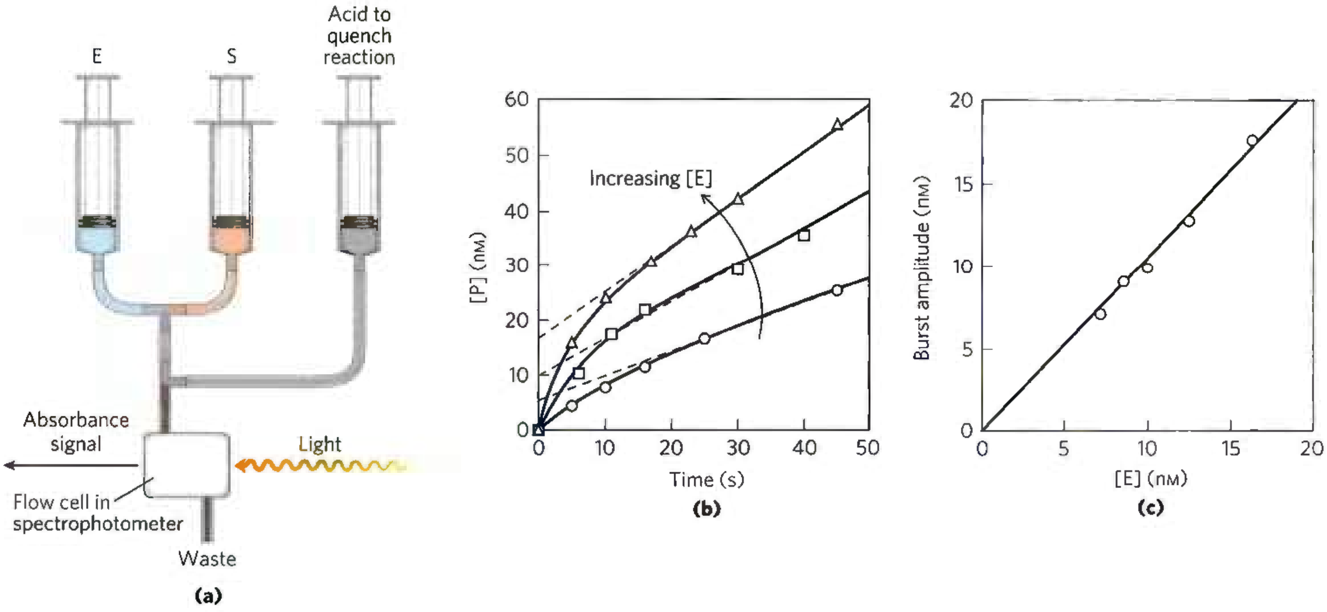


FIGURE 6-18 Pre–steady state kinetics. The transient phase that constitutes the pre–steady state often exists for mere seconds or milliseconds, requiring specialized equipment to monitor it. (a) A simple schematic for a rapid-mixing device, called a stopped-flow device. Enzyme (E) and substrate (S) are mixed with the aid of mechanically operated syringes. The reaction is quenched at a programmed time by adding a denaturing acid through another syringe, and the amount of product formed is measured, in this case with a spectrophotometer. (b) Experimental data for an enzyme reaction show the pre–steady state occurring in the first 5 to 10 seconds. This is a relatively slow reaction and is used as an example because the steady state can be conveniently monitored. The slope of the lines after 15 seconds reflects the steady state. Extrapolating this slope back to zero time (dashed lines) gives the amplitude of the burst phase. The progress of the reaction during the pre–steady state primarily reflects the chemical steps in the reaction (details of which are not shown). The presence of a burst implies that a step following the chemical step that produces P is rate-limiting — in this case, the product-release step. Notice that the extrapolated intercept at time = 0 increases as [E] increases. (c) A plot of burst amplitude (the intercepts from (b)) versus [E] shows that one molecule of P is formed in each active site during the burst (pre–steady state) phase. This provides evidence that product release is the rate-limiting step, because it is the only step following product formation in this simple enzymatic reaction. The enzyme used in this experiment was RNase P, one of the catalytic RNAs described in Chapter 26. [(b, c) Data from J. Hsieh et al., *RNA* 15:224, 2009.]

shown in Equation 6-28. This example also involves an enzyme that catalyzes a relatively slow reaction, so the pre–steady state is more conveniently observed.

For many enzymes, dissociation of product is rate-limiting. In this example (Fig. 6-18b, c), the rate of dissociation of the product (k_3) is slower than the rate of its formation (k_2). Product dissociation therefore dictates the rates observed in the steady state. How do we know that k_3 is rate-limiting? A slow k_3 gives rise to a burst of product formation in the pre–steady state, because the preceding steps are relatively fast. The burst reflects the rapid conversion of one molecule of substrate to one molecule of product at each enzyme active site. The observed rate of product formation soon slows to the steady-state rate as the bound product is slowly released. Each enzymatic turnover after the first one must proceed through the slow product-release step. However, the rapid generation of product in that first turnover provides much information. The amplitude of the burst—when one molecule of product is generated per molecule of enzyme present (Fig. 6-18c), measured by extrapolating the steady-state progress line back to zero time—is the highest amplitude possible. This provides one piece of evidence that product release is, indeed, rate-limiting. The rate constant for the chemical reaction step, k_2, can be derived from the observed rate of the burst phase.

Of course, enzymes do not always conform to the simple reaction scheme of Equation 6-28. Formally, the observation of a burst indicates that a rate-limiting step (typically, product release, or an enzyme conformational change, or another chemical step) occurs after formation of the product being monitored. Additional experiments and analysis can often define the rates of each step in a multistep enzymatic reaction. Some examples of the application of pre–steady state kinetics are included in the descriptions of specific enzymes in Section 6.4.

Enzymes Are Subject to Reversible or Irreversible Inhibition

Enzyme inhibitors are molecules that interfere with catalysis, slowing or halting enzymatic reactions. Enzymes catalyze virtually all cellular processes, so it should not be surprising that enzyme inhibitors are among the most important pharmaceutical agents known. For example, aspirin (acetylsalicylate) inhibits the enzyme that catalyzes the first step in the synthesis of prostaglandins, compounds involved in many processes, including some that produce pain. The study of enzyme inhibitors also has provided valuable information about enzyme mechanisms and has helped define some metabolic pathways. There are two broad classes of enzyme inhibitors: reversible and irreversible.

Reversible Inhibition One common type of **reversible inhibition** is called competitive (**Fig. 6-19a**). A **competitive inhibitor** competes with the substrate for the active site of an enzyme. While the inhibitor (I) occupies the active site, the substrate is excluded, and vice versa. Many competitive inhibitors are structurally similar to the substrate and combine with the enzyme to form an unreactive EI complex. Even fleeting combinations of this type will reduce the efficiency of an enzyme. Competitive inhibition can be analyzed quantitatively by steady-state kinetics. In the presence of a competitive inhibitor, the Michaelis-Menten equation (Eqn 6-9) becomes

$$V_0 = \frac{V_{\max}[S]}{\alpha K_m + [S]} \qquad (6\text{-}31)$$

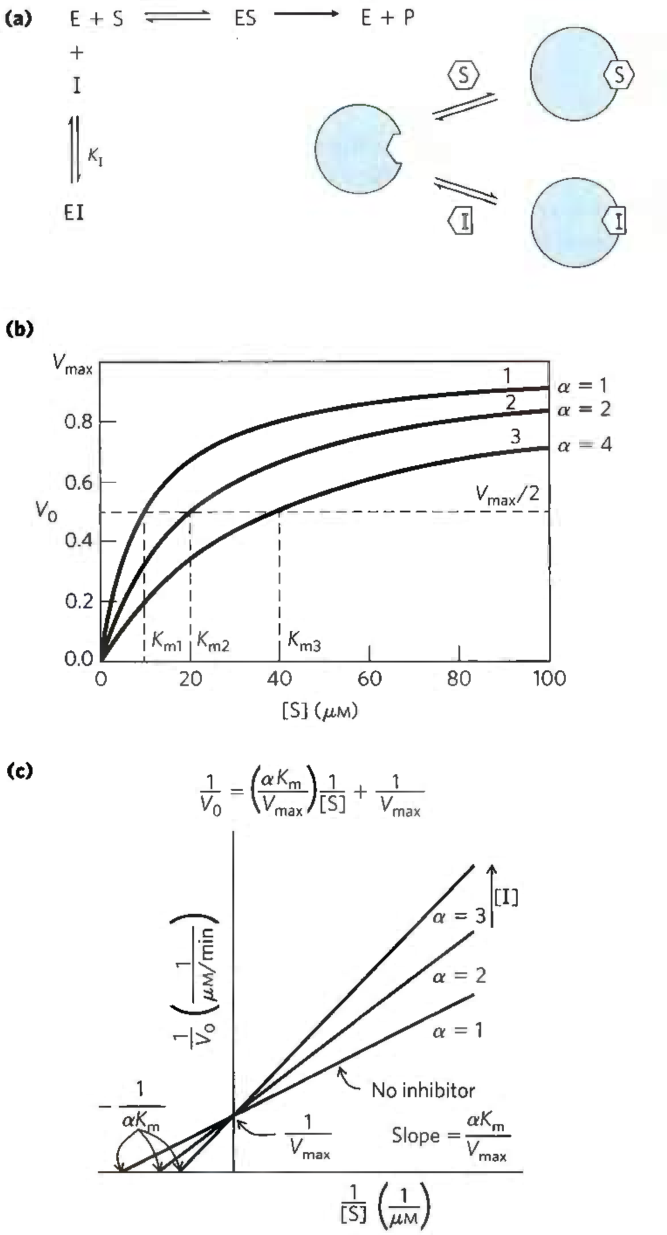

(a)
E + S ⇌ ES ⟶ E + P
+
I
K_I
EI
S
I
(b)
V_{max}
0.8
0.6
V_0
0.4
0.2
0.0
0
20
40
60
80
100
[S] (μM)
1
2
3
α = 1
α = 2
α = 4
$V_{max}/2$
K_{m1}
K_{m2}
K_{m3}
(c)
$\frac{1}{V_0} = \left(\frac{\alpha K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$
$\frac{1}{V_0}\left(\frac{1}{\mu M/min}\right)$
[I]
α = 3
α = 2
α = 1
No inhibitor
$-\frac{1}{\alpha K_m}$
$\frac{1}{V_{max}}$
Slope = $\frac{\alpha K_m}{V_{max}}$
$\frac{1}{[S]}\left(\frac{1}{\mu M}\right)$

FIGURE 6-19 **Competitive inhibition.** (a) Competitive inhibitors bind to the enzyme's active site; K_I is the equilibrium dissociation constant for inhibitor binding to E. (b) Competitive inhibitors affect the observed K_m, but not the V_{max}. This is readily evident in the plot of V_0 versus [S]. (c) In a Lineweaver-Burk plot, the lines generated + and − inhibitor intersect on the *y* axis, which reflects $1/V_{max}$. As the observed K_m increases in the presence of an inhibitor, the intercept on the *x* axis ($-1/K_m$) moves to the right.

where

$$\alpha = 1 + \frac{[\mathrm{I}]}{K_\mathrm{I}} \qquad \text{and} \qquad K_\mathrm{I} = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{EI}]}$$

Equation 6-31 describes the important features of competitive inhibition. The experimentally determined variable αK_m, the K_m observed in the presence of the inhibitor, is often called the "apparent" K_m.

Bound inhibitor does not inactivate the enzyme. When the inhibitor dissociates, substrate can bind and react. Because the inhibitor binds reversibly to the enzyme, the competition can be biased to favor the substrate simply by adding more substrate. When [S] far exceeds [I], the probability that an inhibitor molecule will bind to the enzyme is minimized and the reaction exhibits its normal V_max. However, in the presence of inhibitor, the [S] at which $V_0 = \frac{1}{2} V_\mathrm{max}$, the apparent K_m, increases in the presence of inhibitor by the factor α (Fig. 6-19b). This effect on apparent K_m, combined with the absence of an effect on V_max, is diagnostic of competitive inhibition and is readily revealed in a double-reciprocal plot (Fig. 6-19c). The equilibrium constant for inhibitor binding, K_I, can be obtained from the same plot.

A medical therapy based on competition at the active site is used to treat patients who have ingested methanol, a solvent found in gas-line antifreeze. The liver enzyme alcohol dehydrogenase converts methanol to formaldehyde, which is damaging to many tissues. Blindness is a common result of methanol ingestion, because the eyes are particularly sensitive to formaldehyde. Ethanol competes effectively with methanol as an alternative substrate for alcohol dehydrogenase. The effect of ethanol is much like that of a competitive inhibitor, with the distinction that ethanol is also a substrate for alcohol dehydrogenase and its concentration will decrease over time as the enzyme converts it to acetaldehyde. The therapy for methanol poisoning is slow intravenous infusion of ethanol, at a rate that maintains a controlled concentration in the blood for several hours. This slows the formation of formaldehyde, lessening the danger while the kidneys filter out the methanol to be excreted harmlessly in the urine. ■

Two other types of reversible inhibition, uncompetitive and mixed, can be defined in terms of one-substrate enzymes, but in practice are observed only with enzymes having two or more substrates. An **uncompetitive inhibitor** (**Fig. 6-20a**) binds at a site distinct from the substrate active site and, unlike a competitive inhibitor, binds only to the ES complex. In the presence of an uncompetitive inhibitor, the Michaelis-Menten equation is altered to

$$V_0 = \frac{V_\mathrm{max}[\mathrm{S}]}{K_\mathrm{m} + \alpha'[\mathrm{S}]} \tag{6-32}$$

where

$$\alpha' = 1 + \frac{[\mathrm{I}]}{K'_\mathrm{I}} \qquad \text{and} \qquad K'_\mathrm{I} = \frac{[\mathrm{ES}][\mathrm{I}]}{[\mathrm{ESI}]}$$

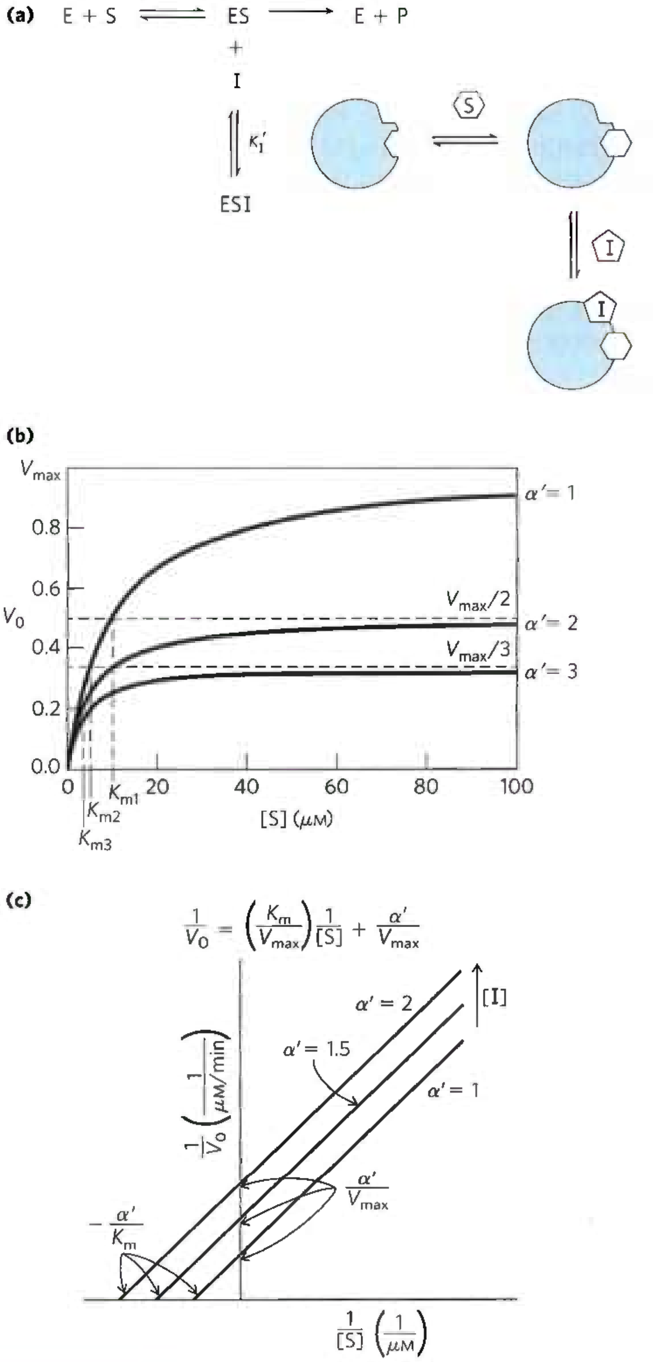


FIGURE 6-20 Uncompetitive inhibition. (a) Uncompetitive inhibitors bind at a separate site, but bind only to the ES complex; K'_I is the equilibrium constant for an inhibitor binding to ES. (b) In the presence of an uncompetitive inhibitor, both the K_m and the V_max decline, and by equivalent factors. In the V_0 versus [S] plot, the decline in V_max is somewhat easier to discern than the decline in K_m. (c) The Lineweaver-Burk plot for an uncompetitive inhibitor is quite diagnostic, as the lines generated in the presence and absence of an inhibitor are parallel. Note that the lines seen in the presence of an inhibitor are always above the line generated in the absence of an inhibitor, reflecting the decline in both K_m and V_max brought about by the inhibitor (i.e., the intercepts move up on the *y* axis and to the left on the *x* axis).

As described by Equation 6-32, at high concentrations of substrate, V_0 approaches V_max/α'. Thus, an uncompetitive inhibitor lowers the measured V_max. Apparent K_m also decreases, because the [S] required to reach one-half V_{max} decreases by the factor α' (Fig. 6-20b, c). This behavior can be explained as follows. Because the enzyme is inactive when the uncompetitive inhibitor is bound, but the inhibitor is not competing with substrate for binding, the inhibitor effectively removes some fraction of the enzyme molecules from the reaction. Given that V_{max} depends on [E], the observed V_{max} decreases. Given that the inhibitor binds only to the ES complex, only ES (not free enzyme) is deleted from the reaction, so the [S] needed to reach $\frac{1}{2}V_{max}$—that is, K_m—declines by the same amount.

A **mixed inhibitor** (Fig. 6-21a) also binds at a site distinct from the substrate active site, but it binds to either E or ES. The rate equation describing mixed inhibition is

$$V_0 = \frac{V_{max}[S]}{\alpha K_m + \alpha'[S]} \tag{6-33}$$

where α and α' are defined as above. A mixed inhibitor usually affects both K_m and V_{max} (Fig. 6-21b, c). V_{max} is affected because the inhibitor renders some fraction of the available enzyme molecules inactive, lowering the effective [E] on which V_{max} depends. The K_m may increase or decrease, depending on which enzyme form, E or ES, the inhibitor binds to most strongly. The special case of $\alpha = \alpha'$, rarely encountered in experiments, historically has been defined as **noncompetitive inhibition**. Examine Equation 6-33 to see why a noncompetitive inhibitor would affect the V_{max} but not the K_m.

Equation 6-33 is a general expression for the effects of reversible inhibitors, simplifying to the expressions for competitive inhibition and uncompetitive inhibition when $\alpha' = 1.0$ or $\alpha = 1.0$, respectively. From this expression we can summarize the effects of inhibitors on individual kinetic parameters. For all reversible inhibitors, the apparent $V_{max} = V_{max}/\alpha'$, because the right side of Equation 6-33 always simplifies to V_{max}/α' at sufficiently high substrate concentrations. For competitive inhibitors, $\alpha' = 1.0$ and can thus be ignored. Taking this expression for apparent V_{max}, we can also derive a general expression for apparent K_m to show how this parameter changes in the presence of reversible inhibitors. Apparent K_m, as always, equals the [S] at which V_0 is one-half apparent V_{max} or, more generally, when $V_0 = V_{max}/2\alpha'$. This condition is met when $[S] = \alpha K_m/\alpha'$. Thus, apparent $K_m = \alpha K_m/\alpha'$. The terms α and α' reflect the binding of inhibitor to E and ES, respectively. Thus, the term $\alpha K_m/\alpha'$ is a mathematical expression of the relative affinity of inhibitor for the two enzyme forms. This expression is simpler when either α or α' is 1.0 (for uncompetitive or competitive inhibitors), as summarized in Table 6-9.

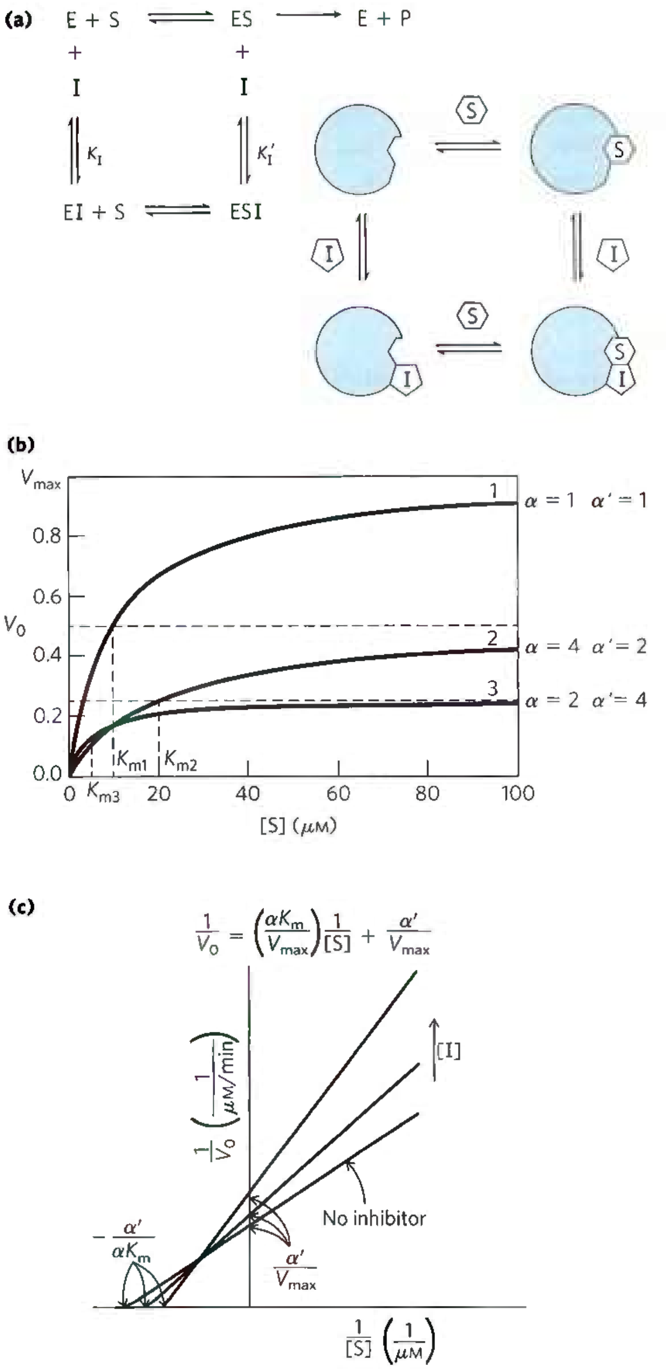


FIGURE 6-21 Mixed inhibition. (a) Mixed inhibitors bind at a separate site, but they may bind to either E or ES. (b) The kinetic patterns brought about by a mixed inhibitor are complex. The V_{max} always declines. The K_m may increase or decrease, depending upon the relative values of α and α'. (c) In a Lineweaver-Burk plot, the lines generated + and − inhibitor always intersect, but not on an axis. The intercepts on the *y* axis always move up, as the V_{max} declines. The lines may intersect either above or below the *x* axis. When they intersect above, as shown here, $\alpha > \alpha'$ and the observed K_m is increasing. When the lines intersect below the *x* axis (not shown), $\alpha < \alpha'$ and the observed K_m is decreasing.

In practice, uncompetitive inhibition and mixed inhibition are observed only for enzymes with two or more substrates—say, S_1 and S_2—and are very important in the experimental analysis of such enzymes. If an inhibitor binds to the site normally occupied by S_1, it may act as a competitive inhibitor in experiments in which $[S_1]$ is varied. If an inhibitor binds to the site normally occupied by S_2, it may act as a mixed or uncompetitive inhibitor of S_1. The actual inhibition patterns

TABLE 6-9 Effects of Reversible Inhibitors on Apparent V_{max} and Apparent K_m

Inhibitor type	Apparent V_{max}	Apparent K_m
None	V_{max}	K_m
Competitive	V_{max}	αK_m
Uncompetitive	V_{max}/α'	K_m/α'
Mixed	V_{max}/α'	$\alpha K_m/\alpha'$

observed depend on whether the S_1- and S_2-binding events are ordered or random, and thus the order in which substrates bind and products leave the active site can be determined. Product inhibition experiments in which one of the reaction products is provided as an inhibitor are often particularly informative. If only one of two reaction products is present, no reverse reaction can take place. However, a product generally binds to some part of the active site and can thus serve as an effective inhibitor. Enzymologists can combine steady-state kinetic studies involving different combinations and amounts of products and inhibitors with pre–steady state analysis to develop a detailed picture of the mechanism of a bisubstrate reaction.

WORKED EXAMPLE 6-3 *Effect of Inhibitor on K_m*

The researchers working on happyase (see Worked Examples 6-1 and 6-2) discover that the compound STRESS is a potent competitive inhibitor of happyase. Addition of 1 nM STRESS increases the measured K_m for SAD by a factor of 2. What are the values for α and α' under these conditions?

SOLUTION: Recall that the apparent K_m, the K_m measured in the presence of a competitive inhibitor, is defined as αK_m. Because K_m for SAD increases by a factor of 2 in the presence of 1 nM STRESS, the value of α must be 2. The value of α' for a competitive inhibitor is 1, by definition.

Irreversible Inhibition The **irreversible inhibitors** bind covalently with or destroy a functional group on an enzyme that is essential for the enzyme's activity, or they form a highly stable noncovalent association. Formation of a covalent link between an irreversible inhibitor and an enzyme is a particularly effective way to inactivate an enzyme. Irreversible inhibitors are another useful tool for studying reaction mechanisms. Amino acids with key catalytic functions in the active site can sometimes be identified by determining which residue is covalently linked to an inhibitor after the enzyme is inactivated. An example is shown in Figure 6-22.

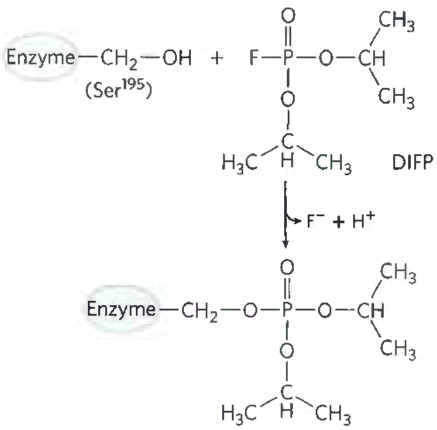


FIGURE 6-22 Irreversible inhibition. Reaction of chymotrypsin with diisopropylfluorophosphate (DIFP) modifies Ser^{195} and irreversibly inhibits the enzyme. This has led to the conclusion that Ser^{195} is the key active-site Ser residue in chymotrypsin.

A special class of irreversible inhibitors is the **suicide inactivators**. These compounds are relatively unreactive until they bind to the active site of a specific enzyme. A suicide inactivator undergoes the first few chemical steps of the normal enzymatic reaction, but instead of being transformed into the normal product, the inactivator is converted to a very reactive compound that combines irreversibly with the enzyme. These compounds are also called **mechanism-based inactivators**, because they hijack the normal enzyme reaction mechanism to inactivate the enzyme. Suicide inactivators play a significant role in *rational drug design*, an approach to obtaining new pharmaceutical agents in which chemists synthesize novel substrates based on knowledge of substrates and reaction mechanisms. A well-designed suicide inactivator is specific for a single enzyme and is unreactive until it is within that enzyme's active site, so drugs based on this approach can offer the important advantage of few side effects (Box 6-1).

An irreversible inhibitor need not bind covalently to the enzyme. Noncovalent binding is enough, if that binding is so tight that the inhibitor dissociates only rarely. How does a chemist develop a tight-binding inhibitor? **P4** Recall that enzymes evolve to bind most tightly to the transition states of the reactions that they catalyze. In principle, if one can design a molecule that looks like that reaction transition state, it should bind tightly to the enzyme. Even though transition states cannot be observed directly, chemists can often predict the approximate structure of a transition state based on accumulated knowledge about reaction mechanisms. Although the transition state is by definition transient and thus unstable, in some cases stable molecules can be designed that resemble transition states. These are called **transition-state analogs**. They bind to an enzyme more tightly than does the substrate in the ES complex, because they fit into the active site better (that is, they form a greater number of weak interactions) than the substrate itself.

BOX 6-1 MEDICINE

Curing African Sleeping Sickness with a Biochemical Trojan Horse

African sleeping sickness, or African trypanosomiasis, is caused by protists (single-celled eukaryotes) called trypanosomes (Fig. 1). This disease (and related trypanosome-caused diseases) is medically and economically significant in many developing nations. Until the late twentieth century, the disease was virtually incurable. Vaccines are ineffective because the parasite has a novel mechanism to evade the host immune system.

The cell coat of trypanosomes is covered with a single protein, which is the antigen to which the human immune system responds. Every so often, however, by a process of genetic recombination (see Table 28-1), a few cells in the population of infecting trypanosomes switch to a new protein coat, not recognized by the immune system. This process of "changing coats" can occur hundreds of times. The result is a chronic cyclic infection: the human host develops a fever, which subsides as the immune system beats back the first infection; trypanosomes with changed coats then become the seed for a second infection, and the fever recurs. This cycle can repeat for weeks, and the weakened person eventually dies.

One approach to treating African sleeping sickness uses pharmaceutical agents designed as mechanism-based enzyme inactivators (suicide inactivators). A vulnerable point in trypanosome metabolism is the pathway of polyamine biosynthesis. The polyamines spermine and spermidine, involved in DNA packaging, are required in large amounts in rapidly dividing cells. The first step in their synthesis is catalyzed by ornithine decarboxylase, an enzyme that requires for its function a coenzyme called pyridoxal phosphate. Pyridoxal phosphate (PLP), derived from vitamin B_6, forms a covalent bond with the amino acid substrates of the reactions it is involved in and acts as an electron sink to facilitate a variety of reactions (see Fig. 22-32). In mammalian cells, ornithine decarboxylase undergoes rapid turnover—that is, a rapid, constant round of enzyme degradation and synthesis. In some trypanosomes, however, the enzyme (for reasons not well understood) is stable, not readily replaced by newly synthesized enzyme. An inhibitor of ornithine decarboxylase that binds permanently to the enzyme thus adversely affects the parasite, but has little effect on human cells, which can rapidly replace inactivated enzyme.

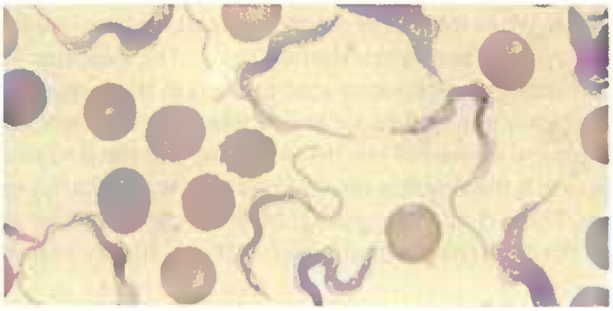


FIGURE 1 *Trypanosoma brucei rhodesiense*, one of several trypanosomes known to cause African sleeping sickness. [John Mansfield, University of Wisconsin–Madison, Department of Bacteriology.]

The first few steps of the normal reaction catalyzed by ornithine decarboxylase are shown in Figure 2. Once CO_2 is released, the electron movement is reversed and putrescine is produced (see Fig. 22-32). Based on this mechanism, several suicide inactivators have been designed, one of which is

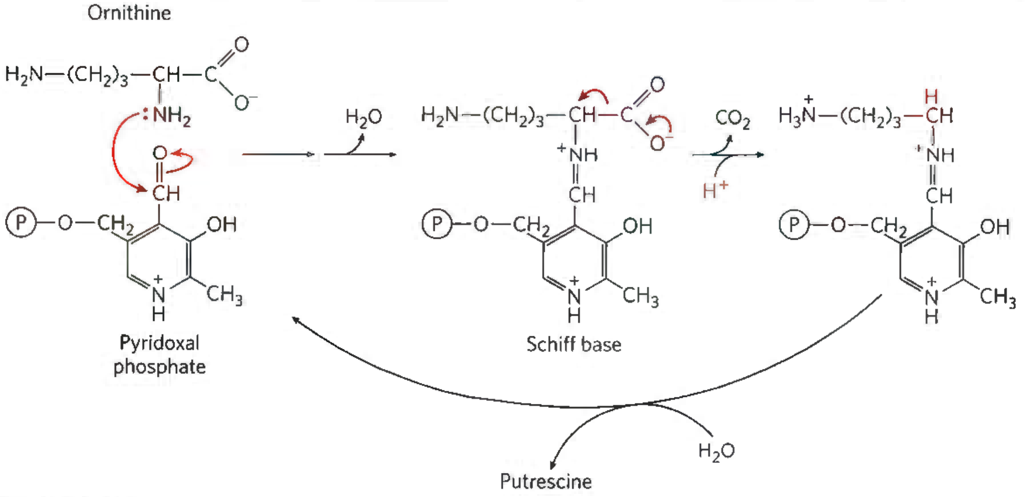


FIGURE 2 Mechanism of ornithine decarboxylase reaction.

(*Continued on next page*)

BOX 6-1 **MEDICINE**

Curing African Sleeping Sickness with a Biochemical Trojan Horse (*Continued*)

difluoromethylornithine (DFMO). DFMO is relatively inert in solution. When it binds to ornithine decarboxylase, however, the enzyme is quickly inactivated (Fig. 3). The inhibitor acts by providing an alternative electron sink in the form of two strategically placed fluorine atoms, which are excellent leaving groups. Instead of electrons moving into the ring structure of PLP, the reaction results in displacement of a fluorine atom. The —S of a Cys residue at the enzyme's active site then forms a covalent complex with the highly reactive PLP-inhibitor adduct, in an essentially irreversible reaction. In this way, the inhibitor makes use of the enzyme's own reaction mechanisms to kill it.

DFMO has proved highly effective in treating African sleeping sickness caused by *Trypanosoma brucei gambiense*. Approaches such as this show great promise for treating a wide range of diseases. The design of drugs based on enzyme mechanism and structure can complement the more traditional trial-and-error methods of developing pharmaceuticals.

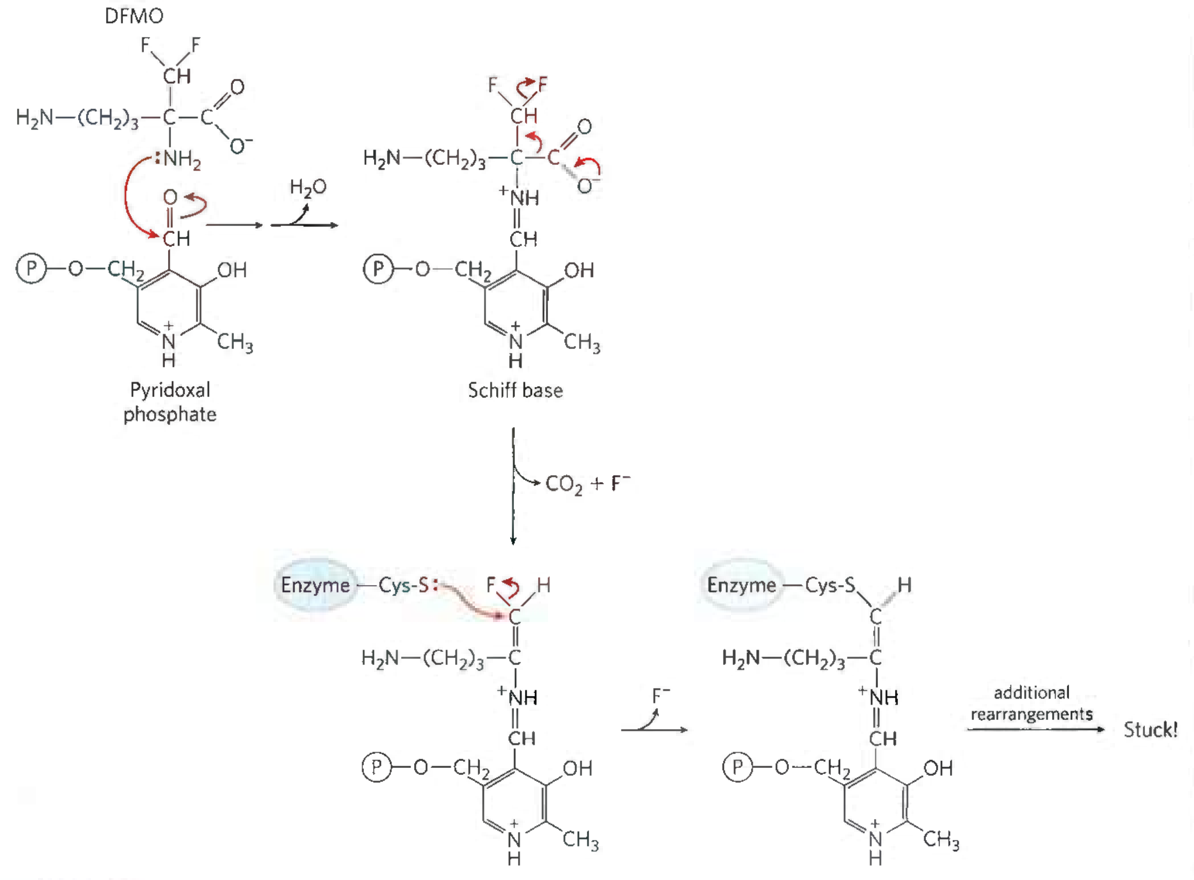


FIGURE 3 Inhibition of ornithine decarboxylase by DFMO.

The idea of transition-state analogs was suggested by Linus Pauling in the 1940s, and it has been explored using a variety of enzymes. For example, transition-state analogs designed to inhibit the glycolytic enzyme aldolase bind to that enzyme more than four orders of magnitude more tightly than do its substrates (**Fig. 6-23**). A transition-state analog cannot perfectly mimic a transition state. Some analogs, however, bind to a target enzyme 10^2 to 10^8 times more tightly than does the normal substrate, providing good evidence that enzyme active sites are indeed complementary to transition states. The concept of transition-state analogs is important to the design of new pharmaceutical agents. As we shall see in Section 6.4, the powerful anti-HIV drugs called protease inhibitors were designed in part as tight-binding transition-state analogs.

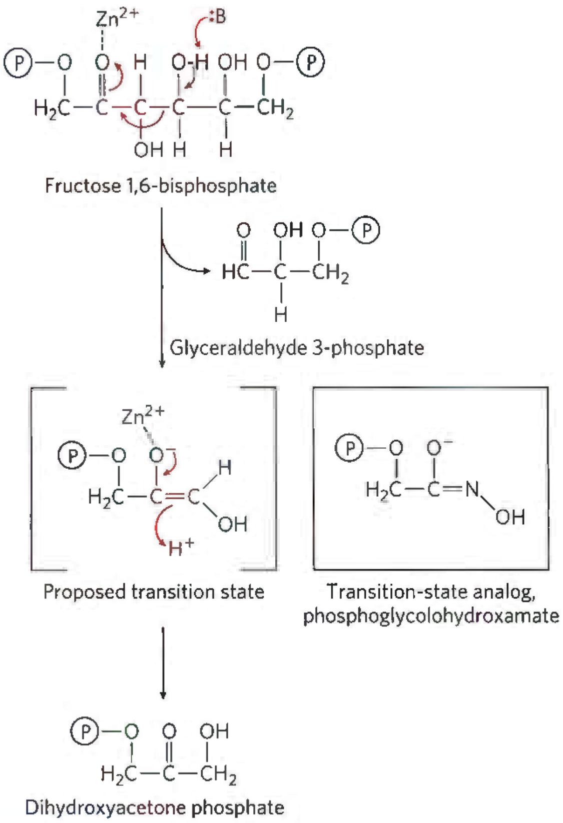

Zn2+
:B
Fructose 1,6-bisphosphate
Glyceraldehyde 3-phosphate
Zn2+
H+
Proposed transition state
Transition-state analog, phosphoglycolohydroxamate
Dihydroxyacetone phosphate

FIGURE 6-23 A transition-state analog. In glycolysis, a class II aldolase (found in bacteria and fungi) catalyzes the cleavage of fructose 1,6-bisphosphate to form glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The reaction proceeds via a reverse aldol-like mechanism. The compound phosphoglycolohydroxamate, which resembles the proposed enediolate transition state, binds to the enzyme nearly 10,000 times better than does the dihydroxyacetone phosphate product.

SUMMARY 6.3 *Enzyme Kinetics as an Approach to Understanding Mechanism*

- Most enzymes have certain kinetic properties in common. When substrate is added to an enzyme, the reaction rapidly achieves a steady state in which the rate at which the ES complex forms balances the rate at which it breaks down. As [S] increases, the steady-state activity of a fixed concentration of enzyme increases in a hyperbolic fashion to approach a characteristic maximum rate, V_{max}, at which essentially all the enzyme has formed a complex with substrate.

- The Michaelis-Menten equation

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

relates initial velocity to [S] and V_{max} through the Michaelis constant, K_m. Michaelis-Menten kinetics is also called steady-state kinetics. K_m and V_{max} have different meanings for different enzymes. However, the K_m is always equal to the substrate concentration that results in a reaction rate equal to one-half V_{max}.

- The values of V_{max} and K_m can be determined by using transformations of the Michaelis-Menten equation that allow linear plotting of data and extrapolation (for example, Lineweaver-Burk), or (more accurately) by using nonlinear regression.

- The limiting rate of an enzyme-catalyzed reaction at saturation is described by the constant k_{cat}, the turnover number. The ratio k_{cat}/K_m provides a good measure of catalytic efficiency.

- Most enzymes catalyze reactions involving multiple substrates, with the majority having two substrates and two products. The Michaelis-Menten equation is applicable to bisubstrate reactions, which occur by ternary complex or Ping-Pong (double-displacement) pathways.

- Every enzyme has an optimum pH (or pH range) at which it has maximal activity. The pH-rate profile can provide mechanistic clues.

- Pre–steady state kinetics can provide added insight into enzymatic reaction mechanisms.

- Reversible inhibition of an enzyme may be competitive, uncompetitive, or mixed. Competitive inhibitors compete with substrate by binding reversibly to the active site, but they are not transformed by the enzyme. Uncompetitive inhibitors bind only to the ES complex, at a site distinct from the active site. Mixed inhibitors bind to either E or ES, again at a site distinct from the active site. In irreversible inhibition, an inhibitor binds permanently to an active site by forming a covalent bond or a very stable noncovalent interaction. Inhibition patterns can elucidate mechanism.

6.4 Examples of Enzymatic Reactions

Thus far we have focused on the general principles of catalysis and on introducing some of the kinetic parameters used to describe enzyme action. We now turn to several examples of specific enzyme reaction mechanisms.

To understand the complete mechanism of action of a purified enzyme, we need to identify all substrates, cofactors, products, and regulators. We also need to know (1) the temporal sequence in which enzyme-bound reaction intermediates form, (2) the structure of each intermediate and each transition state, (3) the rates of interconversion between intermediates, (4) the structural relationship of the enzyme to each intermediate, and (5) the energy that all reacting and interacting groups contribute to the intermediate complexes and transition states. There are still only a few enzymes for which we have an understanding that meets all these requirements.

Here we present the mechanisms for three enzymes: chymotrypsin, hexokinase, and enolase. These examples are not intended to cover all possible classes of enzyme chemistry. They have been chosen in part because they are among the best-understood enzymes and in part because they clearly illustrate some general principles outlined in this chapter. We present the chymotrypsin example in order to review some of the conventions used to depict enzyme mechanisms. Much mechanistic detail and experimental evidence is necessarily omitted; no one book could completely document the rich experimental history of these enzymes. In addition, we consider only briefly the special contribution of coenzymes to the catalytic activity of many enzymes. The function of coenzymes is chemically varied, and we describe each coenzyme in detail as it is encountered in our discussion of metabolism in Part II of this book.

The Chymotrypsin Mechanism Involves Acylation and Deacylation of a Ser Residue

Bovine pancreatic chymotrypsin (M_r 25,191) is a **protease**, an enzyme that catalyzes the hydrolytic cleavage of peptide bonds. This protease is specific for peptide bonds adjacent to aromatic amino acid residues (Trp, Phe, Tyr). The three-dimensional structure of chymotrypsin is shown in **Figure 6-24**, with functional groups in the active site emphasized. The reaction catalyzed by this enzyme illustrates the principle of transition-state stabilization and also provides a classic example of general acid-base catalysis and covalent catalysis.

Chymotrypsin enhances the rate of peptide bond hydrolysis by a factor of at least 10^9. It does not catalyze a direct attack of water on the peptide bond; instead, a transient covalent acyl-enzyme intermediate is formed. The reaction thus has two distinct phases. In the acylation phase, the peptide bond is cleaved and an ester linkage is formed between the peptide carbonyl carbon and the enzyme. In the deacylation phase, the ester linkage is hydrolyzed and the nonacylated enzyme is regenerated.

The first evidence for a covalent acyl-enzyme intermediate came from a classic application of pre–steady state kinetics. In addition to its action on polypeptides, chymotrypsin catalyzes the hydrolysis of small esters and amides. These reactions are much slower than hydrolysis of peptides because less binding energy is available with smaller substrates (the pre–steady state is also correspondingly longer), thus simplifying the analysis of the resulting reactions. In their investigations in 1954, B. S. Hartley and B. A. Kilby found that chymotrypsin hydrolysis of the ester *p*-nitrophenylacetate, as measured by release of *p*-nitrophenol, proceeds with a rapid burst before leveling off to a slower rate (**Fig. 6-25**). By extrapolating back to zero time, they concluded that the burst phase corresponded to the release of just under one molecule of *p*-nitrophenol for every enzyme molecule present (a small fraction of their enzyme molecules were inactive). Recall from Figure 6-18 that a burst implies that the rate-limiting step of catalysis occurs after release of the product being monitored. Hartley and Kilby interpreted the burst to reflect a rapid release of *p*-nitrophenol during a rapid acylation of all the enzyme molecules. They suggested that turnover of the enzyme was limited by a subsequent, slower deacylation step. Later work substantiated their hypothesis. The observation of a burst phase provides yet another example of the use of kinetics to break down a reaction into its constituent steps.

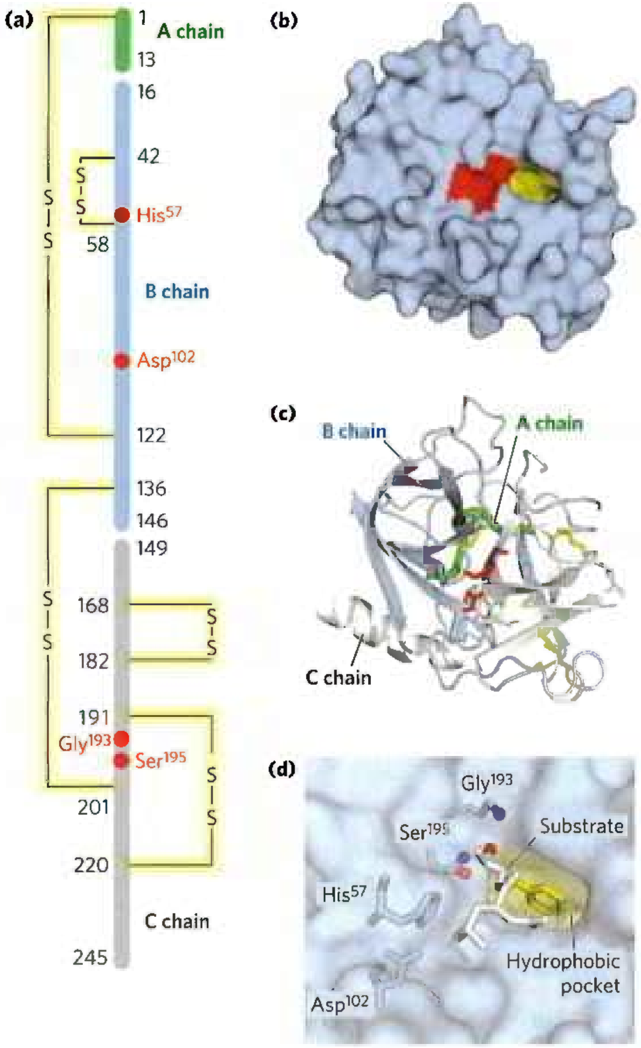


FIGURE 6-24 Structure of chymotrypsin. (a) A representation of primary structure, showing disulfide bonds and the amino acid residues crucial to catalysis. The protein consists of three polypeptide chains linked by disulfide bonds. (The numbering of residues in chymotrypsin, with "missing" residues 14, 15, 147, and 148, is explained in Fig. 6-42.) The active-site amino acid residues are grouped together in the three-dimensional structure. (b) A depiction of the enzyme emphasizing its surface. The hydrophobic pocket in which the aromatic amino acid side chain of the substrate is bound is shown in yellow. Key active-site residues, including Ser^{195}, His^{57}, and Asp^{102}, are red. The roles of these residues in catalysis are illustrated in Figure 6-27. (c) The polypeptide backbone as a ribbon structure. Disulfide bonds are yellow; the three chains are colored as in part (a). (d) A close-up of the active site with a substrate (white and yellow) bound. The hydroxyl of Ser^{195} attacks the carbonyl group of the substrate (the oxygens are red); the developing negative charge on the oxygen is stabilized by the oxyanion hole (amide nitrogens from Ser^{195} and Gly^{193}, in blue), as explained in Figure 6-27. The aromatic amino acid side chain of the substrate (yellow) sits in the hydrophobic pocket. The amide nitrogen of the peptide bond to be cleaved (protruding toward the viewer and projecting the path of the rest of the substrate polypeptide chain) is shown in white. [(b, c, d) Data from PDB ID 7GCH, K. Brady et al., *Biochemistry* 29:7600, 1990.]

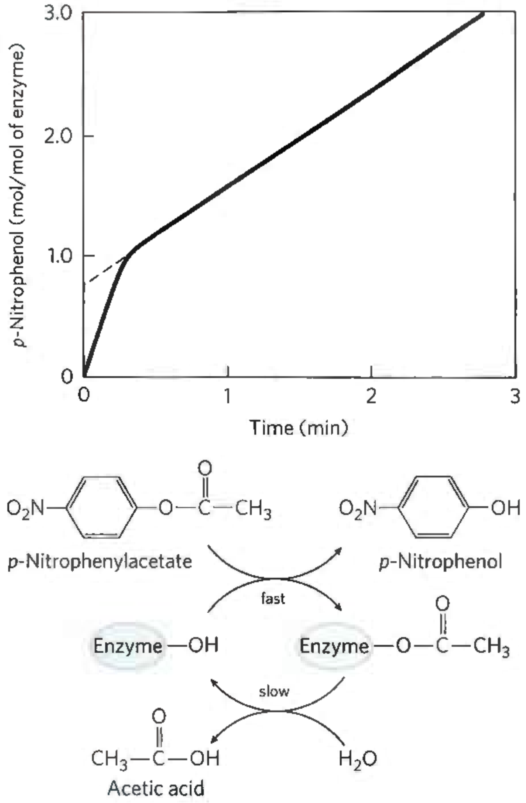


FIGURE 6-25 Pre-steady state kinetic evidence for an acyl-enzyme intermediate. The hydrolysis of *p*-nitrophenylacetate by chymotrypsin is measured by release of *p*-nitrophenol (a colored product). Initially, there is a rapid burst of *p*-nitrophenol release nearly stoichiometric with the amount of enzyme present. This reflects the fast acylation phase of the reaction. The subsequent rate is slower, because enzyme turnover is limited by the rate of the slower deacylation phase.

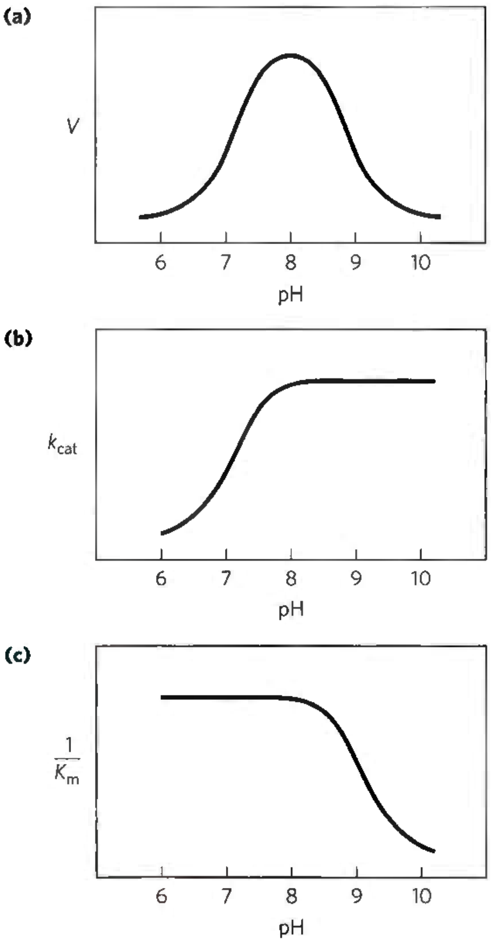
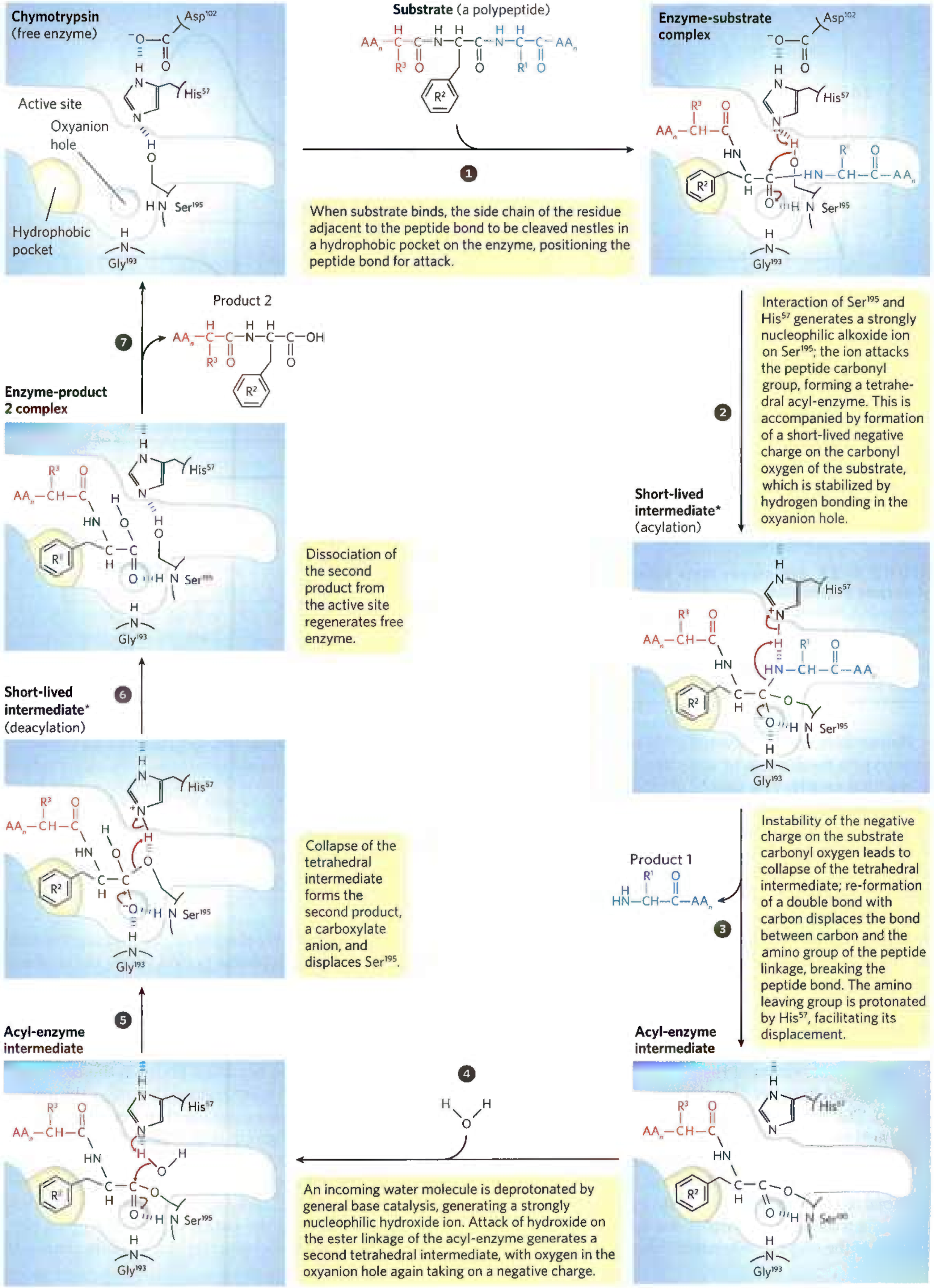


FIGURE 6-26 The pH dependence of chymotrypsin-catalyzed reactions. (a) The rates of chymotrypsin-mediated cleavage produce a bell-shaped pH-rate profile with an optimum at pH 8.0. The rate (V) plotted here is that at low substrate concentrations and thus reflects the term k_{cat}/K_m. The plot can be broken down to its components by using kinetic methods to determine the terms k_{cat} and K_m separately at each pH. When this is done (b, c), it becomes clear that the transition just above pH 7 is due to changes in k_{cat}, whereas the transition above pH 8.5 is due to changes in $1/K_m$. Kinetic and structural studies have shown that the transitions illustrated in (b) and (c) reflect the ionization states of the His^{57} side chain (when substrate is not bound) and the α-amino group of Ile^{16} (at the amino terminus of the B chain), respectively. For optimal activity, His^{57} must be unprotonated and Ile^{16} must be protonated.

Researchers have discovered additional features of the chymotrypsin mechanism by analyzing the dependence of the reaction on pH. The rate of chymotrypsin-catalyzed cleavage generally exhibits a bell-shaped pH-rate profile (**Fig. 6-26**). The rates plotted in Figure 6-26a are obtained at low (subsaturating) substrate concentrations and therefore represent k_{cat}/K_m (see Eqn 6-30, p. 193). A more complete analysis of the rates at different substrate concentrations at each pH allows researchers to determine the individual contributions of the k_{cat} and K_m terms. After obtaining the maximum rates at each pH, one can plot the k_{cat} alone versus pH (Fig. 6-26b); after obtaining the K_m at each pH, researchers can then plot $1/K_m$ versus pH (Fig. 6-26c). Kinetic and structural analyses have revealed that the change in k_{cat} reflects the ionization state of His^{57}. The decline in k_{cat} at low pH results from protonation of His^{57} (so that it can no longer extract a proton from Ser^{195} in the first chemical step of the reaction). This rate reduction illustrates the importance of general acid and general base catalysis in the mechanism for chymotrypsin. The changes in the $1/K_m$ term reflect the ionization of the α-amino group of Ile^{16} (at the amino-terminal end of one of the enzyme's three polypeptide chains). This group forms a salt bridge to Asp^{194}, stabilizing the active conformation of the enzyme. When this group loses its proton at high pH, the salt bridge is eliminated, and a conformational change closes the hydrophobic pocket where the aromatic amino acid side chain of the substrate inserts (Fig. 6-24). Substrates can no longer bind properly, which is measured kinetically as an increase in K_m.

The chymotrypsin reaction is detailed in **Figure 6-27**. The nucleophile in the acylation phase is the oxygen of Ser^{195}. (Proteases with a Ser residue that plays this role in reaction mechanisms are called **serine proteases**.) The pK_a of a Ser hydroxyl group is generally too high for the unprotonated form to be present in significant concentrations at physiological pH. However, in chymotrypsin, Ser^{195} is linked to His^{57} and Asp^{102} in a hydrogen-bonding network referred to as the **catalytic triad**. When a peptide substrate binds to chymotrypsin, a subtle change in


Chymotrypsin
(free enzyme)
Asp102
His57
Active site
Oxyanion hole
Hydrophobic pocket
Ser195
Gly193
Substrate (a polypeptide)
Enzyme-substrate complex
Short-lived intermediate* (acylation)
Acyl-enzyme intermediate
Product 1
Acyl-enzyme intermediate
Short-lived intermediate* (deacylation)
Enzyme-product 2 complex
Product 2
R¹
R²
R³
AAₙ

① When substrate binds, the side chain of the residue adjacent to the peptide bond to be cleaved nestles in a hydrophobic pocket on the enzyme, positioning the peptide bond for attack.

② Interaction of Ser^{195} and His^{57} generates a strongly nucleophilic alkoxide ion on Ser^{195}; the ion attacks the peptide carbonyl group, forming a tetrahedral acyl-enzyme. This is accompanied by formation of a short-lived negative charge on the carbonyl oxygen of the substrate, which is stabilized by hydrogen bonding in the oxyanion hole.

③ Instability of the negative charge on the substrate carbonyl oxygen leads to collapse of the tetrahedral intermediate; re-formation of a double bond with carbon displaces the bond between carbon and the amino group of the peptide linkage, breaking the peptide bond. The amino leaving group is protonated by His^{57}, facilitating its displacement.

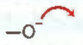
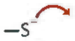
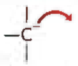
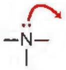
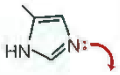
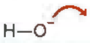
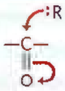
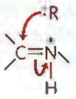
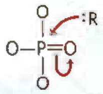
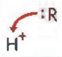
④ An incoming water molecule is deprotonated by general base catalysis, generating a strongly nucleophilic hydroxide ion. Attack of hydroxide on the ester linkage of the acyl-enzyme generates a second tetrahedral intermediate, with oxygen in the oxyanion hole again taking on a negative charge.

⑤ Collapse of the tetrahedral intermediate forms the second product, a carboxylate anion, and displaces Ser^{195}.

⑥ Dissociation of the second product from the active site regenerates free enzyme.

MECHANISM FIGURE 6-27 Hydrolytic cleavage of a peptide bond by chymotrypsin. The reaction has two phases. In the acylation phase (steps ① to ④), formation of a covalent acyl-enzyme intermediate is coupled to cleavage of the peptide bond. In the deacylation phase (steps ⑤ to ⑦), deacylation regenerates the free enzyme; this is essentially the reverse of the acylation phase, with water mirroring, in reverse, the role of the amine component of the substrate.

*The short-lived tetrahedral intermediate following step ② and the second tetrahedral intermediate that forms later, following step ⑤, are sometimes referred to as transition states, but this terminology can cause confusion. An *intermediate* is any chemical species with a finite lifetime, "finite" being defined as longer than the time required for a molecular vibration (~10^{-13} second). A *transition state* is simply the maximum-energy species formed on the reaction coordinate, and it does not have a finite lifetime. The tetrahedral intermediates formed in the chymotrypsin reaction closely resemble, both energetically and structurally, the transition states leading to their formation and breakdown. However, the intermediate represents a committed stage of completed bond formation, whereas the transition state is part of the process of reaction. In the case of chymotrypsin, given the close relationship between the intermediate and the actual transition state, the distinction between them is routinely glossed over. Furthermore, the interaction of the negatively charged oxygen with the amide nitrogens in the oxyanion hole, often referred to as transition-state stabilization, also serves to stabilize the intermediate in this case. Not all intermediates are so short-lived that they resemble transition states. The chymotrypsin acyl-enzyme intermediate is much more stable and more readily detected and studied, and it is never confused with a transition state.

Nucleophiles	Electrophiles
 Negatively charged oxygen (as in an unprotonated hydroxyl group or an ionized carboxylic acid)  Negatively charged sulfhydryl Carbanion   Uncharged amine group Imidazole  Hydroxide ion 	 Carbon atom of a carbonyl group (the more electronegative oxygen of the carbonyl group pulls electrons away from the carbon)  Protonated imine group (activated for nucleophilic attack at the carbon by protonation of the imine) Phosphorus of a phosphate group  Proton 

How to Read Reaction Mechanisms—A Refresher

Chemical reaction mechanisms, which trace the formation and breakage of covalent bonds, are communicated with dots and curved arrows, a convention known informally as "electron pushing." A covalent bond consists of a shared pair of electrons. Nonbonded electrons important to the reaction mechanism are designated by dots ($^-\ddot{O}H$). Curved arrows (↷) represent the movement of electron pairs. For movement of a single electron (as in a free radical reaction), a single-headed (fishhook-type) arrow is used (⇀). Most reaction steps involve an unshared electron pair (as in the chymotrypsin mechanism).

Some atoms are more electronegative than others; that is, they more strongly attract electrons. The relative electronegativities of atoms encountered in this text are F > O > N > C ≈ S > P ≈ H. For example, the two electron pairs making up a $C{=}O$ (carbonyl) bond are not shared equally; the carbon is relatively electron-deficient as the oxygen draws away the electrons. Many reactions involve an electron-rich atom (a nucleophile) reacting with an electron-deficient atom (an electrophile). Some common nucleophiles and electrophiles in biochemistry are shown at right.

In general, a reaction mechanism is initiated at an unshared electron pair of a nucleophile. In mechanism diagrams, the base of the electron-pushing arrow originates near the electron-pair dots, and the head of the arrow points directly at the electrophilic center being attacked. Where the unshared electron pair confers a formal negative charge on the nucleophile, the negative charge symbol itself can represent the unshared electron pair and serves as the base of the arrow. In the chymotrypsin mechanism, the nucleophilic electron pair in the ES complex between steps ① and ② is provided by the oxygen of the Ser^{195} hydroxyl group. This electron pair (2 of the 8 valence electrons of the hydroxyl oxygen) provides the base of the curved arrow. The electrophilic center under attack is the carbonyl carbon of the peptide bond to be cleaved. The C, O, and N atoms have a maximum of 8 valence electrons, and H has a maximum of 2. These atoms are occasionally found in unstable states with less than their maximum allotment of electrons, but C, O, and N cannot have more than 8. Thus, when the electron pair from chymotrypsin's Ser^{195} attacks the substrate's carbonyl carbon, an electron pair is displaced from the carbon valence shell (you cannot have 5 bonds to carbon!). These electrons move toward the more electronegative carbonyl oxygen. The oxygen has 8 valence electrons both before and after this chemical process, but the number shared with the carbon is reduced from 4 to 2, and the carbonyl oxygen acquires a negative charge. In step ③, the electron pair conferring the negative charge on the oxygen moves back to re-form a bond with carbon and reestablish the carbonyl linkage. Again, an electron pair must be displaced from the carbon, and this time it is the electron pair shared with the amino group of the peptide linkage. This breaks the peptide bond. The remaining steps follow a similar pattern.

conformation compresses the hydrogen bond between His^{57} and Asp^{102}, resulting in a stronger interaction, called a low-barrier hydrogen bond. This enhanced interaction increases the pK_a of His^{57} from ~7 (for free histidine) to >12, allowing the His residue to act as an enhanced general base that can remove the proton from the Ser^{195} hydroxyl group. Deprotonation prevents development of a highly unstable positive charge on the Ser^{195} hydroxyl and makes the Ser side chain a stronger nucleophile. At later reaction stages, His^{57} also acts as a proton donor, protonating the amino group in the displaced portion of the substrate (the leaving group).

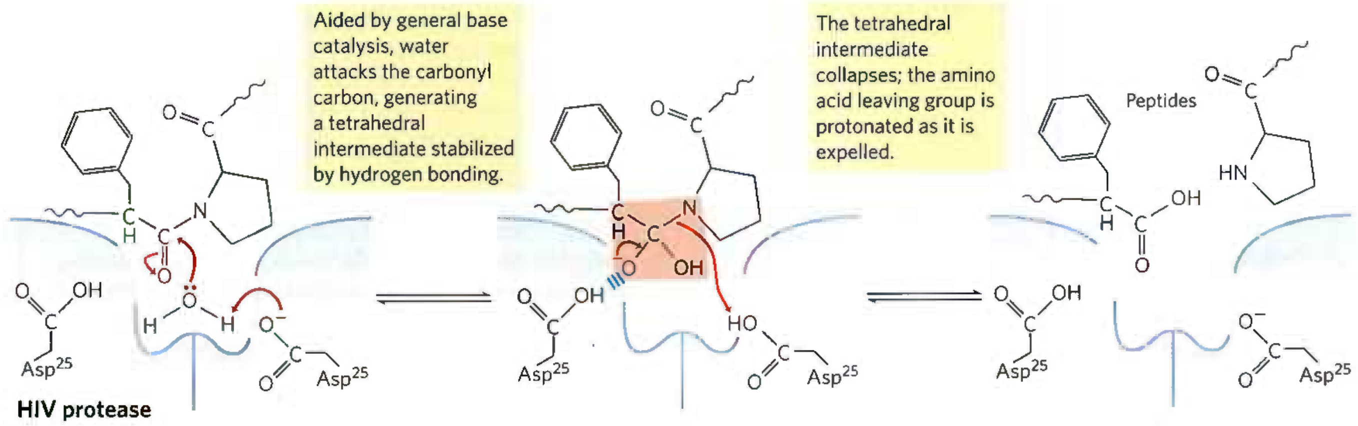
As the Ser^{195} oxygen attacks the carbonyl group of the substrate (Fig. 6-27, step ❷), a very short-lived tetrahedral intermediate is formed in which the carbonyl oxygen acquires a negative charge. This charge, forming within a pocket on the enzyme called the oxyanion hole, is stabilized by hydrogen bonds contributed by the amide groups of two peptide bonds in the chymotrypsin backbone. One of these hydrogen bonds (contributed by Gly^{193}) is present only in this intermediate and in the transition states for its formation and breakdown; it reduces the energy required to reach these states. P4 This is an example of the use of binding energy in catalysis through enzyme–transition state complementarity. The intermediate collapses in step ❸, breaking the peptide bond. The amino group of the first product is protonated by His^{57}, now acting as a general acid catalyst. Water is the second substrate, entering the active site in step ❹. As water attacks the carbon in the ester linkage in step ❺, and the resulting intermediate collapses to break the ester linkage and generate the second product in step ❻, His^{57} again acts—first as a general base to deprotonate the water, and then as a general acid to protonate the Ser oxygen as it leaves. Dissociation of the second product (step ❼) completes the reaction cycle.

An Understanding of Protease Mechanisms Leads to New Treatments for HIV Infection

New pharmaceutical agents are almost always designed to inhibit an enzyme. The extremely successful therapies developed to treat HIV infection provide a case in point. The human immunodeficiency virus (HIV) is the agent that causes acquired immune deficiency syndrome (AIDS). In 2018, 38 million people worldwide were living with HIV infection, with about 1.7 million new infections that year and approximately 770,000 fatalities. AIDS first surfaced as a worldwide epidemic in the 1980s; HIV was discovered soon after and was identified as a **retrovirus**. Retroviruses possess (1) an RNA genome and (2) an enzyme, reverse transcriptase, that is capable of using RNA to direct the synthesis of a complementary DNA. Efforts to understand HIV and develop therapies for HIV infection benefited from decades of basic research, both on enzyme mechanisms and on the properties of other retroviruses.

A retrovirus such as HIV has a relatively simple life cycle (see Fig. 26-29). Its RNA genome is converted to duplex DNA in several steps catalyzed by the reverse transcriptase (described in Chapter 26). The duplex DNA is then inserted into a chromosome in the nucleus of the host cell by the enzyme integrase (described in Chapter 25). The integrated copy of the viral genome can remain dormant indefinitely. Alternatively, it can be transcribed back into RNA, which can then be translated into proteins to construct new virus particles. Most of the viral genes are translated into large polyproteins, which are cut by an HIV protease into the individual proteins needed to make the virus (see Fig. 26-30). Only three key enzymes operate in this cycle: the reverse transcriptase, the integrase, and the protease. These enzymes thus represent the most promising drug targets.

There are four major subclasses of proteases. The serine proteases, such as chymotrypsin and trypsin, and the cysteine proteases (in which a Cys residue serves a catalytic role similar to that of Ser in the active site) form covalent enzyme-substrate complexes; the aspartyl proteases and metalloproteases do not. The HIV protease is an aspartyl protease. Two active-site Asp residues facilitate the direct attack of a water molecule on the carbonyl group of the peptide bond to be cleaved (**Fig. 6-28**). The initial product of this attack is an unstable tetrahedral intermediate, much like that in the chymotrypsin reaction.



Aided by general base catalysis, water attacks the carbonyl carbon, generating a tetrahedral intermediate stabilized by hydrogen bonding.

The tetrahedral intermediate collapses; the amino acid leaving group is protonated as it is expelled.

MECHANISM FIGURE 6-28 Mechanism of action of HIV protease. Two active-site Asp residues (from different subunits) act as general acid-base catalysts, facilitating the attack of water on the peptide bond. The unstable tetrahedral intermediate in the reaction pathway is shaded light red.

P4 This intermediate is close in structure and energy to the reaction transition state. The drugs that have been developed as HIV protease inhibitors form noncovalent complexes with the enzyme, but they bind to it so tightly that they can be considered irreversible inhibitors. The tight binding is derived in part from their design as transition-state analogs. The success of these drugs makes a point worth emphasizing: the catalytic principles we have studied in this chapter are not simply abstruse ideas to be memorized—their application saves lives.

The HIV protease is most efficient at cleaving peptide bonds between Phe and Pro residues. The active site has a pocket that binds an aromatic group next to the bond to be cleaved. Several HIV protease inhibitors are shown in **Figure 6-29**. Although the structures appear varied, they all share a core structure: a main chain with a hydroxyl group positioned next to a branch containing a benzyl group. This arrangement targets the benzyl group to an aromatic (hydrophobic) binding pocket. The adjacent hydroxyl group mimics the negatively charged oxygen in the tetrahedral intermediate in the normal reaction, providing a transition-state analog that facilitates very tight binding. The remainder of each inhibitor structure was designed to fit into and bind to various crevices along the surface of the enzyme, enhancing overall binding. The availability of these effective drugs has vastly increased the life span and quality of life of millions of people with HIV and AIDS. In 2018, 23.3 million of the 38 million people living with HIV infection were receiving antiretroviral therapy. ●

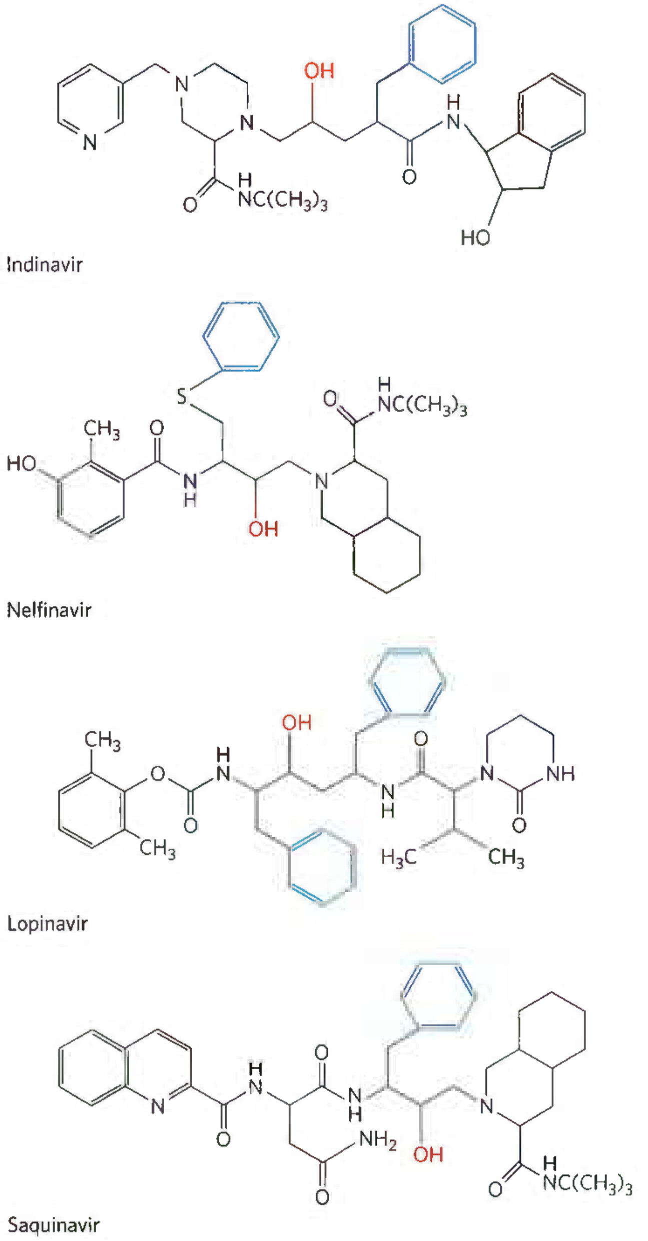
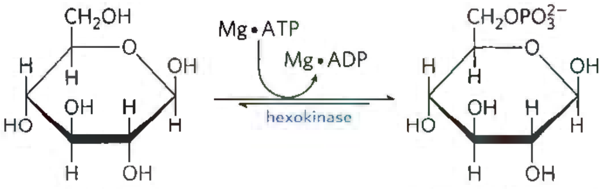


FIGURE 6-29 **HIV protease inhibitors.** The hydroxyl group (red) acts as a transition-state analog, mimicking the oxygen of the tetrahedral intermediate. The adjacent benzyl group (blue) helps to properly position the drug in the active site.

Hexokinase Undergoes Induced Fit on Substrate Binding

Yeast hexokinase (M_r 107,862) is a bisubstrate enzyme that catalyzes this reversible reaction:



β-D-Glucose Glucose 6-phosphate

ATP and ADP always bind to enzymes as a complex with the metal ion Mg^{2+}.

In the hexokinase reaction, the γ-phosphoryl of ATP is transferred to the hydroxyl at C-6 of glucose. This hydroxyl is similar in chemical reactivity to water, and water freely enters the enzyme active site. Yet hexokinase favors the reaction with glucose by a factor of 10^6. The enzyme can discriminate between glucose and water because of a conformational change in the enzyme when the correct substrate binds (**Fig. 6-30**). Hexokinase thus provides a good example of induced fit. When glucose is not present, the enzyme is in an inactive conformation, with the active-site amino acid side chains out of position for reaction. When glucose (but not water) and Mg·ATP bind, the binding energy derived from this interaction induces a conformational change in hexokinase to the catalytically active form.

This model has been reinforced by kinetic studies. The five-carbon sugar xylose, stereochemically similar to glucose but one carbon shorter, binds to hexokinase but in a position where it cannot be phosphorylated. Nevertheless, addition of xylose to the reaction mixture increases the rate of ATP hydrolysis. Evidently, the binding of xylose is sufficient to induce a change in hexokinase to its active conformation, and the enzyme is thereby "tricked" into phosphorylating water. The hexokinase reaction also illustrates that

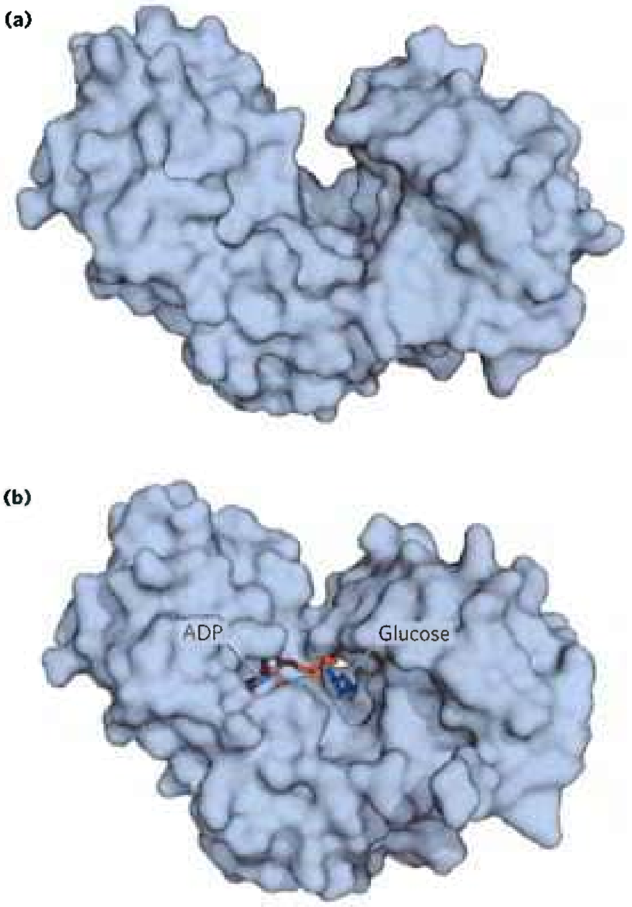
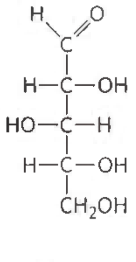
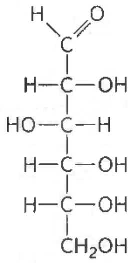


FIGURE 6-30 Induced fit in hexokinase. (a) Hexokinase has a U-shaped structure. (b) The ends pinch toward each other in a conformational change induced by binding D-glucose. [Data from (a) PDB ID 2YHX, C. M. Anderson et al., *J. Mol. Biol.* 123:15, 1978. (b) PDB ID 2E2O, modeled with ADP derived from PDB ID 2E2Q, H. Nishimasu et al., *J. Biol. Chem.* 282:9923, 2007.]

enzyme specificity is not always a simple matter of binding one compound but not another. In the case of hexokinase, specificity is observed not in the formation of the ES complex but in the relative rates of subsequent catalytic steps. Reaction rates increase greatly in the presence of a substrate, glucose, that is able to accept a phosphoryl group.

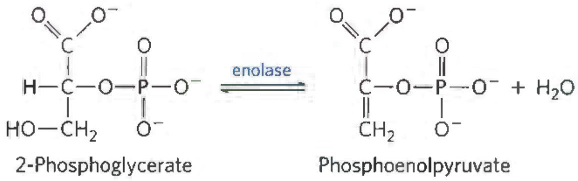

Xylose


Glucose

P4 Induced fit is only one aspect of the catalytic mechanism of hexokinase—like chymotrypsin, hexokinase uses several catalytic mechanisms. For example, the active-site amino acid residues (those brought into position by the conformational change that follows substrate binding) participate in general acid-base catalysis and transition-state stabilization.

The Enolase Reaction Mechanism Requires Metal Ions

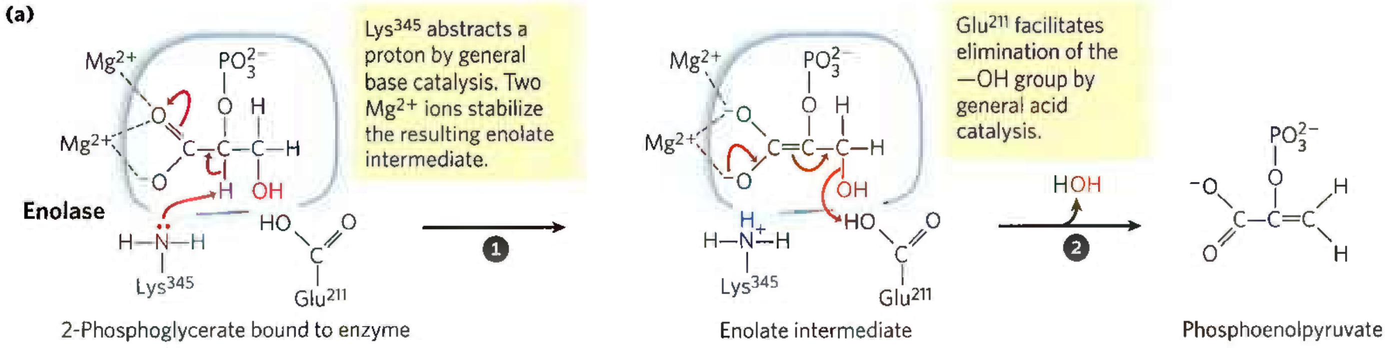
Another glycolytic enzyme, enolase, catalyzes the reversible dehydration of 2-phosphoglycerate to phosphoenolpyruvate:

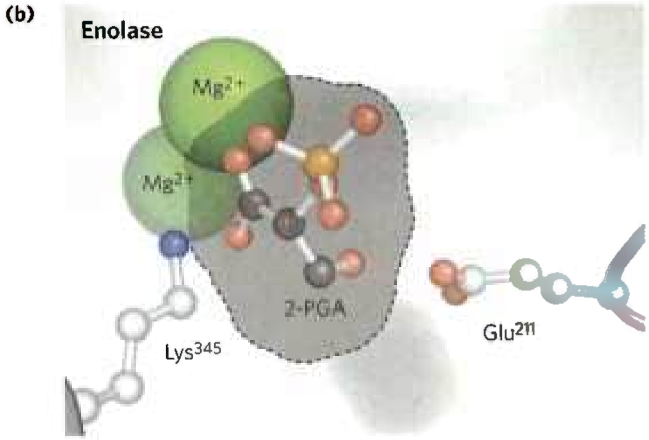


The reaction provides an example of the use of an enzymatic cofactor, in this case a metal ion (another example of coenzyme function is provided in Box 6-1). Yeast enolase (M_r 93,316) is a dimer with 436 amino acid residues per subunit. The enolase reaction illustrates one type of metal ion catalysis and provides an additional example of general acid-base catalysis and transition-state stabilization. The reaction occurs in two steps (**Fig. 6-31a**). First, Lys^{345} acts as a general base catalyst, abstracting a proton from C-2 of 2-phosphoglycerate; then Glu^{211} acts as a general acid catalyst, donating a proton to the —OH leaving group. The proton at C-2 of 2-phosphoglycerate is not acidic and thus is quite resistant to its removal by Lys^{345}. However, the electronegative oxygen atoms of the adjacent carboxyl group pull electrons away from C-2, making the attached protons somewhat more labile. In the active site, the carboxyl group of 2-phosphoglycerate undergoes strong ionic interactions with two bound Mg^{2+} ions (Fig. 6-31b), greatly enhancing the electron withdrawal by the carboxyl. Together, these effects render the C-2 protons sufficiently acidic (lowering the pK_a) that one proton can be abstracted to initiate the reaction. As the unstable enolate intermediate is formed, the metal ions further act to shield the two negative charges (on the carboxyl oxygen atoms) that transiently exist in close proximity to each other. Hydrogen bonding to other active-site amino acid residues also contributes to the overall mechanism. The various interactions effectively stabilize both the enolate intermediate and the transition state preceding its formation.

An Understanding of Enzyme Mechanism Produces Useful Antibiotics

Penicillin was discovered in 1928 by Alexander Fleming, but another 15 years passed before this


(a)
Enolase
Mg2+
Mg2+
PO32−
O
H
C
C
C
H
H
OH
H—N—H
Lys345
HO
O
C
Glu211
2-Phosphoglycerate bound to enzyme
Lys345 abstracts a proton by general base catalysis. Two Mg2+ ions stabilize the resulting enolate intermediate.
1
Enolate intermediate
Glu211 facilitates elimination of the —OH group by general acid catalysis.
HOH
2
Phosphoenolpyruvate


(b)
Enolase
Mg2+
Mg2+
2-PGA
Lys345
Glu211

MECHANISM FIGURE 6-31 Two-step reaction catalyzed by enolase. (a) The mechanism by which enolase converts 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate. The carboxyl group of 2-PGA is coordinated by two Mg^{2+} ions at the active site. (b) The substrate, 2-PGA, in relation to the Mg^{2+}, Lys^{345}, and Glu^{211} in the enolase active site (gray outline). Nitrogen is shown in blue, phosphorus in orange; hydrogen atoms are not shown. [(b) Data from PDB ID 1ONE, T. M. Larsen et al., *Biochemistry* 35:4349, 1996.]

relatively unstable compound was understood well enough for it to be used as a pharmaceutical agent to treat bacterial infections. Penicillin interferes with the synthesis of peptidoglycan, the major component of the rigid cell wall that protects bacteria from osmotic lysis. Peptidoglycan consists of polysaccharides and peptides cross-linked in several steps that include a transpeptidase reaction (**Fig. 6-32**). It is this reaction that is inhibited by penicillin and related compounds (**Fig. 6-33a**), all of which are irreversible inhibitors of transpeptidase. They bind to the active site of transpeptidase through a segment that mimics one conformation of the D-Ala–D-Ala segment of the peptidoglycan precursor. The peptide bond in the precursor is replaced by a highly reactive β-lactam ring in the antibiotic. When penicillin binds to the transpeptidase, an active-site Ser attacks the carbonyl of the β-lactam ring and generates a covalent adduct between penicillin and the enzyme. The leaving group remains attached, however, because it is linked by the remnant of the β-lactam ring (Fig. 6-33b). The covalent complex irreversibly inactivates the enzyme. This, in turn, blocks synthesis of the bacterial cell wall, and most bacteria die as the fragile inner membrane bursts under osmotic pressure.

Human use of penicillin and its derivatives has led to the evolution of strains of pathogenic bacteria that express **β-lactamases** (**Fig. 6-34a**), enzymes that cleave β-lactam antibiotics, rendering them inactive. The bacteria thereby become resistant to the antibiotics. The genes for these enzymes have spread rapidly through bacterial populations under the selective pressure imposed by the use (and often overuse) of β-lactam antibiotics. Human medicine responded with the development of compounds such as clavulanic acid, a suicide inactivator, which irreversibly inactivates the β-lactamases (Fig. 6-34b). Clavulanic acid mimics the structure of a β-lactam antibiotic and forms a covalent adduct with a Ser in the β-lactamase active site. This leads to a rearrangement that creates a much more reactive derivative, which is subsequently attacked by another nucleophile in the active site to irreversibly acylate the enzyme and inactivate it. Amoxicillin and clavulanic acid are combined in a widely used pharmaceutical formulation with the trade name Augmentin. The cycle of chemical warfare between humans and bacteria continues unabated. Strains of disease-causing bacteria that are resistant to both amoxicillin and clavulanic acid have been discovered. Mutations in β-lactamase within these strains render it unreactive to clavulanic acid. The development of new antibiotics promises to be a growth industry for the foreseeable future. ■

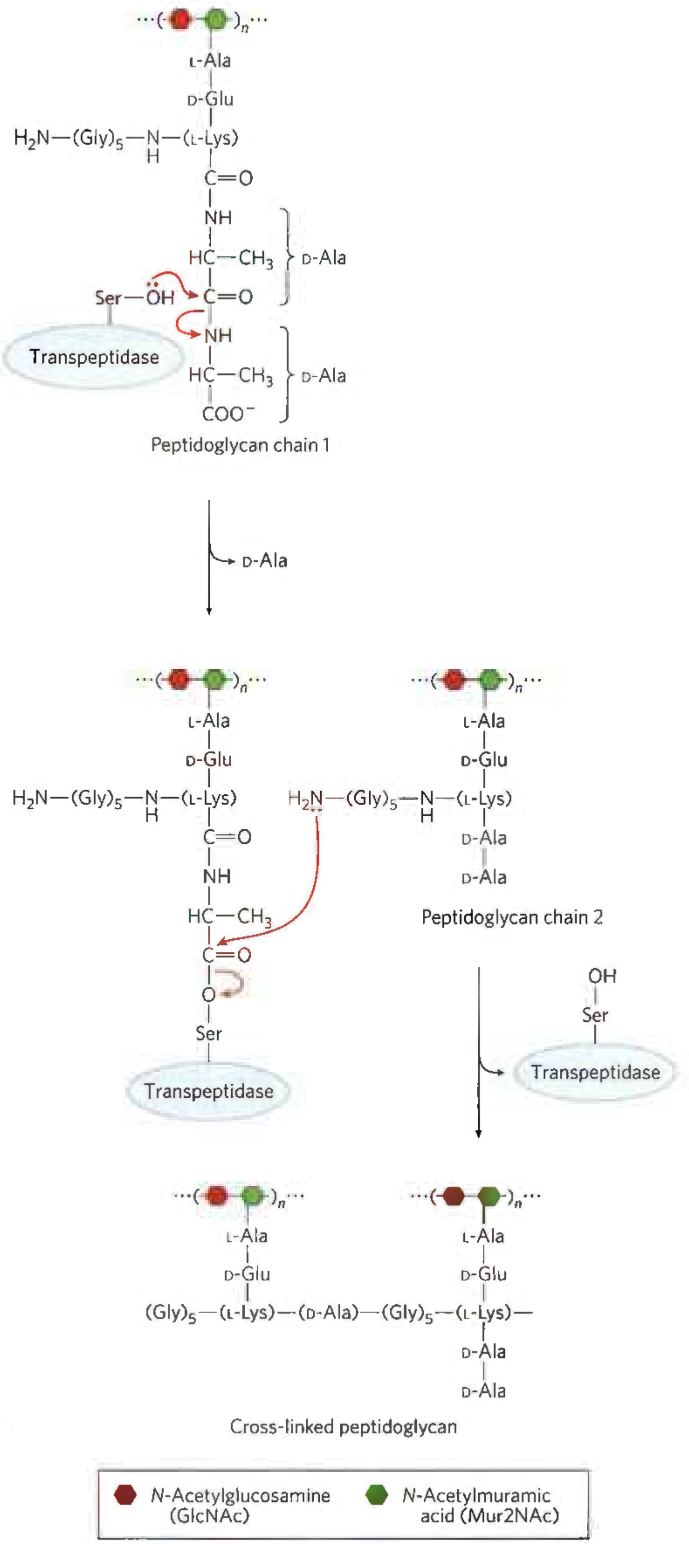

···(●-●)ₙ···
L-Ala
D-Glu
H₂N—(Gly)₅—N—(L-Lys)
H
C=O
NH
HC—CH₃
D-Ala
Ser—ÖH
C=O
NH
Transpeptidase
HC—CH₃
D-Ala
COO⁻
Peptidoglycan chain 1
D-Ala
H₂N—(Gly)₅—N—(L-Lys)
D-Ala
D-Ala
O
Ser
Transpeptidase
Peptidoglycan chain 2
OH
Ser
Transpeptidase
(Gly)₅—(L-Lys)—(D-Ala)—(Gly)₅—(L-Lys)—
Cross-linked peptidoglycan
N-Acetylglucosamine (GlcNAc)
N-Acetylmuramic acid (Mur2NAc)

FIGURE 6-32 The transpeptidase reaction. This reaction, which links two peptidoglycan precursors into a larger polymer, is facilitated by an active-site Ser and a covalent catalytic mechanism similar to that of chymotrypsin. Note that peptidoglycan is one of the few places in nature where D-amino acid residues are found. The active-site Ser attacks the carbonyl of the peptide bond between the two D-Ala residues, creating a covalent ester linkage between the substrate and the enzyme, with release of the terminal D-Ala residue. An amino group from the second peptidoglycan precursor then attacks the ester linkage, displacing the enzyme and cross-linking the two precursors.

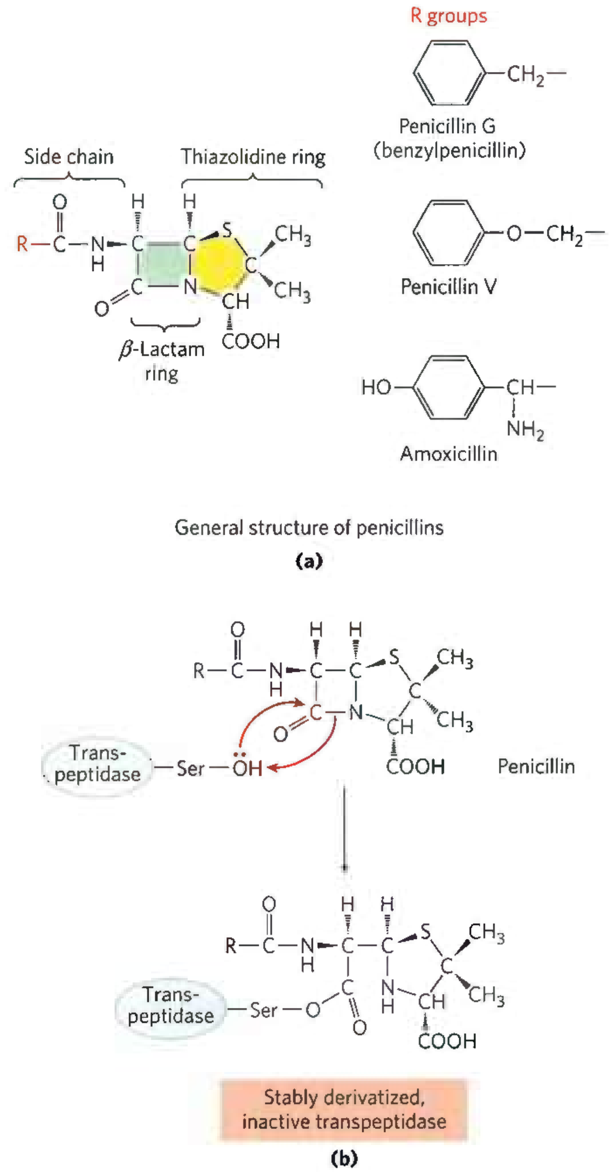
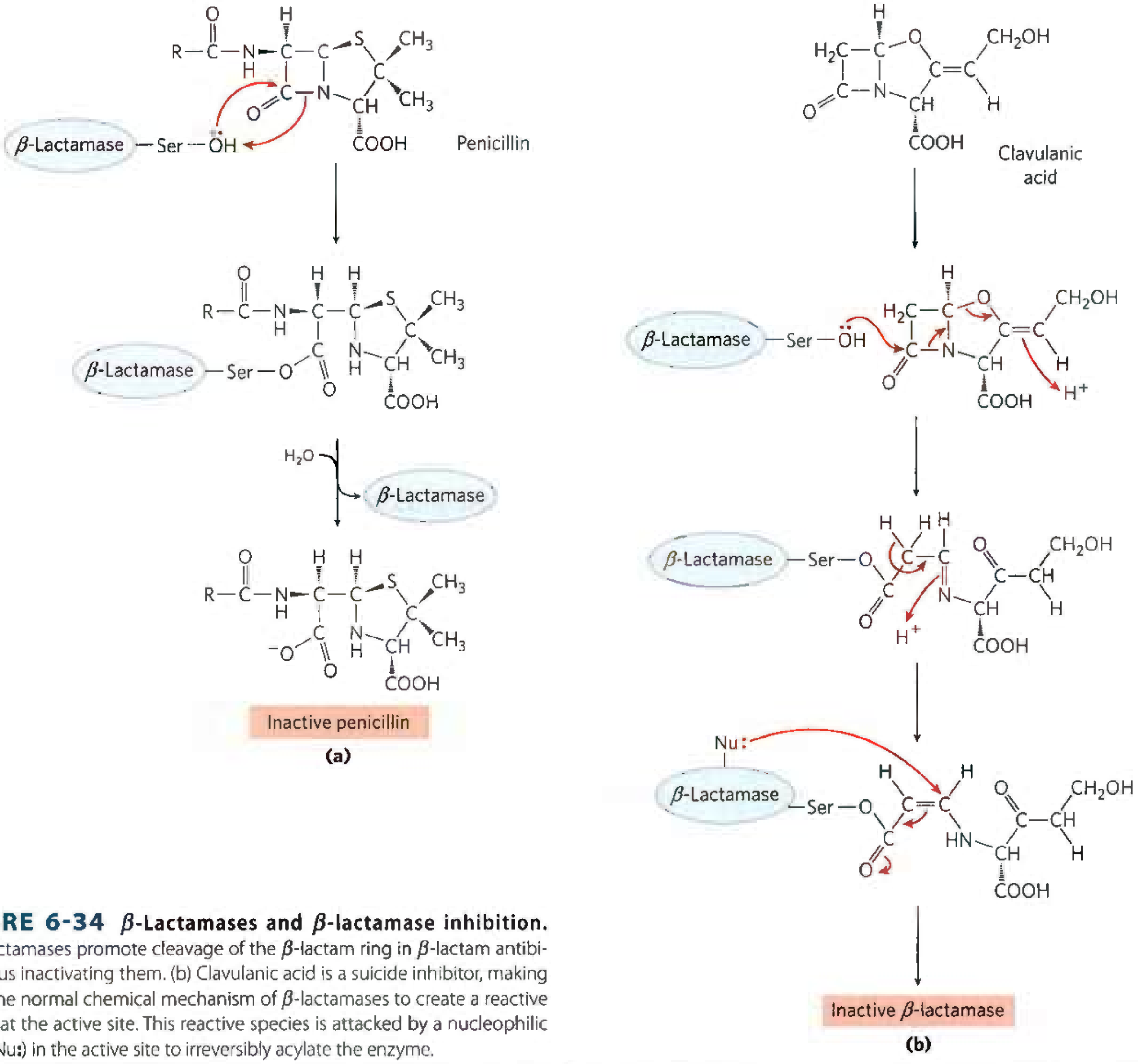

R groups
Penicillin G (benzylpenicillin)
Penicillin V
HO
NH₂
Amoxicillin
Side chain
Thiazolidine ring
R—C—N
S
CH₃
COOH
β-Lactam ring
General structure of penicillins
(a)
Trans-peptidase
—Ser—ÖH
Penicillin
Trans-peptidase
—Ser—O
Stably derivatized, inactive transpeptidase
(b)

FIGURE 6-33 Transpeptidase inhibition by β-lactam antibiotics. (a) β-Lactam antibiotics have a five-membered thiazolidine ring fused to a four-membered β-lactam ring. The latter ring is strained and includes an amide moiety that plays a critical role in the inactivation of peptidoglycan synthesis. The R group differs with the type of penicillin. Penicillin G was the first to be isolated and remains one of the most effective, but it is degraded by stomach acid and must be administered by injection. Penicillin V is nearly as effective and is acid stable, so it can be administered orally. Amoxicillin has a broad range of effectiveness, is readily administered orally, and thus is the most widely prescribed β-lactam antibiotic. (b) Attack on the amide moiety of the β-lactam ring by a transpeptidase active-site Ser results in a covalent acyl-enzyme product. This is hydrolyzed so slowly that adduct formation is practically irreversible, and the transpeptidase is inactivated.



(a)

(b)

FIGURE 6-34 β-Lactamases and β-lactamase inhibition. (a) β-Lactamases promote cleavage of the β-lactam ring in β-lactam antibiotics, thus inactivating them. (b) Clavulanic acid is a suicide inhibitor, making use of the normal chemical mechanism of β-lactamases to create a reactive species at the active site. This reactive species is attacked by a nucleophilic group (Nu:) in the active site to irreversibly acylate the enzyme.

SUMMARY 6.4 ***Examples of Enzymatic Reactions***

- Chymotrypsin is a serine protease with a well-understood mechanism, featuring general acid-base catalysis, covalent catalysis, and transition-state stabilization.
- Hexokinase provides an excellent example of induced fit as a means of using substrate binding energy.
- The enolase reaction proceeds via metal ion catalysis.
- Understanding enzyme mechanism allows for the development of drugs to inhibit enzyme action.

6.5 Regulatory Enzymes

In cellular metabolism, groups of enzymes work together in sequential pathways to carry out a given metabolic process, such as the multireaction breakdown of glucose to lactate or the multireaction synthesis of an amino acid from simpler precursors. Each separate reaction is catalyzed by a different enzyme. In such enzyme systems, the reaction product of one enzyme becomes the substrate of the next. This functional compartmentalization of cellular chemistry does more than accelerate individual reactions; it provides opportunities for the exquisitely precise regulation of all cellular processes.

Most of the enzymes in each metabolic pathway follow the kinetic patterns we have already described. Each pathway, however, includes one or more enzymes that have a greater effect on the rate of the overall sequence. The catalytic activity of these **regulatory enzymes** increases or decreases in response to certain signals. **P5** Adjustments in the rate of reactions catalyzed by regulatory enzymes, and therefore in the rate of entire metabolic sequences, allow the cell to meet changing needs for energy and for biomolecules required in growth and repair.

The activities of regulatory enzymes are modulated in a variety of ways. **Allosteric enzymes** function through reversible, noncovalent binding of regulatory compounds called **allosteric modulators** or **allosteric effectors**, which are generally small metabolites or cofactors. Other enzymes are regulated by reversible **covalent modification**. Both classes of regulatory enzymes tend to be multisubunit proteins, and in some cases the regulatory site(s) and the active site are on separate subunits. Metabolic systems have at least two other mechanisms of enzyme regulation. Some enzymes are stimulated or inhibited when they are bound by separate **regulatory proteins**. Others are activated when peptide segments are removed by **proteolytic cleavage**; unlike effector-mediated regulation, regulation by proteolytic cleavage is irreversible. Important examples of both mechanisms are found in physiological processes such as digestion, blood clotting, hormone action, and vision.

Cell growth and survival depend on efficient use of resources, and this efficiency is made possible by regulatory enzymes. No single rule governs which of the various types of regulation occur in different systems. Several types of regulation may occur in a single regulatory enzyme. The remainder of this chapter is devoted to a discussion of these major mechanisms of enzyme regulation.

Allosteric Enzymes Undergo Conformational Changes in Response to Modulator Binding

As we saw in Chapter 5, allosteric proteins are those having "other shapes" or conformations induced by the binding of modulators. The same concept applies to certain regulatory enzymes, as conformational changes induced by one or more modulators interconvert more-active and less-active forms of the enzyme. The modulators for allosteric enzymes may be inhibitory or stimulatory. The modulator can be the substrate itself; regulation in which substrate and modulator are identical is referred to as **homotropic**. The effect is similar to that of O_2 binding to hemoglobin (Chapter 5): binding of the ligand—or substrate, in the case of enzymes—causes conformational changes that affect the subsequent activity of other sites on the protein. In most cases, the conformational change converts a relatively inactive conformation (often referred to as a T state—a convention based on the early hemoglobin literature) to a more active conformation (an R state). When the modulator is a molecule other than the substrate, the enzyme is said to be **heterotropic**. Note that heterotropic modulators should not be confused with uncompetitive and mixed inhibitors. Although the latter bind at a second site on the enzyme, they do not necessarily mediate conformational changes between active and inactive forms, and the kinetic effects are distinct.

The properties of allosteric enzymes are significantly different from those of simple nonregulatory enzymes. Some of the differences are structural. In addition to active sites, allosteric enzymes often have one or more regulatory, or allosteric, sites for binding to each heterotropic modulator (**Fig. 6-35**). Just as an enzyme's active site is specific for its substrate, each regulatory site is specific for its modulator. Enzymes with several modulators generally have different specific binding sites for each. In homotropic enzymes, the active site and regulatory site are the same.

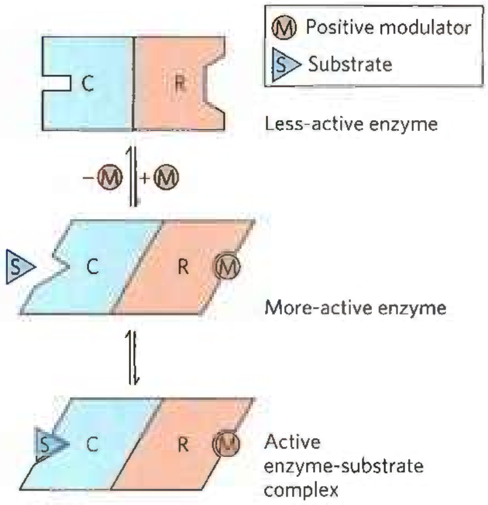
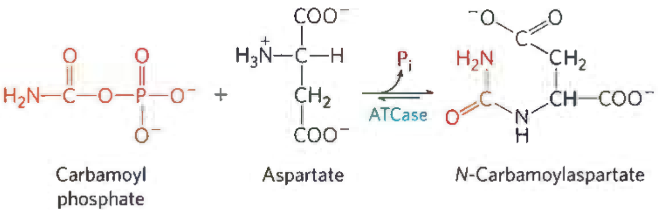


FIGURE 6-35 Subunit interactions in an allosteric enzyme, and interactions with inhibitors and activators. In many allosteric enzymes, the substrate-binding site and the modulator-binding site(s) are on different subunits: the catalytic (C) and regulatory (R) subunits, respectively. Binding of the positive (stimulatory) modulator (M) to its specific site on the regulatory subunit is communicated to the catalytic subunit through a conformational change. This change renders the catalytic subunit active and capable of binding the substrate (S) with higher affinity. Upon dissociation of the modulator from the regulatory subunit, the enzyme reverts to its inactive or less-active form.

Allosteric enzymes are typically larger and more complex than nonallosteric enzymes, with two or more subunits. A classic example is aspartate transcarbamoylase (often abbreviated ATCase), which catalyzes an early step in the biosynthesis of pyrimidine nucleotides, the reaction of carbamoyl phosphate and aspartate to form carbamoyl aspartate:



ATCase has 12 polypeptide chains organized into 6 catalytic subunits (organized as 2 trimeric complexes) and 6 regulatory subunits (organized as 3 dimeric complexes). **Figure 6-36** shows the quaternary structure of this enzyme, deduced from x-ray analysis. The enzyme exhibits allosteric behavior as detailed below, as the catalytic subunits function cooperatively. The regulatory subunits have binding sites for ATP and CTP, which function as positive and negative regulators, respectively. CTP is one of the end products of the pathway, and negative

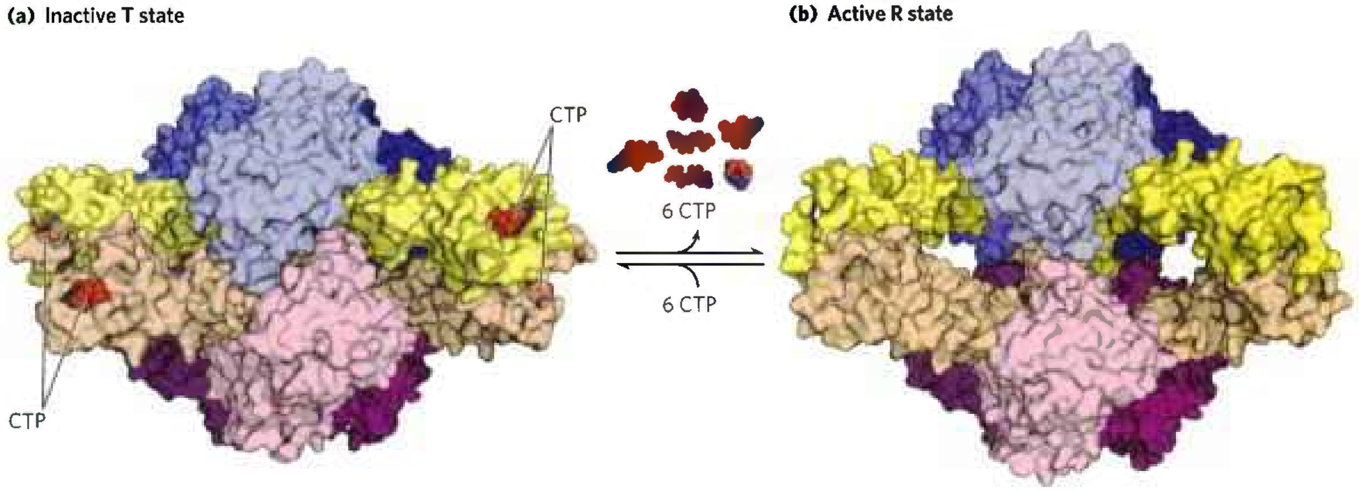


FIGURE 6-36 The regulatory enzyme aspartate transcarbamoylase. (a) The inactive T state and (b) the active R state of the enzyme are shown. This allosteric regulatory enzyme has two stacked catalytic clusters, each with three catalytic polypeptide chains (in shades of blue and purple), and three regulatory clusters, each with two regulatory polypeptide chains (in beige and yellow). The regulatory clusters form the points of a triangle (not evident in this side view) surrounding the catalytic subunits. Binding sites for allosteric modulators (including CTP) are on the regulatory subunits. Modulator binding produces large changes in enzyme conformation and activity. The role of this enzyme in nucleotide synthesis, and details of its regulation, are discussed in Chapter 22. [Data from (a) PDB ID 1RAB, R. P. Kosman et al., *Proteins* 15:147, 1993; (b) PDB ID 1F1B, L. Jin et al., *Biochemistry* 39:8058, 2000.]

regulation by CTP serves to limit ATCase action under conditions when CTP is abundant. On the other hand, high concentrations of ATP indicate that cellular metabolism is robust, that the cell is growing, and that additional pyrimidine nucleotides may be needed to support RNA transcription and DNA replication.

The Kinetic Properties of Allosteric Enzymes Diverge from Michaelis-Menten Behavior

Allosteric enzymes show relationships between V_0 and [S] that differ from Michaelis-Menten kinetics. They do exhibit saturation with the substrate when [S] is sufficiently high, but for allosteric enzymes, plots of V_0 versus [S] (**Fig. 6-37**) usually produce a sigmoid saturation curve, rather than the hyperbolic curve typical of nonregulatory enzymes. On the sigmoid saturation curve we can find a value of [S] at which V_0 is half-maximal, but we cannot refer to it with the designation K_m, because the enzyme does not follow the hyperbolic Michaelis-Menten relationship. Instead, the symbol $[S]_{0.5}$ or $K_{0.5}$ is often used to represent the substrate concentration giving half-maximal velocity of the reaction catalyzed by an allosteric enzyme (Fig. 6-37).

Sigmoid kinetic behavior reflects cooperative interactions between multiple protein subunits. In other words, changes in the structure of one subunit are translated into structural changes in adjacent subunits, an effect mediated by noncovalent interactions at the interface between subunits. Sigmoid kinetic behavior for enzymes is well explained by the concerted and sequential models for subunit interactions we previously encountered when considering O_2 binding to hemoglobin (see Fig. 5-14).

ATCase effectively illustrates both homotropic and heterotropic allosteric kinetic behavior. Binding of the substrates, aspartate and carbamoyl phosphate, to the enzyme gradually brings about a transition from the relatively inactive T state to the more active R state. This accounts for the sigmoid rather than hyperbolic change in V_0 with increasing [S]. One characteristic of sigmoid kinetics is that small changes in the concentration of a modulator can be associated with large changes in activity. As exemplified in Figure 6-37a, a relatively small increase in [S] in the steep part of the curve causes a comparatively large increase in V_0.

The heterotropic allosteric regulation of ATCase is brought about by its interactions with ATP and CTP. For heterotropic allosteric enzymes, an activator may cause the curve to become more nearly hyperbolic, with a decrease in $K_{0.5}$ but no change in V_{max}, resulting in an increased reaction velocity at a fixed substrate concentration. For ATCase, the interaction with ATP brings this about, and the enzyme exhibits a V_0 versus [S] curve that is characteristic of the active R state at sufficiently high ATP concentrations (V_0 is higher for any value of [S]; Fig. 6-37b). A negative modulator (an inhibitor) may produce a *more* sigmoid substrate-saturation curve, with an increase in $K_{0.5}$, as illustrated by the effects of CTP on ATCase kinetics (see curves for a negative modulator in Fig. 6-37b). Other heterotropic allosteric enzymes respond to an activator by an increase in V_{max} with little change in $K_{0.5}$ (Fig. 6-37c). Heterotropic allosteric enzymes therefore show different kinds of responses in their substrate-activity curves, because some have inhibitory modulators, some have activating modulators, and some (like ATCase) have both.

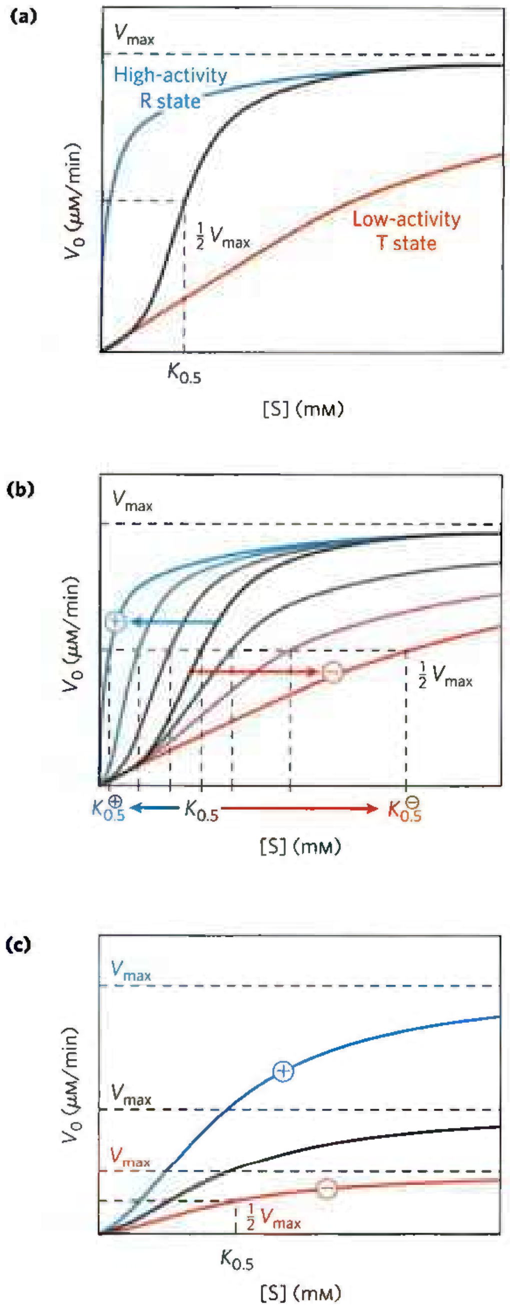


FIGURE 6-37 Substrate-activity curves for representative allosteric enzymes. Three examples of complex responses of allosteric enzymes to their modulators. (a) The sigmoid curve of a homotropic enzyme, in which the substrate also serves as a positive (stimulatory) modulator, or activator. Notice the resemblance to the oxygen-saturation curve of hemoglobin (see Fig. 5-12). The sigmoid curve is a hybrid curve in which the enzyme is present primarily in the relatively inactive T state at low substrate concentration, and primarily in the more active R state at high substrate concentration. The curves for the pure T and R states are plotted separately in color. ATCase exhibits a kinetic pattern similar to this. (b) The effects of several different concentrations of a positive modulator (+) or a negative modulator (−) on an allosteric enzyme in which $K_{0.5}$ is altered without a change in V_{max}. The central curve shows the substrate-activity relationship without a modulator. For ATCase, CTP is a negative modulator and ATP is a positive modulator. (c) A less common type of modulation, in which V_{max} is altered and $K_{0.5}$ is nearly constant.

Some Enzymes Are Regulated by Reversible Covalent Modification

In another important class of regulatory enzymes, activity is modulated by covalent modification of one or more of the amino acid residues in the enzyme molecule. Over 500 different types of covalent modification have been found in proteins. Common modifying groups include phosphoryl, acetyl, adenylyl, uridylyl, methyl, amide, carboxyl, myristoyl, palmitoyl, prenyl, hydroxyl, sulfate, and adenosine diphosphate ribosyl groups (**Fig. 6-38**). There are even entire proteins that function as specialized modifying groups. These include ubiquitin and SUMO (small *u*biquitin-like *mo*difier), which are attached and detached from other proteins to regulate their activity in some way. All of these groups, small and large, are linked to and removed from a regulated enzyme by separate enzymes. When an amino acid residue in an enzyme is modified, a novel amino acid with altered properties has effectively been introduced into the enzyme. Introduction of a charge can alter the local properties of the enzyme and induce a change in conformation. Introduction of a hydrophobic group can trigger association with a membrane. The changes are often substantial and can be critical to the function of the altered enzyme.

Although there are too many examples of covalent modification of proteins to cover in detail, a few examples are instructive. One enzyme that is regulated by methylation is the methyl-accepting chemotaxis protein of bacteria. This protein is part of a system that permits a bacterium to swim toward an attractant (such as a sugar) in solution and away from repellent chemicals. The methylating agent is *S*-adenosylmethionine (adoMet) (see Fig. 18-18). Acetylation is another common modification, with approximately 80% of the soluble proteins in eukaryotes, including many enzymes, acetylated at their amino terminus. Ubiquitin is added to proteins as a tag that destines them for proteolytic degradation (see Fig. 27-47). Ubiquitination can also have a regulatory function. SUMO is found attached to many eukaryotic nuclear proteins and has roles in the regulation of transcription, chromatin structure, and DNA repair.

ADP-ribosylation is an especially interesting reaction; the ADP-ribose is derived from nicotinamide adenine dinucleotide (NAD) (see Fig. 8-41). This type of modification occurs for the bacterial enzyme dinitrogenase reductase, resulting in regulation of the important process of biological nitrogen fixation. Diphtheria toxin and cholera toxin are enzymes that catalyze the ADP-ribosylation (and inactivation) of key cellular enzymes or other proteins.

By far the most common type of regulatory modification is phosphorylation. About one-third of all proteins in a eukaryotic cell are phosphorylated, and one or (often) many phosphorylation events are part of virtually every regulatory process. Some proteins have only one

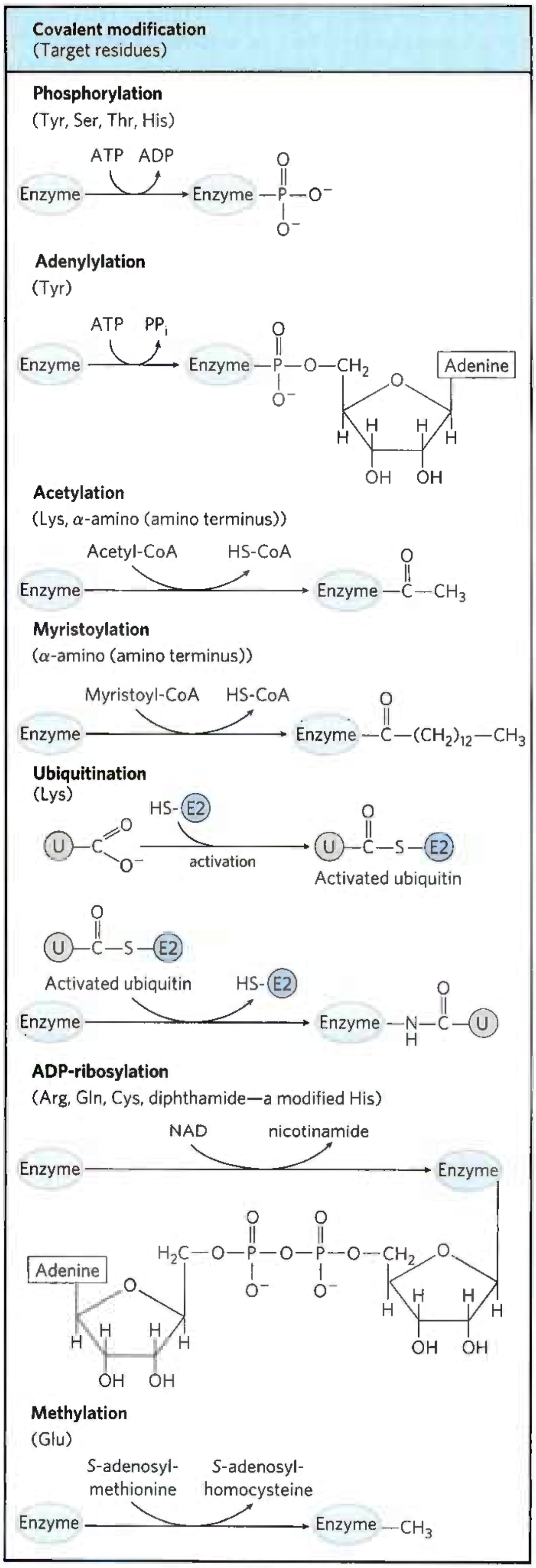

Covalent modification
(Target residues)
Phosphorylation
(Tyr, Ser, Thr, His)
ATP ADP
Enzyme
Enzyme
Adenylylation
(Tyr)
ATP PPi
Adenine
Acetylation
(Lys, α-amino (amino terminus))
Acetyl-CoA HS-CoA
Myristoylation
(α-amino (amino terminus))
Myristoyl-CoA HS-CoA
Ubiquitination
(Lys)
HS-E2
activation
Activated ubiquitin
U
E2
ADP-ribosylation
(Arg, Gln, Cys, diphthamide—a modified His)
NAD nicotinamide
Methylation
(Glu)
S-adenosyl-methionine S-adenosyl-homocysteine

FIGURE 6-38 **Some enzyme modification reactions.**

phosphorylated residue, others have several, and a few have dozens of sites for phosphorylation. This mode of covalent modification is central to a large number of regulatory pathways. We discuss it in some detail here, and again in Chapter 12.

We will encounter all of these types of enzyme modification again in later chapters.

Phosphoryl Groups Affect the Structure and Catalytic Activity of Enzymes

The attachment of phosphoryl groups to specific amino acid residues of a protein is catalyzed by **protein kinases**. More than 500 genes encoding these critical enzymes are found in the human genome. In the reactions they catalyze, the γ-phosphoryl group derived from a nucleoside triphosphate (usually ATP) is transferred to a particular Ser, Thr, or Tyr residue (occasionally His as well) on the target protein. This introduces a bulky, charged group into a region of the target protein that was only moderately polar prior to modification. The oxygen atoms of a phosphoryl group can hydrogen-bond with one or several groups in a protein, commonly the amide groups of the peptide backbone at the start of an α helix or the charged guanidinium group of an Arg residue. The two negative charges on a phosphorylated side chain can also repel neighboring negatively charged (Asp or Glu) residues. When the modified side chain is located in a region of an enzyme critical to its three-dimensional structure, phosphorylation can have dramatic effects on enzyme conformation and thus on substrate binding and catalysis. Removal of phosphoryl groups from these same target proteins is catalyzed by **phosphoprotein phosphatases**, also called simply **protein phosphatases**.

An important example of enzyme regulation by phosphorylation is the case of glycogen phosphorylase (M_r 94,500) of muscle and liver (Chapter 15), which catalyzes the reaction

$$\underset{\text{Glycogen}}{(\text{Glucose})_n} + \text{P}_\text{i} \longrightarrow \underset{\substack{\text{Shortened}\\ \text{glycogen chain}}}{(\text{glucose})_{n-1}} + \text{glucose 1-phosphate}$$

The glucose 1-phosphate so formed can be used for ATP synthesis in muscle or converted to free glucose in the liver. Note that glycogen phosphorylase, though it adds a phosphate to a substrate, is not itself a kinase, because it does not utilize ATP or any other nucleotide triphosphate as a phosphoryl donor in its catalyzed reaction. It is, however, the substrate for a protein kinase that phosphorylates it. In the discussion below, the phosphoryl groups we are concerned with are those involved in regulation of the enzyme, as distinguished from its catalytic function.

Glycogen phosphorylase occurs in two forms: the more active phosphorylase *a* and the less active phosphorylase *b* (**Fig. 6-39**). Phosphorylase *a* has two subunits, each with a specific Ser residue (Ser^{14}) that is phosphorylated at its hydroxyl group. Phosphorylase *b* is covalently transformed into active phosphorylase *a* by

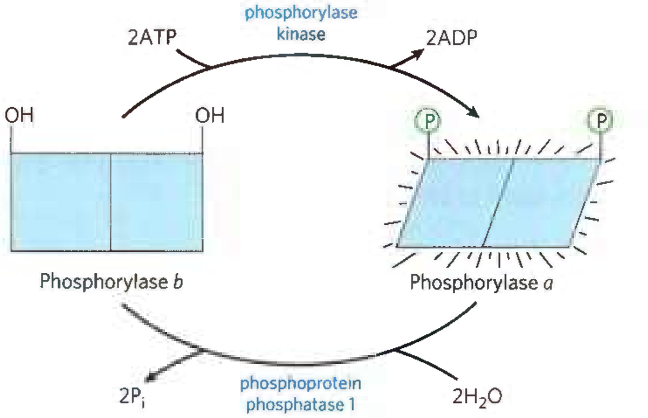


FIGURE 6-39 Regulation of muscle glycogen phosphorylase activity by phosphorylation. In the more-active form of the enzyme, phosphorylase *a*, specific Ser residues, one on each subunit, are phosphorylated. Phosphorylase *a* is converted to the less active phosphorylase *b* by enzymatic loss of these phosphoryl groups, promoted by phosphoprotein phosphatase 1 (PP1). Phosphorylase *b* can be reconverted (reactivated) to phosphorylase *a* by the action of phosphorylase kinase.

another enzyme, phosphorylase kinase, which catalyzes the transfer of phosphoryl groups from ATP to the hydroxyl groups of the two specific Ser residues in phosphorylase *b*:

$$2\text{ATP} + \underset{\text{(less active)}}{\text{phosphorylase } b} \longrightarrow 2\,\text{ADP} + \underset{\text{(more active)}}{\text{phosphorylase } a}$$

These phosphoserine residues are required for maximal activity of the enzyme.

To serve as an effective regulatory mechanism, phosphorylation must be reversible. In general, phosphoryl groups are added and removed by different enzymes, and the processes can therefore be separately regulated. The phosphoryl groups of phosphorylase *a* are hydrolytically removed by a separate enzyme called phosphoprotein phosphatase 1 (PP1):

$$\underset{\text{(more active)}}{\text{Phosphorylase } a} + 2\text{H}_2\text{O} \longrightarrow \underset{\text{(less active)}}{\text{phosphorylase } b} + 2\text{P}_\text{i}$$

In this reaction, phosphorylase *a* is converted to phosphorylase *b* by the cleavage of two phosphoserine covalent bonds, one on each subunit of glycogen phosphorylase.

The regulation of glycogen phosphorylase by phosphorylation illustrates the effects on both structure and catalytic activity of adding a phosphoryl group (**Fig. 6-40**). In the unphosphorylated state (phosphorylase *b*), each subunit of this enzyme is folded so as to bring the 20 residues at its amino terminus, including some basic residues, into a region containing several acidic amino acids; this produces an electrostatic interaction that stabilizes the conformation. Phosphorylation of Ser^{14} interferes with this interaction, forcing the amino-terminal domain out of the acidic environment and into a conformation that allows interaction between the Ⓟ–Ser and several Arg side chains (phosphorylase *a*). In this conformation, the enzyme is much more active.

As we shall see in Chapter 15, phosphorylation is just one of the regulatory mechanisms by which the activity of glycogen phosphorylase is controlled. Allosteric regulation and additional hormonal responses also contribute to the overall precision of control needed to meet moment-to-moment cellular demands for glucose and ATP.

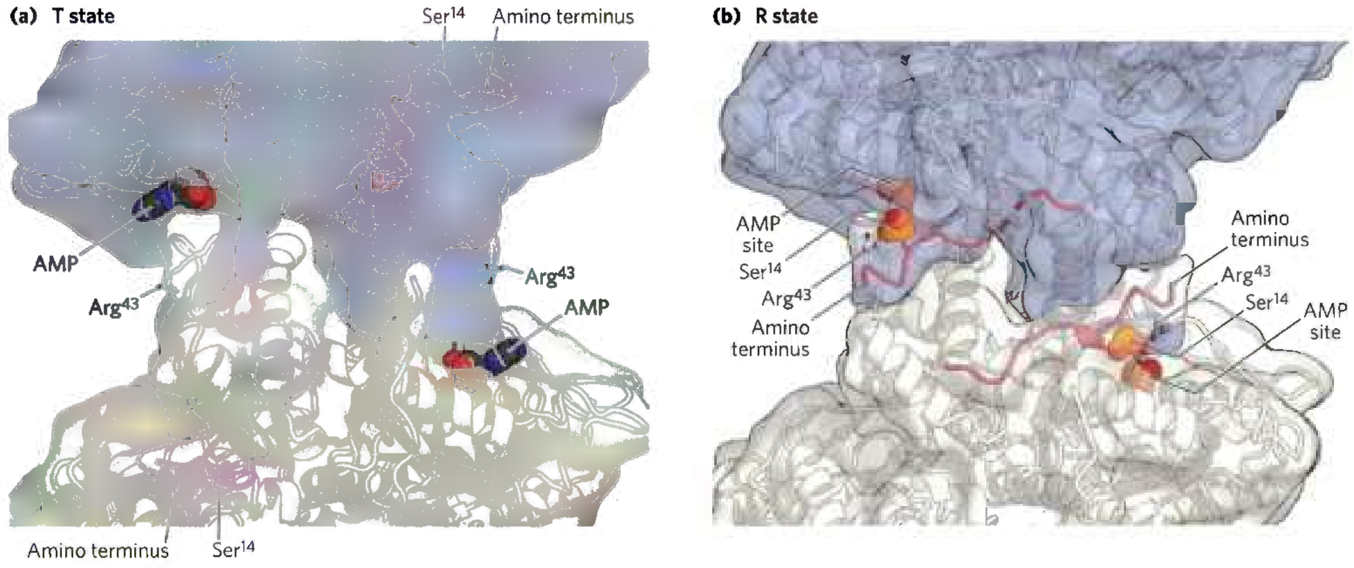


FIGURE 6-40 The conformation change brought about by phosphorylation in glycogen phosphorylase from rabbit muscle. Twenty amino acid residues at the amino terminus of each subunit are shown in red, including the residue phosphorylated in phosphorylase *a* (Ser^{14}). This peptide segment interacts with different parts of the protein depending upon the phosphorylation state of Ser^{14}, stabilizing the different conformations that characterize phosphorylase *a* and *b*. [Data from (a) PDB ID 8GPB, (b) PDB ID 1GPA, D. Barford et al., *J. Mol. Biol.* 218:233, 1991.]

TABLE 6-10 Consensus Recognition Sequences for a Few Protein Kinases

Protein kinase	Consensus sequence and phosphorylated residue
Protein kinase A	-x-R-[RK]-x-**[ST]**-B-
Protein kinase G	-x-R-[RK]-x-**[ST]**-X-
Protein kinase C	-[RK](2)-x-**[ST]**-B-[RK](2)-
Protein kinase B	R-x-x-R-x-**[ST]**-x-ψ[a]-
Ca^{2+}/calmodulin kinase I	-B-x-R-x(2)-**[ST]**-x(3)-B-
Ca^{2+}/calmodulin kinase II	-B-x-[RK]-x(2)-**[ST]**-x(2)-
Myosin light chain kinase (smooth muscle)	-K(2)-R-x(2)-**S**-x-B(2)-
Phosphorylase *b* kinase	*-K-R-K-Q-I-**S**-V-R-*
Extracellular signal-regulated kinase (ERK)	-P-x-**[ST]**-P(2)-
Cyclin-dependent protein kinase (cdc2)	-x-**[ST]**-P-x-[KR]-
Casein kinase I	-[SpTp]-x(2)-**[ST]**-B[b]
Casein kinase II	-x-**[ST]**-x(2)-[ED]-x-
β-Adrenergic receptor kinase	-[DE](*n*)-**[ST]**-x(3)
Rhodopsin kinase	-x(2)-**[ST]**-E(*n*)-vABL-[YLV]-**Y**-$X_{1\text{-}3}$-[PF]-
Epidermal growth factor (EGF) receptor kinase	*-E(4)-**Y**-F-E-L-V-*

Information from L. A. Pinna and M. H. Ruzzene, *Biochim. Biophys. Acta* 1314:191, 1996; B. E. Kemp and R. B. Pearson, *Trends Biochem. Sci.* 15:342, 1990; P. J. Kennelly and E. G. Krebs, *J. Biol. Chem.* 266:15,555, 1991; T. P. Cujec, P. F. Madeiros, P. Hammond, C. Rise, and B. L. Kreider, *Chem. Biol.* 9:253, 2002

Note: Shown here are deduced consensus sequences (in roman type) and actual sequences from known substrates (italic). The Ser (S), Thr (T), or Tyr (Y) residue that undergoes phosphorylation is in bold red; all amino acid residues are shown as their one-letter abbreviations (see Table 3-1). An x represents any amino acid; B, any hydrophobic amino acid; Sp and Tp are Ser and Thr residues that must already be phosphorylated for the kinase to recognize the site.

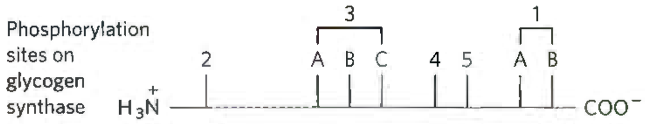
[a] Ψ denotes any amino acid with a bulky hydrophobic side chain.

[b] The best target site has two amino acid residues separating the phosphorylated and target Ser/Thr residues; target sites with one or three intervening residues function at a reduced level.

Multiple Phosphorylations Allow Exquisite Regulatory Control

The Ser, Thr, or Tyr residues that are typically phosphorylated in regulated proteins occur within common structural motifs, called consensus sequences, that are recognized by specific protein kinases (**Table 6-10**). Some kinases are basophilic, preferentially phosphorylating a residue that has basic neighbors; others have different substrate preferences, such as for a residue near a Pro residue. However, amino acid sequence is not the only important factor in determining whether a given residue will be phosphorylated. Protein folding brings together residues that are distant in the primary sequence; the resulting three-dimensional structure can determine whether a protein kinase has access to a given residue and can recognize it as a substrate.

Regulation by phosphorylation is often complicated. Some proteins have consensus sequences recognized by several different protein kinases, each of which can phosphorylate the protein and alter its enzymatic activity. In some cases, phosphorylation is hierarchical: a certain residue can be phosphorylated only if a neighboring residue has already been phosphorylated. For example, glycogen synthase, the enzyme that catalyzes the condensation of glucose monomers to form glycogen (Chapter 15), is inactivated by phosphorylation of specific Ser residues and is also modulated by at least four other protein kinases that phosphorylate four other sites in the enzyme (**Fig. 6-41**). The enzyme does not become



Kinase	Phosphorylation sites	Degree of synthase inactivation
Protein kinase A	1A, 1B, 2, 4	+
Protein kinase G	1A, 1B, 2	+
Protein kinase C	1A	+
Ca^{2+}/calmodulin kinase	1B, 2	+
Phosphorylase *b* kinase	2	+
Casein kinase I	At least nine	+ + + +
Casein kinase II	5	0
Glycogen synthase kinase 3	3A, 3B, 3C	+ + +
Glycogen synthase kinase 4	2	+

FIGURE 6-41 Multiple regulatory phosphorylations. The enzyme glycogen synthase has at least nine separate sites in five designated regions that are susceptible to phosphorylation by one of the cellular protein kinases. Thus, regulation of this enzyme is a matter not of binary (on/off) switching but of finely tuned modulation of activity over a wide range in response to a variety of signals.

a substrate for glycogen synthase kinase 3 until one site has been phosphorylated by casein kinase II. Some phosphorylations inhibit glycogen synthase more than others, and some combinations of phosphorylations are cumulative. These multiple regulatory phosphorylations provide the potential for extremely subtle modulation of enzyme activity.

As is the case for kinases, there are many different enzymes that remove phosphoryl groups from proteins. Cells contain a family of phosphoprotein phosphatases that each hydrolyze specific Ⓟ–Ser, Ⓟ–Thr, and Ⓟ–Tyr esters, releasing P_i. The phosphoprotein phosphatases generally act on only a subset of phosphorylated proteins, but they show less substrate specificity than protein kinases.

Some Enzymes and Other Proteins Are Regulated by Proteolytic Cleavage of an Enzyme Precursor

For some enzymes, an inactive precursor called a **zymogen** is cleaved to form the active enzyme. Many proteolytic enzymes (proteases) of the stomach and pancreas are regulated in this way. Chymotrypsin and trypsin are initially synthesized as chymotrypsinogen and trypsinogen (**Fig. 6-42**). Specific cleavage causes conformational changes that expose the enzyme active site. This type of activation is irreversible, and additional regulation requires a different mechanism. When necessary, a previously activated protease is inactivated by inhibitor proteins that bind very tightly to the enzyme active site. For example, pancreatic trypsin inhibitor (M_r 6,000) binds to and inhibits trypsin. α_1-Antiproteinase (M_r 53,000) primarily inhibits neutrophil elastase (neutrophils are a type of leukocyte, or white blood cell; elastase is a protease that acts on elastin, a component of some connective tissues). An insufficiency of α_1-antiproteinase, which can be caused by exposure to cigarette smoke, has been associated with lung damage, including emphysema.

Proteases are not the only proteins activated by proteolysis. In other cases, however, the precursors are called not zymogens but, more generally, **proproteins** or **proenzymes**, as appropriate. For example, the connective tissue protein collagen is initially synthesized as the soluble precursor procollagen.

A Cascade of Proteolytically Activated Zymogens Leads to Blood Coagulation

The formation of a blood clot provides a well-studied example of a **regulatory cascade**, a mechanism that allows a very sensitive response to—and amplification of—a molecular signal. The blood coagulation pathways also bring together several other types of regulation, including proteolytic activation and regulatory proteins. We have not yet encountered the latter type of regulation. It employs proteins whose only function is to regulate the activity of other proteins by interacting with them noncovalently.

In a regulatory cascade, a signal leads to the activation of protein X. Protein X catalyzes the activation of protein Y. Protein Y catalyzes the activation of protein Z, and so on. Since proteins X, Y, and Z are catalysts and activate multiple copies of the next protein in the chain, the signal is amplified in each step. In some cases, the activation steps involve proteolytic cleavage and are thus effectively irreversible. In other cases, activation entails readily reversible protein modification steps such as phosphorylation. Regulatory cascades govern a wide range of biological processes, including, besides blood coagulation, some aspects

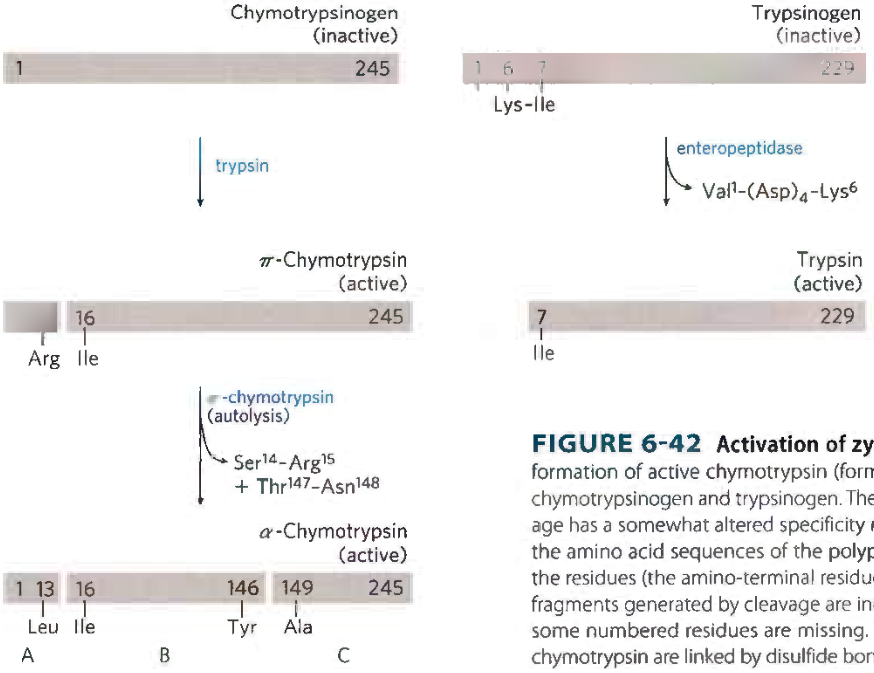


FIGURE 6-42 Activation of zymogens by proteolytic cleavage. Shown here is the formation of active chymotrypsin (formally, α-chymotrypsin) and trypsin from their zymogens, chymotrypsinogen and trypsinogen. The π-chymotrypsin intermediate generated by trypsin cleavage has a somewhat altered specificity relative to the mature α-chymotrypsin. The bars represent the amino acid sequences of the polypeptide chains, with numbers indicating the positions of the residues (the amino-terminal residue is number 1). Residues at the termini of the polypeptide fragments generated by cleavage are indicated below the bars. Note that in the final active forms, some numbered residues are missing. Recall that the three polypeptide chains (A, B, and C) of chymotrypsin are linked by disulfide bonds (see Fig. 6-24).

of cell fate determination during development, the detection of light by retinal rods, and programmed cell death (apoptosis). A regulatory cascade is also one of the strategies governing the overall activity of glycogen phosphorylase (see Fig. 15-13).

A blood clot is an aggregate of specialized cell fragments that lack nuclei, called **platelets**. They are cross-linked and stabilized by proteinaceous fibers consisting mainly of the protein **fibrin** (**Fig. 6-43a**). Fibrin is derived from a soluble zymogen called **fibrinogen**. After albumins and globulins, fibrinogen is usually the third most abundant type of protein in blood plasma. Blood clotting begins with the activation of circulating platelets at the site of a wound. Tissue damage causes collagen molecules present beneath the epithelial cell layer that lines each blood vessel to become exposed to the blood. Platelet activation is primarily triggered by interaction with this collagen. Activation leads to the presentation of anionic phospholipids on the surface of each platelet and the release of signaling molecules such as **thromboxanes** (p. 355) that help stimulate the activation of additional platelets. The activated platelets aggregate at the site of a wound, forming a loose clot. Stabilization of the clot requires fibrin, generated by fibrinogen cleavage as the endpoint of regulatory cascades.

Fibrinogen is a dimer of heterotrimers ($A\alpha_2B\beta_2\gamma_2$) with three different but evolutionarily related types of subunits (Fig. 6-43b). Fibrinogen is converted to fibrin ($\alpha_2\beta_2\gamma_2$), and thereby activated for blood clotting, by the proteolytic removal of 16 amino acid residues from the amino-terminal end (the A peptide) of each α subunit and 14 amino acid residues from the amino-terminal end (the B peptide) of each β subunit. Peptide removal is catalyzed by the serine protease **thrombin**. The newly exposed amino termini of the α and β subunits fit neatly into binding sites in the carboxyl-terminal globular portions of the γ and β subunits, respectively, of another fibrin protein. Fibrin thus polymerizes into a gel-like matrix to generate a soft clot. Covalent cross-links between the associated fibrins are generated by the condensation of particular Lys residues in one fibrin heterotrimer with Gln residues in another, catalyzed by a transglutaminase, **factor XIIIa**. The covalent cross-links convert the soft clot into a hard clot.

Fibrinogen activation to produce fibrin is the end point of not one but two parallel but intertwined regulatory cascades (**Fig. 6-44**). One of these is referred to as

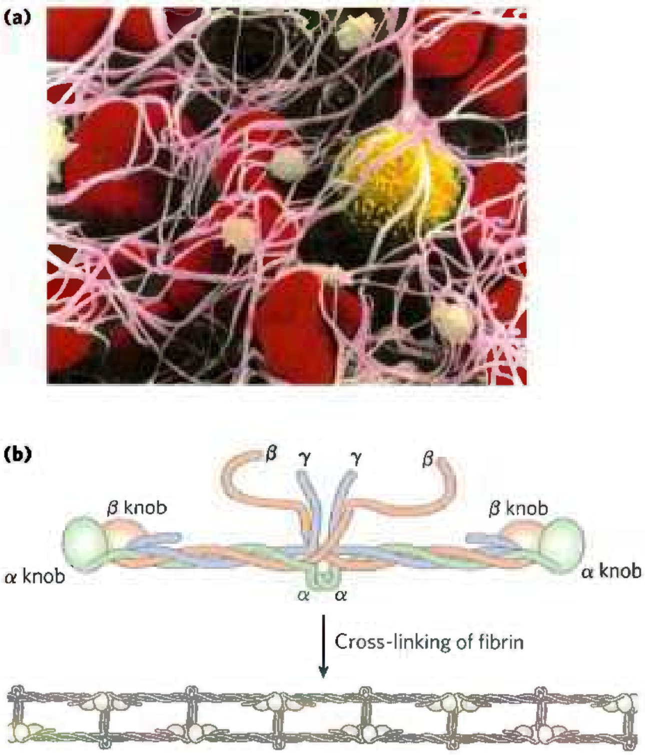


FIGURE 6-43 The function of fibrin in blood clots. (a) A blood clot consists of aggregated platelets (small, light-colored cells) tied together with strands of cross-linked fibrin. Erythrocytes (red in this colorized scanning electron micrograph) are also trapped in the matrix. (b) The soluble plasma protein fibrinogen consists of two complexes of α, β, and γ subunits ($\alpha_2\beta_2\gamma_2$). The removal of amino-terminal peptides from the α and β subunits (not shown) leads to the formation of higher-order complexes and eventual covalent cross-linking that results in the formation of fibrin fibers. The "knobs" are globular domains at the ends of the proteolyzed subunits. [(a) CNRI/Science Source.]

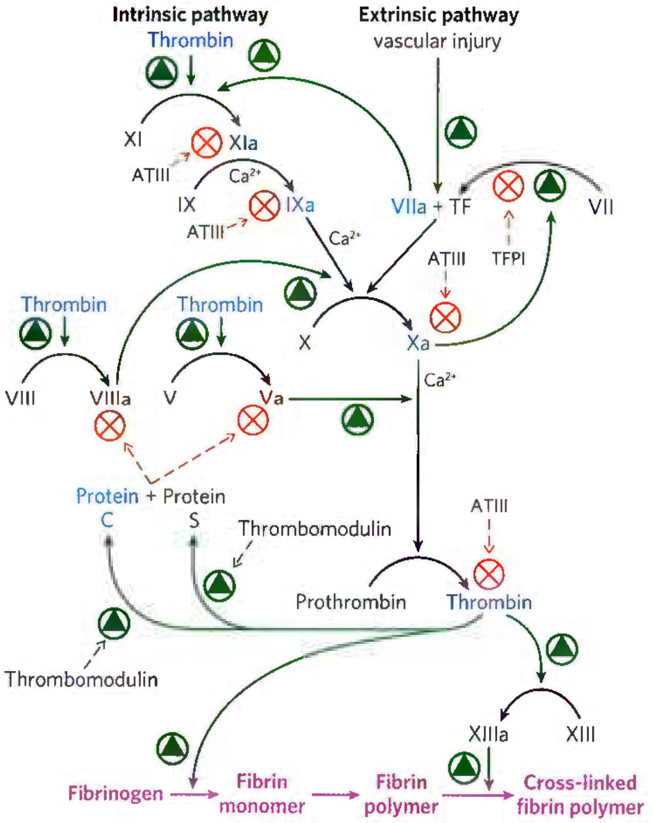


FIGURE 6-44 The coagulation cascades. The interlinked intrinsic and extrinsic pathways leading to the cleavage of fibrinogen to form active fibrin are shown. Active serine proteases in the pathways are shown in blue. Green arrows denote activating steps, and red arrows indicate inhibitory processes.

the contact activation pathway ("contact" refers to interaction of key components of this system with anionic phospholipids presented on the surface of platelets at the site of a wound). As all components of this pathway are found in the blood plasma, it is also called the **intrinsic pathway**. The second path is the tissue factor or **extrinsic pathway**. A major component of this pathway, the protein **tissue factor (TF)**, is not present in the blood. Most of the protein factors in both pathways are designated by roman numerals. Many of these factors are either chymotrypsin-like serine proteases or regulatory proteins, most with zymogen precursors that are synthesized in the liver and exported to the blood. For those with both inactive and active forms, the roman numeral designates the inactive zymogen (VII, X, for example). An "a" is added to designate the cleaved and active form (VIIa, Xa). The regulatory proteins bind to particular serine proteases and help to activate them.

The extrinsic pathway comes into play first. Tissue damage exposes the blood plasma to TF embedded largely in the membranes of fibroblasts and smooth muscle cells beneath the endothelial layer. An initiating complex is formed between TF and factor VII, present in the blood plasma. **Factor VII** is a zymogen of a serine protease, and TF is a regulatory protein required for its function. Factor VII is converted to its active form, **factor VIIa**, by proteolytic cleavage catalyzed by **factor Xa** (another serine protease). The TF-VIIa complex then cleaves **factor X**, creating the active form, factor Xa.

If TF-VIIa is needed to cleave X, and Xa is needed to cleave TF-VII, how does the process ever get started? A very small amount of factor VIIa is present in the blood at all times, enough to form a small amount of the active TF-VIIa complex immediately after tissue is damaged. This allows formation of factor Xa and establishes the initiating feedback loop. Once levels of factor Xa begin to build up, Xa (in a complex with another regulatory protein, factor Va) cleaves prothrombin to form active thrombin, and thrombin cleaves fibrinogen.

The extrinsic pathway thus provides a burst of thrombin. However, the TF-VIIa complex is quickly shut down by the protein **tissue factor pathway inhibitor (TFPI)**. Clot formation is sustained by the activation of components of the second cascade, the intrinsic pathway. **Factor IX** is converted to the active serine protease **factor IXa** by the TF-VIIa protease during initiation of the clotting sequence. Factor IXa, in a complex with the regulatory protein **VIIIa**, is relatively stable and provides an alternative enzyme for the proteolytic conversion of factor X to Xa. Activated IXa can also be produced by the serine protease factor XIa. Most of the XIa is generated by cleavage of **factor XI** zymogen by thrombin in a feedback loop.

Left uncontrolled, blood coagulation could eventually lead to blockage of blood vessels, causing heart attacks or strokes. More regulation is thus needed. As a hard clot forms, regulatory pathways are already acting to limit the time during which the coagulation cascade is active. In addition to cleaving fibrinogen, thrombin forms a complex with a protein embedded in the vascular surface of endothelial cells, **thrombomodulin**. The thrombin-thrombomodulin complex cleaves the serine protease zymogen **protein C**. Activated protein C, in a complex with the regulatory **protein S**, cleaves and inactivates factors Va and VIIIa, leading to suppression of the overall cascade. Another protein, **antithrombin III (ATIII)**, is a serine protease inhibitor. ATIII makes a covalent 1:1 complex between an Arg residue on ATIII and the active-site Ser residue of serine proteases, particularly thrombin and factor Xa. These two regulatory systems, in concert with TFPI, help to establish a threshold or level of exposure to TF that is needed to activate the coagulation cascade. Individuals with genetic defects that eliminate or decrease levels of protein C or ATIII in the blood have a greatly elevated risk of thrombosis (inappropriate formation of blood clots).

The control of blood coagulation has important roles in medicine, particularly in the prevention of blood clotting during surgery and in patients at risk for heart attacks or strokes. Several different medical approaches to anticoagulation are available. The first takes advantage of another feature of several proteins in the coagulation cascade that we have not yet considered. The factors VII, IX, X, and prothrombin, along with proteins C and S, have calcium-binding sites that are critical to their function. In each case, the calcium-binding sites are formed by modification of multiple Glu residues near the amino terminus of each protein to **γ-carboxyglutamate** residues (abbreviated **Gla**; p. 76). The Glu-to-Gla modifications are carried out by enzymes that depend on the function of the fat-soluble vitamin K (p. 359). Bound calcium functions to adhere these proteins to the anionic phospholipids that appear on the surface of activated platelets, effectively localizing the coagulation factors to the area where the clot is to form. Vitamin K antagonists such as **warfarin** (Coumadin) have proven highly effective as anticoagulants. A second approach to anticoagulation is the administration of heparins. **Heparins** are highly sulfated polysaccharides (see Fig. 7-19). They act as anticoagulants by increasing the affinity of ATIII for factor Xa and thrombin, thus facilitating the inactivation of key cascade elements (see Figs 7-23 and 7-24). Finally, **aspirin** (acetylsalicylate; Fig. 21-15b) is effective as an anticoagulant. Aspirin inhibits the enzyme cyclooxygenase, required for the production of thromboxanes. As aspirin reduces thromboxane release from platelets, the capacity of the platelets to aggregate declines.

Humans born with a deficiency in any one of most components of the clotting cascade have an increased tendency to bleed that varies from mild to essentially uncontrollable, a fatal condition. Genetic defects in the genes encoding the proteins required for blood clotting result in diseases referred to as hemophilias. Hemophilia B is a sex-linked trait resulting from a deficiency in factor IX. This type of hemophilia affects about one in 25,000 males worldwide. The most famous example of the inheritance

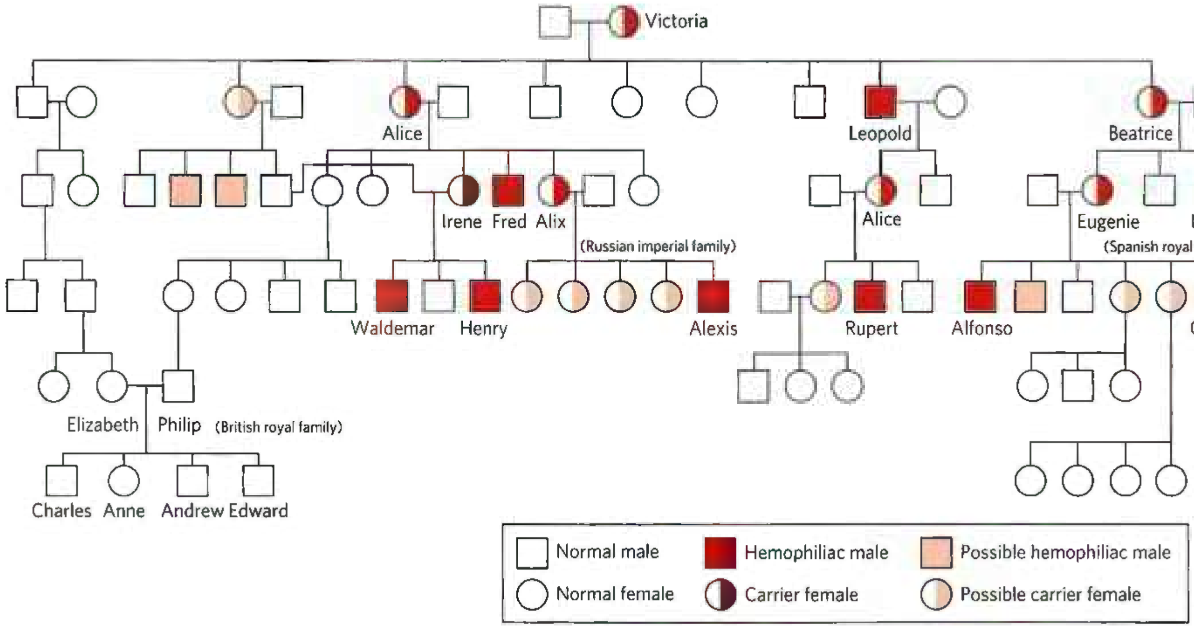


FIGURE 6-45 The royal families of Europe, and inheritance of hemophilia B. Males are indicated by squares and females by circles. Males who suffered from hemophilia are represented by red squares, presumed female carriers by half-red circles.

of hemophilia B occurred among European royalty. Queen Victoria (1819–1901) was evidently a carrier. Prince Leopold, her eighth child, suffered from hemophilia B and died at the age of 31 after a minor fall. At least two of her daughters were carriers and passed the defective gene to other royal families of Europe (**Fig. 6-45**). ■

Some Regulatory Enzymes Use Several Regulatory Mechanisms

Glycogen phosphorylase and the blood clotting cascade are not the only examples of complex regulatory patterns. Additional examples of regulatory complexity are found at key metabolic crossroads. Bacterial glutamine synthetase, which catalyzes a reaction that introduces reduced nitrogen into cellular metabolism (Chapter 22), is among the most complex regulatory enzymes known. It is regulated allosterically, with at least eight different modulators; by reversible covalent modification; and by the association of other regulatory proteins, a mechanism examined in detail when we consider the regulation of specific metabolic pathways in Part II of this book.

What is the advantage of such complexity in the regulation of enzymatic activity? We began this chapter by stressing the central importance of catalysis to the existence of life. P5 The *control* of catalysis is also critical to life. If all possible reactions in a cell were catalyzed simultaneously, macromolecules and metabolites would quickly be broken down to much simpler chemical forms. Instead, cells catalyze only the reactions they need at a given moment. When chemical resources are plentiful, cells synthesize and store glucose and other metabolites. When chemical resources are scarce, cells use these stores to fuel cellular metabolism. Chemical energy is used economically, parceled out to various metabolic pathways as cellular needs dictate. The availability of powerful catalysts, each specific for a given reaction, makes the regulation of these reactions possible. This, in turn, gives rise to the complex, highly regulated symphony we call life.

SUMMARY 6.5 *Regulatory Enzymes*

- The activities of metabolic pathways in cells are regulated by control of the activities of certain enzymes.
- The activity of an allosteric enzyme is adjusted by reversible binding of a specific modulator to a regulatory site. A modulator may be the substrate itself or some other metabolite, and the effect of the modulator may be inhibitory or stimulatory.
- The kinetic behavior of allosteric enzymes reflects cooperative interactions among enzyme subunits.
- Other regulatory enzymes are modulated by covalent modification of a specific functional group necessary for activity.
- The phosphorylation of specific amino acid residues is a particularly common way to regulate enzyme activity.
- Many proteolytic enzymes are synthesized as inactive precursors called zymogens, which are activated by cleavage to release small peptide fragments.
- Blood clotting is mediated by two interlinked regulatory cascades of proteolytically activated zymogens.
- Enzymes at important metabolic intersections may be regulated by complex combinations of effectors, allowing coordination of the activities of interconnected pathways.

KEY TERMS

Terms in bold are defined in the glossary.

enzyme 178
cofactor 178
coenzyme 178
prosthetic group 179
holoenzyme 179
apoenzyme 179
apoprotein 179
active site 179
substrate 180
ground state 180
standard free-energy change, ΔG° 180
biochemical standard free-energy change, $\Delta G'^\circ$ 180
transition state 180
activation energy ($\Delta G^\ddagger$) 181
reaction intermediate 181
rate-limiting step 181
equilibrium constant (K_{eq}) 182
rate constant 182
binding energy (ΔG_B) 183
specificity 185
desolvation 186
induced fit 186
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steady-state kinetics 188
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dissociation constant (K_d) 192
k_{cat} 192
turnover number 193
Cleland nomenclature 195
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competitive inhibition 197
uncompetitive inhibition 198
mixed inhibition 199
noncompetitive inhibition 199
irreversible inhibitors 200
suicide inactivator 200
transition-state analog 200
serine proteases 205
retrovirus 208
regulatory enzyme 213
allosteric enzyme 213
allosteric modulator (allosteric effector) 213
regulatory protein 214
homotropic 214
heterotropic 214
protein kinases 217
protein phosphatases 217
zymogen 220
proproteins (proenzymes) 220
regulatory cascade 220
platelets 221
fibrin 221
fibrinogen 221
thromboxane 221
thrombin 221
intrinsic pathway 222
extrinsic pathway 222

PROBLEMS

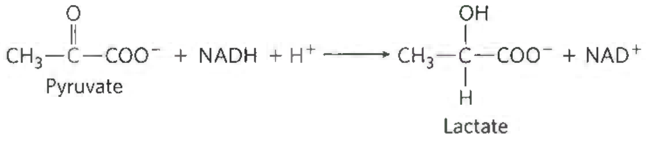
1. Keeping the Sweet Taste of Corn The sweet taste of freshly picked corn (maize) is due to the high level of sugar in the kernels. Store-bought corn (several days after picking) is not as sweet, because about 50% of the free sugar is converted to starch within one day of picking. To preserve the sweetness of fresh corn, the husked ears can be immersed in boiling water for a few minutes ("blanched"), then cooled in cold water. Corn processed in this way and stored in a freezer maintains its sweetness. What is the biochemical basis for this procedure?

2. Intracellular Concentration of Enzymes To approximate the concentration of enzymes in a bacterial cell, assume that the cell contains equal concentrations of 1,000 different enzymes in solution in the cytosol and that each protein has a molecular weight of 100,000. Assume also that the bacterial cell is a cylinder (diameter 1.0 μm, height 2.0 μm), that the cytosol (specific gravity 1.20) is 20% soluble protein by weight, and that the soluble protein consists entirely of enzymes. Calculate the *average* molar concentration of each enzyme in this hypothetical cell.

3. Rate Enhancement by Urease The enzyme urease enhances the rate of urea hydrolysis at pH 8.0 and 20 °C by a factor of 10^{14}. Suppose that a given quantity of urease can completely hydrolyze a given quantity of urea in 5.0 min at 20 °C and pH 8.0. How long would it take for this amount of urea to be hydrolyzed under the same conditions in the absence of urease? Assume that both reactions take place in sterile systems so that bacteria cannot attack the urea.

4. Protection of an Enzyme against Denaturation by Heat When enzyme solutions are heated, there is a progressive loss of catalytic activity over time due to denaturation of the enzyme. A solution of the enzyme hexokinase incubated at 45 °C lost 50% of its activity in 12 min, but when incubated at 45 °C in the presence of a very large concentration of one of its substrates, it lost only 3% of its activity in 12 min. Suggest why thermal denaturation of hexokinase was retarded in the presence of one of its substrates.

5. Quantitative Assay for Lactate Dehydrogenase The muscle enzyme lactate dehydrogenase catalyzes the reaction



$$CH_3-\overset{\overset{O}{\|}}{C}-COO^- + NADH + H^+ \longrightarrow CH_3-\underset{\underset{H}{|}}{\overset{\overset{OH}{|}}{C}}-COO^- + NAD^+$$

NADH and NAD$^+$ are the reduced and oxidized forms, respectively, of the coenzyme NAD. Solutions of NADH, but *not* NAD$^+$, absorb light at 340 nm. This property is used to determine the concentration of NADH in solution by measuring spectrophotometrically the amount of light absorbed at 340 nm (A_{340}) by the solution. Explain how these properties of NADH can be used to design a quantitative assay for lactate dehydrogenase.

6. Effect of Enzymes on Reactions Consider this simple reaction:

$$S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} P \qquad \text{where} \qquad K'_{eq} = \frac{[P]}{[S]}$$

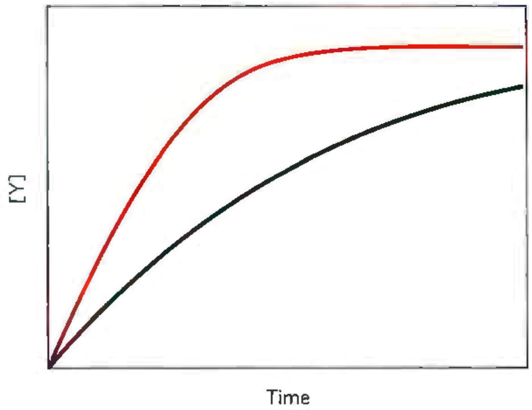
Which of the listed effects would be brought about by an enzyme catalyzing the simple reaction? **(a)** increased k_1 **(b)** increased K'_{eq} **(c)** decreased $\Delta G^\ddagger$ **(d)** more negative $\Delta G'^\circ$ **(e)** increased k_2

7. Relation between Reaction Velocity and Substrate Concentration: Michaelis-Menten Equation The K_m of an enzyme is 5.0 mM.

(a) Calculate the substrate concentration when this enzyme operates at one-quarter of its maximum rate.

(b) Determine the fraction of V_{max} that would be obtained when the substrate concentration, [S], is 0.5 K_m, 2 K_m, and 10 K_m.

(c) An enzyme that catalyzes the reaction X $\rightleftharpoons$ Y is isolated from two bacterial species. The enzymes have the same V_{max} but different K_m values for the substrate X. Enzyme A has a K_m of 2.0 μM, and enzyme B has a K_m of 0.5 μM. Kinetic experiments used the same concentration of each enzyme and 1 μM substrate X. The graph plots the concentration of product Y formed over time. Which curve corresponds to which enzyme?



8. Applying the Michaelis-Menten Equation I An enzyme has a V_{max} of 1.2 μM s^{-1}. The K_m for its substrate is 10 μM. Calculate the initial velocity of the reaction, V_0, when the substrate concentration is **(a)** 2 μM **(b)** 10 μM **(c)** 30 μM.

9. Applying the Michaelis-Menten Equation II An enzyme is present at a concentration of 1 nM and has a V_{max} of 2 μM s^{-1}. The K_m for its primary substrate is 4 μM.
(a) Calculate k_{cat}.
(b) Calculate the apparent (measured) V_{max} and apparent (measured) K_m of this enzyme in the presence of sufficient amounts of an uncompetitive inhibitor to generate an α' of 2. Assume that the enzyme concentration remains at 1 nM.

10. Applying the Michaelis-Menten Equation III A research group discovers a new version of happyase, which they call happyase*, that catalyzes the chemical reaction HAPPY $\rightleftharpoons$ SAD. The researchers begin to characterize the enzyme.
(a) In the first experiment, with [E$_t$] at 4 nM, they find that the V_{max} is 1.6 μM s^{-1}. Based on this experiment, what is the k_{cat} for happyase*? (Include appropriate units.)
(b) In the second experiment, with [E$_t$] at 1 nM and [HAPPY] at 30 μM, the researchers find that V_0 = 300 nM s^{-1}. What is the measured K_m of happyase* for its substrate HAPPY? (Include appropriate units.)
(c) Further research shows that the purified happyase* used in the first two experiments was actually contaminated with a reversible inhibitor called ANGER. When ANGER is carefully removed from the happyase* preparation and the two experiments are repeated, the measured V_{max} in (a) is increased to 4.8 μM s^{-1}, and the measured K_m in (b) is now 15 μM. Calculate the values of α and α' for ANGER.
(d) Based on the information given, what type of inhibitor is ANGER?

11. Applying the Michaelis-Menten Equation IV Researchers discover an enzyme that catalyzes the reaction X $\rightleftharpoons$ Y. They find that the K_m for the substrate X is 4 μM, and the k_{cat} is 20 min^{-1}.
(a) In an experiment, [X] = 6 mM, and V_0 = 480 nM min^{-1}. What was the [E$_t$] used in the experiment?
(b) In another experiment, [E$_t$] = 0.5 μM, and the measured V_0 = 5 μM min^{-1}. What was the [X] used in the experiment?
(c) The researchers discover that compound Z is a very strong competitive inhibitor of the enzyme. In an experiment with the same [E$_t$] as in (a), but a different [X], they add an amount of Z that produces an α of 10 and reduces V_0 to 240 nM min^{-1}. What is the [X] in this experiment?
(d) Based on the kinetic parameters given, has this enzyme evolved to achieve catalytic perfection? Explain your answer briefly, using the kinetic parameter(s) that define catalytic perfection.

12. Estimation of V_{max} and K_m by Inspection Graphical methods are available for accurate determination of the V_{max} and K_m of an enzyme-catalyzed reaction. However, these quantities can sometimes be estimated by inspecting values of V_0 at increasing [S]. Estimate the V_{max} and K_m of the enzyme-catalyzed reaction for which the data in the table were obtained.

[S] (M)	V_0 (μM/min)
2.5×10^{-6}	28
4.0×10^{-6}	40
1×10^{-5}	70
2×10^{-5}	95
4×10^{-5}	112
1×10^{-4}	128
2×10^{-3}	139
1×10^{-2}	140

13. Properties of an Enzyme of Prostaglandin Synthesis Prostaglandins are one class of the fatty acid derivatives called eicosanoids. Prostaglandins produce fever and inflammation, as well as the pain associated with inflammation. The enzyme prostaglandin endoperoxide synthase, a cyclooxygenase, uses oxygen to convert arachidonic acid to PGG$_2$, the immediate precursor of many different prostaglandins (prostaglandin synthesis is described in Chapter 21).

Ibuprofen inhibits prostaglandin endoperoxide synthase, thereby reducing inflammation and pain. The kinetic data given in the table are for the reaction catalyzed by prostaglandin endoperoxide synthase in the absence and presence of ibuprofen.
(a) Based on the data, determine the V_{max} and K_m of the enzyme.

[Arachidonic acid] (mM)	Rate of formation of PGG$_2$ (mM min^{-1})	Rate of formation of PGG$_2$ with 10 mg/mL ibuprofen (mM min^{-1})
0.5	23.5	16.67
1.0	32.2	25.25
1.5	36.9	30.49
2.5	41.8	37.04
3.5	44.0	38.91

(b) Based on the data, determine the type of inhibition that ibuprofen exerts on prostaglandin endoperoxide synthase.

14. Graphical Analysis of $V_{\max}$ and K_m A kinetic study of an intestinal peptidase using glycylglycine as the substrate produced the experimental data shown in the table. The peptidase catalyzes this reaction:

$$\text{Glycylglycine} + H_2O \longrightarrow 2\ \text{glycine}$$

[S] (mM)	Product formed (μmol/min)
1.5	0.21
2.0	0.24
3.0	0.28
4.0	0.33
8.0	0.40
16.0	0.45

Use the Lineweaver-Burk equation to determine the $V_{\max}$ and K_m for this enzyme preparation and substrate.

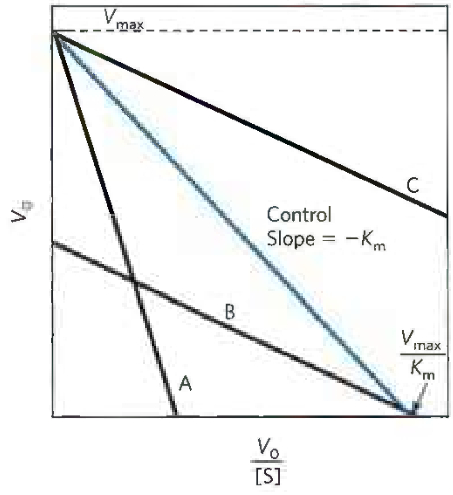
15. The Eadie-Hofstee Equation There are several ways to transform the Michaelis-Menten equation so as to plot data and derive kinetic parameters, each with different advantages depending on the data set being analyzed. One transformation of the Michaelis-Menten equation is the Lineweaver-Burk, or double-reciprocal, equation. Multiplying both sides of the Lineweaver-Burk equation by $V_{\max}$ and rearranging gives the Eadie-Hofstee equation:

$$V_0 = (-K_m)\frac{V_0}{[S]} + V_{\max}$$

Consider the plot of V_0 versus $V_0/[S]$ for an enzyme-catalyzed reaction. The slope of the line is $-K_m$. The x intercept is $V_{\max}/K_m$. The control reactions (the blue line in the plot) did not contain any inhibitor.

(a) Which of the other lines (A, B, or C) depicts this enzyme's activity in the presence of a competitive inhibitor? Hint: See Equation 6-33.

(b) Which line (A, B, or C) depicts this enzyme's activity in the presence of an uncompetitive inhibitor?



16. The Turnover Number of Carbonic Anhydrase Carbonic anhydrase of erythrocytes (M_r 30,000) has one of the highest turnover numbers known. It catalyzes the reversible hydration of CO_2:

$$H_2O + CO_2 \rightleftharpoons H_2CO_3$$

This is an important process in the transport of CO_2 from the tissues to the lungs. If 10.0 μg of pure carbonic anhydrase catalyzes the hydration of 0.30 g of CO_2 in 1 min at 37 °C at $V_{\max}$, what is the turnover number (k_{cat}) of carbonic anhydrase (in units of min^{-1})?

17. Describing Reactions with the Cleland Shorthand The chymotrypsin-catalyzed reaction is diagrammed using the Cleland shorthand. Match the letters in the drawing with each description:

(a) The product that includes the amino group from the cleaved peptide bond

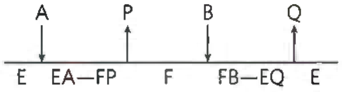
(b) The product that includes the carbonyl group from the cleaved peptide bond

(c) Free chymotrypsin (nothing bound to it)

(d) Water

(e) The peptide substrate

(f) The acyl-enzyme intermediate



18. Kinetic Inhibition Patterns Indicate how the observed K_m of an enzyme would change in the presence of inhibitors having the given effect on α and α':

(a) $\alpha > \alpha'$; $\alpha' = 1.0$

(b) $\alpha' > \alpha$

(c) $\alpha = \alpha'$; $\alpha' > 1.0$

(d) $\alpha = \alpha'$; $\alpha' = 1.0$

19. Deriving a Rate Equation for Competitive Inhibition The Michaelis-Menten rate equation for an enzyme subject to competitive inhibition is

$$V_0 = \frac{V_{\max}[S]}{\alpha K_m + [S]}$$

Beginning with a new definition of total enzyme as

$$[E_t] = [E] + [ES] + [EI]$$

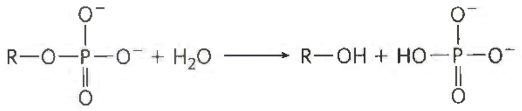
and the definitions of α and K_I provided in the text, derive the first rate equation. Use the derivation of the Michaelis-Menten equation as a guide.

20. Irreversible Inhibition of an Enzyme Many enzymes are inhibited irreversibly by heavy metal ions such as Hg^{2+}, Cu^{2+}, or Ag^+, which can react with essential sulfhydryl groups to form mercaptides:

$$\text{Enz-SH} + Ag^+ \longrightarrow \text{Enz–S–Ag} + H^+$$

The affinity of Ag^+ for sulfhydryl groups is so great that Ag^+ can be used to titrate —SH groups quantitatively. An investigator added just enough $AgNO_3$ to completely inactivate a 10.0 mL solution containing 1.0 mg/mL enzyme. A total of 0.342 μmol of $AgNO_3$ was required. Calculate the minimum molecular weight of the enzyme. Why does the value obtained in this way give only the *minimum* molecular weight?

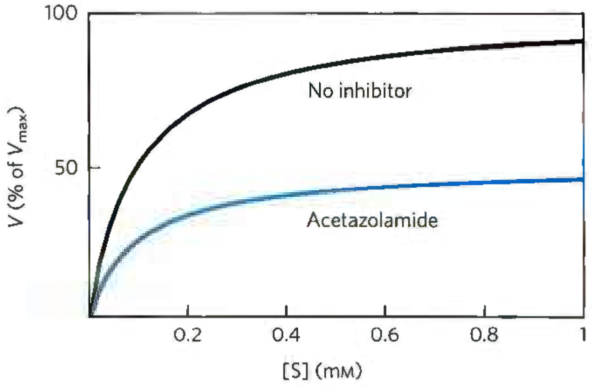
21. Clinical Application of Differential Enzyme Inhibition Human blood serum contains a class of enzymes known as acid phosphatases, which hydrolyze biological phosphate esters under slightly acidic conditions (pH 5.0):



$$R{-}O{-}PO_3^{2-} + H_2O \longrightarrow R{-}OH + HO{-}PO_3^{2-}$$

Acid phosphatases are produced by erythrocytes and by the liver, kidney, spleen, and prostate gland. The enzyme of the prostate gland is clinically important, because its increased activity in the blood can be an indication of prostate cancer. The phosphatase from the prostate gland is strongly inhibited by tartrate ion, but acid phosphatases from other tissues are not. How can this information be used to develop a specific procedure for measuring the activity of prostatic acid phosphatase in human blood serum?

22. Inhibition of Carbonic Anhydrase by Acetazolamide Carbonic anhydrase is strongly inhibited by the drug acetazolamide, which is used as a diuretic (i.e., to increase the production of urine) and to lower excessively high pressure in the eye (due to accumulation of intraocular fluid) in glaucoma. Carbonic anhydrase plays an important role in these and other secretory processes because it participates in regulating the pH and bicarbonate content of several body fluids. Carbonic anhydrase activity can be analyzed using the initial reaction velocity (as percentage of V_{max}) versus [S]. The black curve of the graph shows the uninhibited activity; the blue curve shows activity in the presence of acetazolamide. Based on the data provided, determine the nature of the inhibition by acetazolamide. Explain your reasoning.



23. The Effects of Reversible Inhibitors The Michaelis-Menten rate equation for reversible mixed inhibition is written as

$$V_0 = \frac{V_{max}[S]}{\alpha K_m + \alpha'[S]}$$

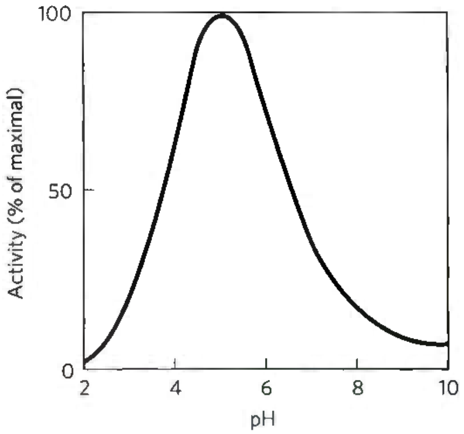
Apparent, or observed, K_m is equivalent to the [S] at which

$$V_0 = \frac{V_{max}}{2\alpha'}$$

Derive an expression for the effect of a reversible inhibitor on apparent K_m from the previous equation.

24. Perturbed pK_a Values in Enzyme Active Sites Alanine racemase is a bacterial enzyme that converts L-alanine to D-alanine, which is needed in small amounts to synthesize the bacterial cell wall. The active site of alanine racemase includes a Tyr residue with a pK_a value of 7.2. The pK_a of free tyrosine is 10. The altered pK_a of this residue is due largely to the presence of a nearby charged amino acid residue. Which amino acid(s) could lower the pK_a of the neighboring Tyr residue? Explain your reasoning.

25. pH Optimum of Lysozyme The enzyme lysozyme hydrolyzes glycosidic bonds in peptidoglycan, an oligosaccharide found in bacterial cell walls. The active site of lysozyme contains two amino acid residues essential for catalysis: Glu^{35} and Asp^{52}. The pK_a values of the carboxyl side chains of these residues are 5.9 and 4.5, respectively. What is the ionization state (protonated or deprotonated) of each residue at pH 5.2, the pH optimum of lysozyme? How can the ionization states of these residues explain the pH-activity profile of lysozyme shown?

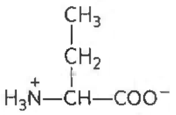


DATA ANALYSIS PROBLEM

26. Mirror-Image Enzymes As noted in Chapter 3, "The amino acid residues in protein molecules are almost all L stereoisomers." It is not clear whether this selectivity is necessary for proper protein function or is an accident of evolution. To explore this question, Milton and colleagues (1992) studied an enzyme made entirely of D stereoisomers. The enzyme they chose was HIV protease, the proteolytic enzyme made by HIV that converts inactive viral pre-proteins to their active forms as described earlier in Figure 6-28.

Previously, Wlodawer and coworkers (1989) had reported the complete chemical synthesis of HIV protease from L-amino acids (the L-enzyme), using the process shown in Figure 3-30. Normal HIV protease contains two Cys residues at positions 67 and 95. Because chemical synthesis of proteins containing Cys is technically difficult, Wlodawer and colleagues substituted the synthetic amino acid L-α-amino-n-butyric acid (Aba) for the two Cys residues in the protein. They did this to "reduce synthetic difficulties associated with Cys deprotection and ease product handling."

(a) The structure of Aba is shown below.



$$H_3\overset{+}{N}{-}CH(CH_2CH_3){-}COO^-$$

Why was this a suitable substitution for a Cys residue?

In their study, Milton and coworkers synthesized HIV protease from D-amino acids, using the same protocol as the earlier study (Wlodawer et al.). Formally, there are three possible outcomes for the folding of the D-protease: (1) the same shape as the L-protease; (2) the mirror image of the L-protease, or (3) something else, possibly inactive.

(b) For each possibility, decide whether or not it is a likely outcome and defend your position.

In fact, the D-protease was active: it cleaved a particular synthetic substrate and was inhibited by specific inhibitors. To examine the structure of the D- and L-enzymes, Milton and coworkers tested both forms for activity with D and L forms of a chiral peptide substrate and for inhibition by D and L forms of a chiral peptide-analog inhibitor. Both forms were also tested for inhibition by the achiral inhibitor Evans blue. The findings are given in the table.

			Inhibition	
	Substrate hydrolysis		**Peptide inhibitor**	
HIV protease	**D form**	**L form**	**D form**	**L form**
L form	–	+	–	+
D form	+	–	+	–

(c) Which of the three models proposed above is supported by these data? Explain your reasoning.

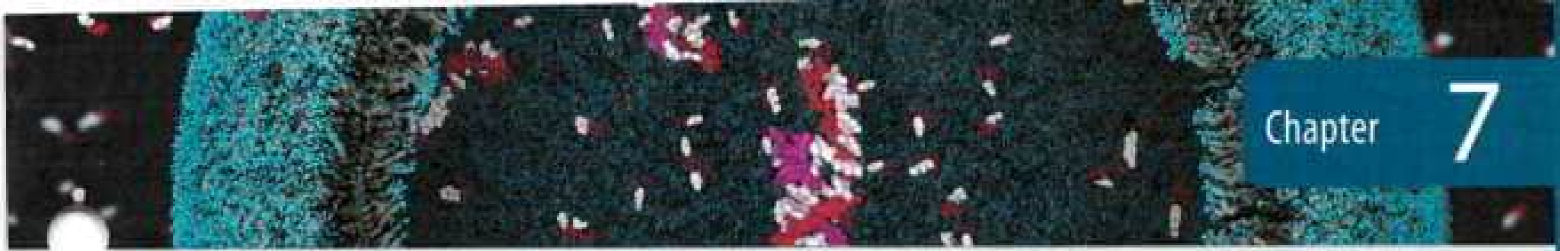
(d) Would you expect chymotrypsin to digest the D-protease? Explain your reasoning.

(e) Would you expect total synthesis from D-amino acids followed by renaturation to yield active enzyme for any enzyme? Explain your reasoning.

References

Milton, R.C., Milton, S.C., and Kent, S.B. 1992. Total chemical synthesis of a D-enzyme: the enantiomers of HIV-1 protease show demonstration of reciprocal chiral substrate specificity. *Science* 256, 1445–1448.

Wlodawer, A., Miller, M., Jaskólski, M., Sathyanarayana, B.K., Baldwin, E., Weber, I.T., Selk, L.M., Clawson, L., Schneider, J., and Kent, S.B. 1989. Conserved folding in retroviral proteases: crystal structure of a synthetic HIV-1 protease. *Science* 245, 616–621.



Chapter 7

CARBOHYDRATES AND GLYCOBIOLOGY

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7.4 Carbohydrates as Informational Molecules: The Sugar Code *254*

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Carbohydrates are the most abundant biomolecules on Earth. Each year, photosynthesis converts more than 100 billion metric tons of CO_2 and H_2O into cellulose and other plant products. The carbohydrates in these plant products are a dietary staple in most parts of the world, and the oxidation of carbohydrates is the central energy-yielding pathway in most nonphotosynthetic cells. Carbohydrate polymers called glycans serve as structural and protective elements in the cell walls of bacteria, fungi, and plants, and in the connective tissues of animals. Other carbohydrate polymers lubricate skeletal joints and participate in cell-cell recognition and adhesion. In addition, some complex carbohydrate polymers covalently attached to proteins or lipids act as signals that determine the intracellular destination or metabolic fate of these hybrid molecules, called glycoconjugates.

Carbohydrates are aldehydes or ketones with at least two hydroxyl groups, or substances that yield such compounds on hydrolysis. Many, but not all, carbohydrates have the empirical formula $(CH_2O)_n$; some also contain nitrogen, phosphorus, or sulfur. There are three major size classes of carbohydrates: monosaccharides, oligosaccharides, and polysaccharides (the word "saccharide" is derived from the Greek *sakcharon*, meaning "sugar"). **Monosaccharides**, or simple sugars, consist of a single polyhydroxy aldehyde or ketone unit. The most abundant monosaccharide in nature is the six-carbon sugar D-glucose, sometimes referred to as dextrose.

Oligosaccharides consist of short chains of monosaccharide units, or residues, joined by characteristic linkages called glycosidic bonds. The most abundant are the **disaccharides**, with two monosaccharide units. Sucrose (table sugar), for example, consists of the six-carbon sugars D-glucose and D-fructose. All common monosaccharides and disaccharides have names ending with the suffix "-ose." In cells, most oligosaccharides consisting of three or more units do not occur as free entities but are joined to nonsugar molecules (lipids or proteins) in glycoconjugates.

The **polysaccharides** are sugar polymers containing more than 10 monosaccharide units; some have hundreds or thousands of units. Some polysaccharides, such as cellulose, are linear chains; others, such as glycogen, are branched. Both cellulose and glycogen consist of recurring units of D-glucose, but they differ in the type of glycosidic linkage and consequently have strikingly different properties and biological roles.

This chapter introduces the major classes of carbohydrates and glycoconjugates and provides examples of their many structural and functional roles. Learning the structures and chemical properties of biomolecules is essential, because they are the vocabulary and the grammar of biochemistry. As you read about carbohydrates, note how specific cases illustrate these general principles that underlie all of biochemistry.

P1 **Carbohydrates can have multiple chiral carbons; the configuration of groups around each carbon atom determines how the compound interacts with other biomolecules.** As we saw for L-amino acids in proteins, with rare exceptions, biological evolution selected one stereochemical series (D-series) for sugars.

P2 **Monomeric subunits, monosaccharides, serve as the building blocks of large carbohydrate polymers.** The specific sugar, the way the units are linked, and whether the polymer is branched determine its properties and thus its function.

P3 **Storage of low molecular weight metabolites in polymeric form avoids the very high osmolarity that would result from storing them as individual monomers.** If the glucose in liver glycogen were monomeric, the glucose concentration in liver would be so high that cells would swell and lyse from the entry of water by osmosis.

P4 **The sequences of complex polysaccharides are determined by the intrinsic properties of the biosynthetic enzymes that add each monomeric unit to the growing polymer.** This is in contrast with DNA, RNA, and proteins, which are synthesized on templates that direct their sequence.

P5 **Polysaccharides assume three-dimensional structures with the lowest-energy conformations, determined by covalent bonds, hydrogen bonds, charge interactions, and steric factors.** Starch folds into a helical structure stabilized by internal hydrogen bonds; cellulose assumes an extended structure in which intermolecular hydrogen bonds are more important.

P6 **Molecular complementarity is central to function.** The recognition of oligosaccharides by sugar-binding proteins (lectins) results from a perfect fit between lectin and ligand.

P7 **An almost infinite variety of discrete structures can be built from a small number of monomeric subunits.** Even short polymers, when arranged in different sequences, joined through different linkages, and branched to specific degrees, present unique faces recognized by their molecular partners.

7.1 Monosaccharides and Disaccharides

The simplest of the carbohydrates, the monosaccharides, are either aldehydes or ketones with two or more hydroxyl groups; the six-carbon monosaccharides glucose and fructose have five hydroxyl groups. Many of the carbon atoms to which the hydroxyl groups are attached are chiral centers, which give rise to the many sugar stereoisomers found in nature. Stereoisomerism in sugars is biologically significant because the enzymes that act on sugars are strictly stereospecific, typically preferring one stereoisomer to another by three or more orders of magnitude, as reflected in K_m values or binding constants. It is as difficult to fit the wrong sugar stereoisomer into an enzyme's binding site as it is to put your left glove on your right hand.

We begin by describing the families of monosaccharides with backbones of three to seven carbons—their structure, their stereoisomeric forms, and the means of representing their three-dimensional structures on paper. We then discuss several chemical reactions of the carbonyl groups of monosaccharides. One such reaction, the addition of a hydroxyl group from within the same molecule, generates cyclic forms with four or more backbone carbons (the forms that predominate in aqueous solution). **P1** This ring closure creates a new chiral center, adding further stereochemical complexity to this class of compounds. The nomenclature for unambiguously specifying the configuration about each carbon atom in a cyclic form and the means of representing these structures on paper are described in some detail; this information will be useful as we discuss the metabolism of monosaccharides in Part II. We also introduce here some important monosaccharide derivatives that will be examined closely in later chapters.

The Two Families of Monosaccharides Are Aldoses and Ketoses

Monosaccharides are colorless, crystalline solids that are freely soluble in water but insoluble in nonpolar solvents. Most have a sweet taste (**Box 7-1**). The backbones of common monosaccharides are unbranched carbon chains in which all the carbon atoms are linked by single bonds. In this open-chain form, one of the carbon atoms is double-bonded to an oxygen atom to form a carbonyl group; each of the other carbon atoms has a hydroxyl group. If the carbonyl group is at an end of the carbon chain (that is, in an aldehyde group), the monosaccharide is an **aldose**; if the carbonyl group is at any other position (in a ketone group), the monosaccharide is a **ketose**. The simplest monosaccharides are the two three-carbon trioses: glyceraldehyde, an aldotriose, and dihydroxyacetone, a ketotriose (**Fig. 7-1a**).

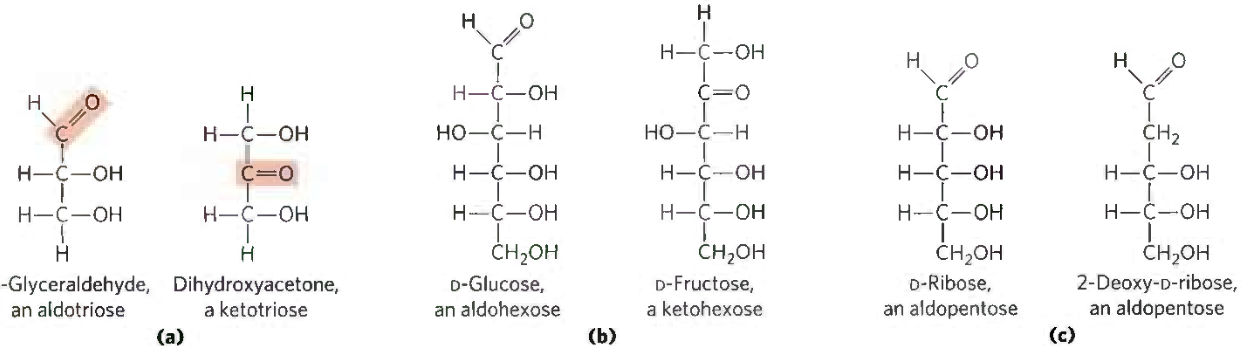

D-Glyceraldehyde, an aldotriose
Dihydroxyacetone, a ketotriose
(a)
D-Glucose, an aldohexose
D-Fructose, a ketohexose
(b)
D-Ribose, an aldopentose
2-Deoxy-D-ribose, an aldopentose
(c)

FIGURE 7-1 Representative monosaccharides. (a) Two trioses: an aldose and a ketose. The carbonyl group in each is shaded. (b) Two common hexoses. (c) The pentose components of nucleic acids. D-Ribose is a component of ribonucleic acid (RNA), and 2-deoxy-D-ribose is a component of deoxyribonucleic acid (DNA).

BOX 7-1 MEDICINE

What Makes Sugar Sweet?

Sweetness is one of the five basic flavors that humans can taste; the others are sour, bitter, salty, and umami. Sweetness is detected by protein receptors in the plasma membranes of cells in the taste buds on the surface of the tongue. In humans, two closely related genes (*TAS1R2* and *TAS1R3*) encode sweet-taste receptors (Fig. 1). When a molecule with a compatible structure binds a sweet-taste receptor's extracellular domain, it triggers a series of events in the cell (including activation of a GTP-binding protein; see Fig. 12-20) that generates an electrical signal to the brain that is interpreted as "sweet."

During evolution, there has probably been selection for the ability to taste compounds found in foods containing important nutrients, such as the carbohydrates that are major fuels for most organisms. Most sugars, including sucrose, glucose, lactose, and fructose, taste sweet, but other classes of compounds also bind sweet-taste receptors. The amino acids glycine, alanine, and serine are mildly sweet and harmless; nitrobenzene and ethylene glycol have a strong sweet taste but are toxic. (See Box 18-2 for a remarkable medical mystery involving ethylene glycol poisoning.) Several natural products are extraordinarily sweet. Stevioside, a sugar derivative isolated from the leaves of the stevia plant (*Stevia rebaudiana* Bertoni), is several hundred times sweeter than an equivalent amount of sucrose. The small protein brazzein (54 amino acids), isolated from berries of the Oubli vine (*Pentadiplandra brazzeana* Baillon) in Gabon and Cameroon, is 17,000 times sweeter than sucrose on a molar basis. Presumably, the sweetness of the berries encourages their consumption by animals that then disperse the seeds so that new plants are established.

In societies where obesity is a major health problem, compounds that give foods a sweet taste without adding the calories found in sugars are common food additives. The artificial sweetener aspartame demonstrates the importance of stereochemistry in biology (Fig. 2). One simple model of binding to the sweet-taste receptor involves three sites: AH^+, B^-, and X. Site AH^+ has a group (an alcohol or an amine) that binds the partially negative oxygen of the carboxylic acid of (*S,S*)-aspartame. Site B^- has a partially negative oxygen available to hydrogen-bond with the amine nitrogen of (*S,S*)-aspartame. Site X is oriented perpendicular to the other two groups and can accommodate the hydrophobic benzene ring of (*S,S*)-aspartame.

When the steric match is correct, as for (*S,S*)-aspartame, on the left in Figure 2, the sweet receptor is stimulated and the signal "sweet" is conducted to the brain. When the match is not correct, as for (*R,S*)-aspartame, the sweet receptor is not stimulated; in fact, (*R,S*)-aspartame stimulates a separate receptor specifically for bitterness. Stereoisomerism really matters!

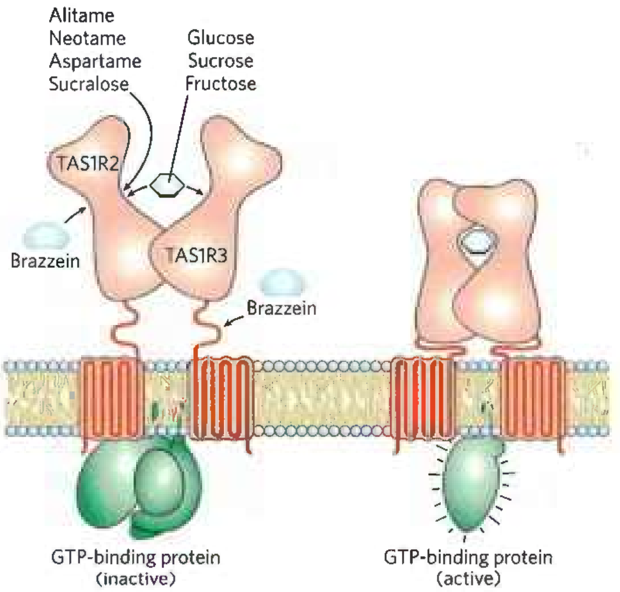


FIGURE 1 The receptor for sweet-tasting substances, showing its regions of interaction (short arrows) with various sweet-tasting compounds. Each receptor subunit has seven transmembrane helices, a common feature of signaling receptors. Artificial sweeteners bind to only one of the two receptor subunits; natural sugars bind to both. TAS1R2 and TAS1R3 are the proteins encoded by the genes *TAS1R2* and *TAS1R3*. [Information from F. M. Assadi-Porter et al., *J. Mol. Biol.* 398:584, 2010, Fig. 1.]

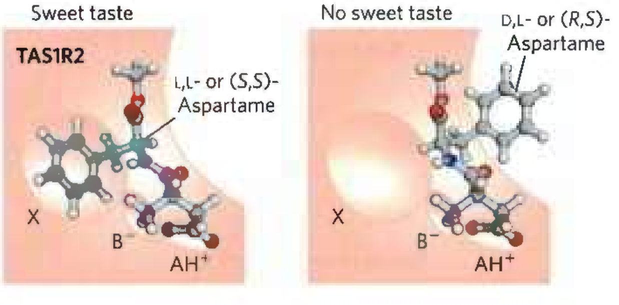


FIGURE 2 Stereochemical basis for the taste of two isomers of aspartame. [Information from http://chemistry.elmhurst.edu/vchembook/549receptor.html, © Charles E. Ophardt, Elmhurst College.]

Monosaccharides with four, five, six, and seven carbon atoms in their backbones are called, respectively, tetroses, pentoses, hexoses, and heptoses. There are aldoses and ketoses of each of these chain lengths: aldotetroses and ketotetroses, aldopentoses and ketopentoses, and so on. The hexoses, which include the aldohexose D-glucose and the ketohexose D-fructose (Fig. 7-1b), are the most common monosaccharides in nature—the products of photosynthesis and key intermediates in the central energy-yielding reaction sequence in most organisms. The aldopentoses D-ribose and 2-deoxy-D-ribose (Fig. 7-1c) are components of nucleotides and nucleic acids (Chapter 8).

Monosaccharides Have Asymmetric Centers

P1 All the monosaccharides except dihydroxyacetone contain one or more asymmetric (chiral) carbon atoms and thus occur in optically active isomeric forms (pp. 16–17). The simplest aldose, glyceraldehyde, contains one chiral center (the middle carbon atom) and therefore has two different optical isomers, or **enantiomers** (Fig. 7-2).

KEY CONVENTION One of the two enantiomers of glyceraldehyde is, by convention, designated the D isomer; the other is the L isomer. As for other biomolecules with chiral centers, the absolute configurations of sugars are known from x-ray crystallography. To represent three-dimensional sugar structures on paper, we often use **Fischer projection formulas** (Fig. 7-2). In these projections, bonds drawn horizontally indicate bonds that project out of the plane of the paper, toward the reader; bonds drawn vertically project behind the plane of the paper, away from the reader. ■

In general, a molecule with n chiral centers can have 2^n stereoisomers. Glyceraldehyde has $2^1 = 2$; the aldohexoses, with four chiral centers, have $2^4 = 16$. The stereoisomers of monosaccharides of each carbon-chain length can be divided into two groups that differ in the configuration about the chiral center *most distant* from the carbonyl carbon. Those in which the configuration at this reference carbon is the same as that of D-glyceraldehyde are designated D isomers, and those with the same configuration as L-glyceraldehyde are L isomers. In other words, when the hydroxyl group on the reference carbon is on the right (*dextro*) in a projection formula that has the carbonyl carbon at the top, the sugar is the D isomer; when the hydroxyl group is on the left (*levo*), it is the L isomer. Of the 16 possible aldohexoses, eight are D forms and eight are L. P1 Most of the hexoses of living organisms are D isomers. Why D isomers? An interesting and unanswered question. Recall that all of the amino acids found in proteins are exclusively one of two possible stereoisomers, L (p. 73). The basis for this initial preference for one isomer during evolution is unknown; however, once one isomer became prevalent, evolving enzymes able to use that isomer efficiently would have a selective advantage.

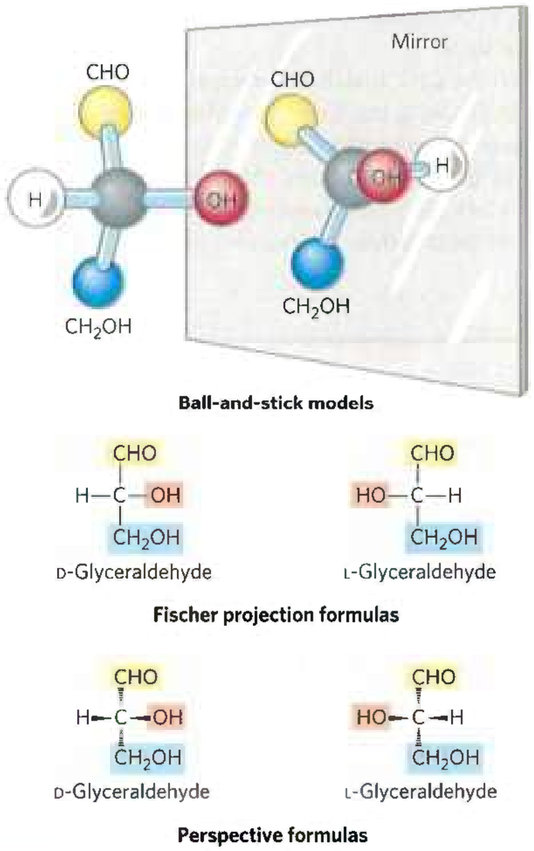
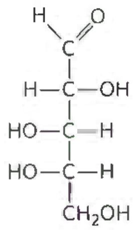


FIGURE 7-2 Three ways to represent the two enantiomers of glyceraldehyde. The enantiomers are mirror images of each other. Ball-and-stick models show the actual configuration of molecules. In Fischer projections, vertical lines point behind the plane of the page, and horizontal lines project above the page. Recall (see Fig. 1-17) that in perspective formulas, the wide end of a solid wedge projects out of the plane of the paper, toward the reader; a dashed wedge extends behind.

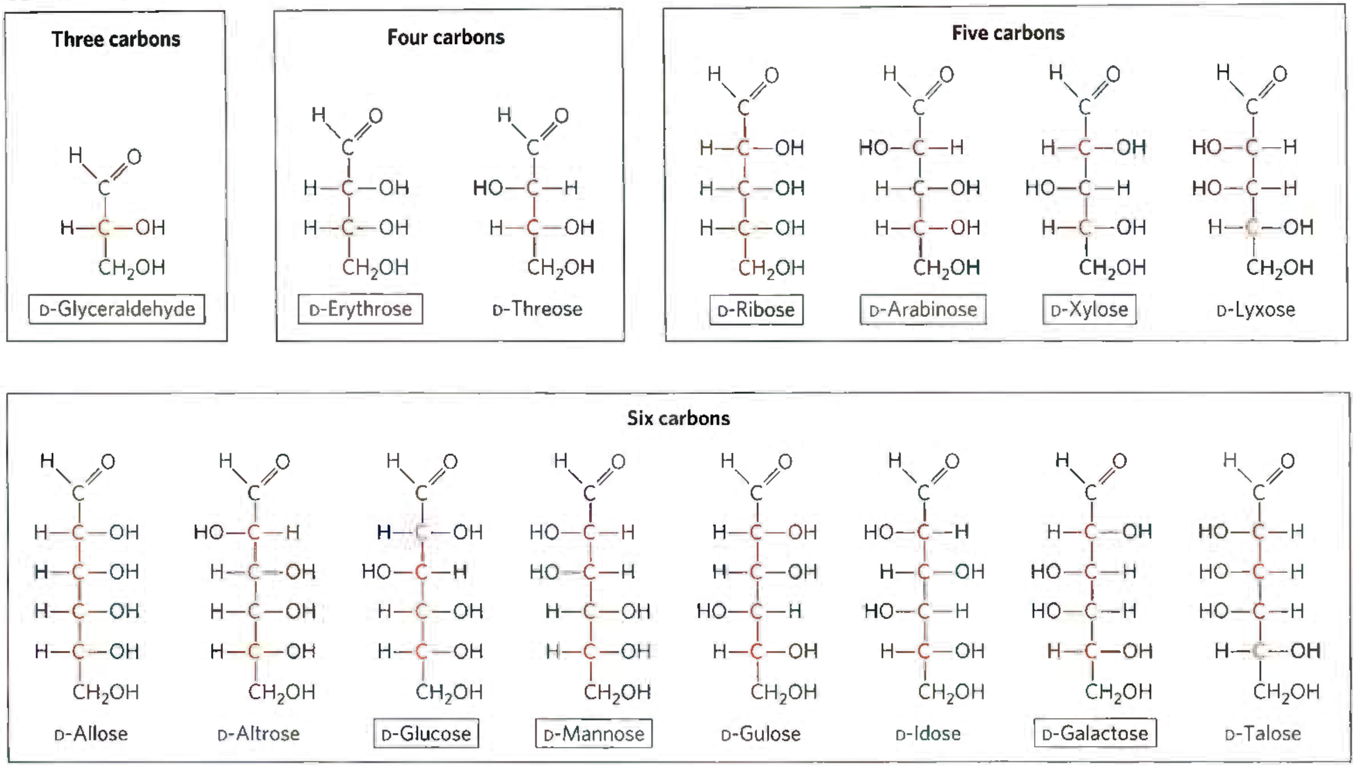
Figure 7-3 shows the structures of the D stereoisomers of all the aldoses and ketoses having three to six carbon atoms. The carbons of a sugar are numbered beginning at the end of the chain nearest the carbonyl group. Each of the eight D-aldohexoses, which differ in the stereochemistry at C-2, C-3, or C-4, has its own name: D-glucose, D-galactose, D-mannose, and so forth (Fig. 7-3a). The four- and five-carbon ketoses are designated by inserting "ul" into the name of a corresponding aldose; for example, D-ribulose is the ketopentose corresponding to the aldopentose D-ribose. (The importance of ribulose will become clear when we discuss the fixation of atmospheric CO_2 by green plants, in Chapter 20.) The ketohexoses are named otherwise: for example, fructose (from the Latin *fructus*, "fruit"; fruits are one source of this sugar) and sorbose (from *Sorbus*, the genus of mountain ash, which has berries rich in the related sugar alcohol sorbitol). Two sugars that differ only in the configuration around one carbon atom are called **epimers**; D-glucose and D-mannose, which differ only in the stereochemistry at C-2, are epimers, as are D-glucose and D-galactose (which differ at C-4) (**Fig. 7-4**).

Some sugars occur naturally in their L form; examples are L-arabinose and the L isomers of some sugar derivatives that are common components of glycoconjugates (Section 7.3).



L-Arabinose

(a) D-Aldoses


Three carbons
D-Glyceraldehyde
Four carbons
D-Erythrose
D-Threose
Five carbons
D-Ribose
D-Arabinose
D-Xylose
D-Lyxose
Six carbons
D-Allose
D-Altrose
D-Glucose
D-Mannose
D-Gulose
D-Idose
D-Galactose
D-Talose

(b) D-Ketoses

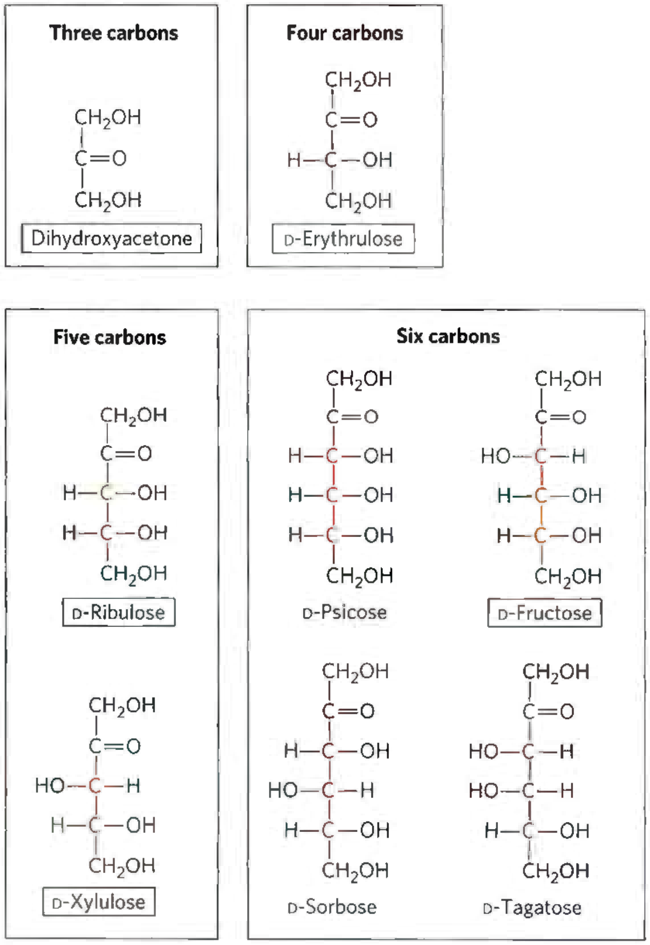

Three carbons
Dihydroxyacetone
Four carbons
D-Erythrulose
Five carbons
D-Ribulose
D-Xylulose
Six carbons
D-Psicose
D-Fructose
D-Sorbose
D-Tagatose

FIGURE 7-3 Aldoses and ketoses. The series of (a) D-aldoses and (b) D-ketoses having from three to six carbon atoms, shown as projection formulas. The carbon atoms in red are chiral centers. In all of these D isomers, the chiral carbon *most distant from the carbonyl carbon* has the same configuration as the chiral carbon in D-glyceraldehyde. Shown are the most common sugars in nature; you will encounter these again in this and later chapters.

The Common Monosaccharides Have Cyclic Structures

For simplicity, we have thus far represented the structures of aldoses and ketoses as straight-chain molecules (Figs 7-3, 7-4). In fact, in aqueous solution, aldotetroses and all monosaccharides with five or more carbon atoms in the backbone occur predominantly as cyclic (ring) structures in which the carbonyl group has formed a covalent bond with the oxygen of a hydroxyl group in the same sugar molecule. The formation of these ring

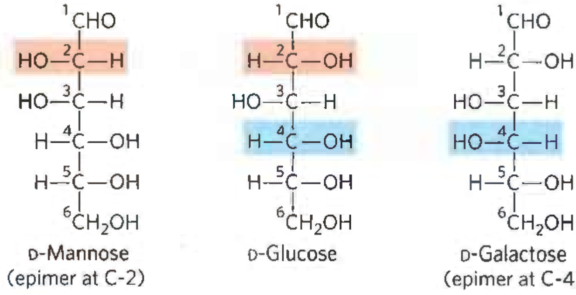

D-Mannose
(epimer at C-2)
D-Glucose
D-Galactose
(epimer at C-4)

FIGURE 7-4 Epimers. D-Glucose and two of its epimers are shown as projection formulas. Each epimer differs from D-glucose in the configuration at one chiral center (shaded light red or blue).

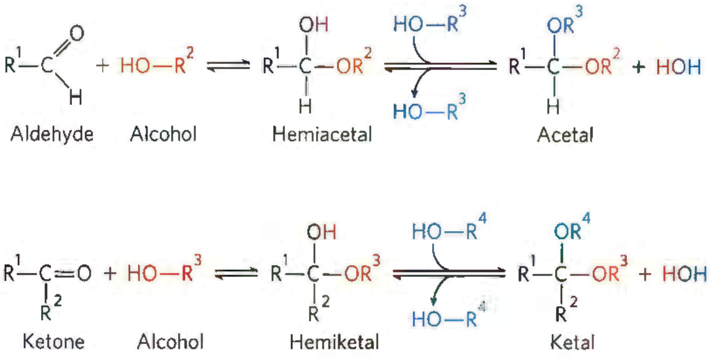
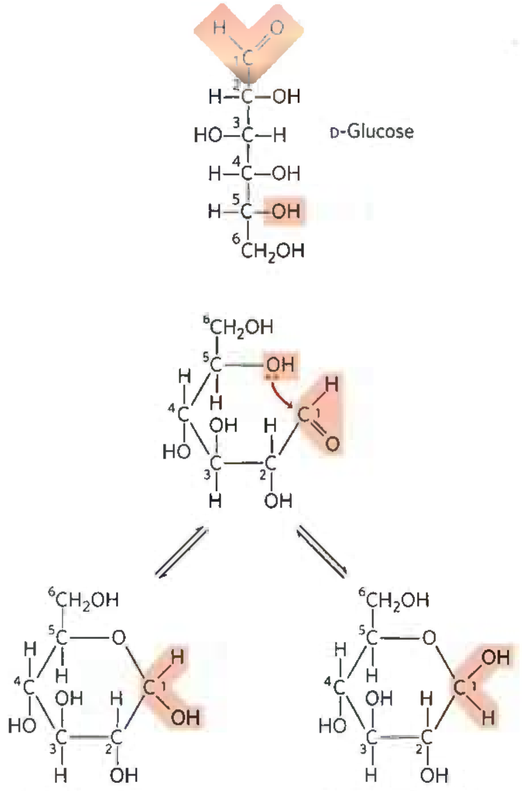


FIGURE 7-5 Formation of hemiacetals and hemiketals. An aldehyde or a ketone can react with an alcohol in a 1:1 ratio to yield a hemiacetal or a hemiketal, respectively, creating a new chiral center at the carbonyl carbon. Substitution of a second alcohol molecule produces an acetal or a ketal. When the second alcohol is part of another sugar molecule, the bond produced is a glycosidic bond.

structures is the result of a general reaction between alcohols and aldehydes or ketones to form derivatives called **hemiacetals** or **hemiketals**. Two molecules of an alcohol can add to a carbonyl carbon; the product of the first addition is a hemiacetal (for addition to an aldose) or a hemiketal (for addition to a ketose). If the —OH and carbonyl groups are on the same molecule, a five- or six-membered ring results. Addition of the second molecule of alcohol produces the full **acetal** or **ketal** (Fig. 7-5), and the linkage formed is a glycosidic bond. When the two molecules that react are monosaccharides, the acetal or ketal formed is a disaccharide.

The reaction with the first molecule of alcohol creates an additional chiral center (the carbonyl carbon). Because the alcohol can add in either of two ways, attacking either the "front" or the "back" of the carbonyl carbon, the reaction can produce either of two stereoisomeric configurations, denoted α and β. For example, D-glucose exists in solution as an intramolecular hemiacetal in which the free hydroxyl group at C-5 has reacted with the aldehydic C-1, rendering the latter carbon asymmetric and producing two possible stereoisomers, designated α and β (Fig. 7-6). Isomeric forms of monosaccharides that differ only in their configuration about the hemiacetal or hemiketal carbon atom are called **anomers**, and the carbonyl carbon atom is called the **anomeric carbon**.

Six-membered ring compounds are called **pyranoses** because they resemble the six-membered ring compound pyran (shown in Fig. 7-7). The systematic names for the two ring forms of D-glucose are therefore α-D-glucopyranose and β-D-glucopyranose. Ketohexoses (such as fructose) also occur as cyclic compounds with α and β anomeric forms. In these compounds, the hydroxyl group at C-5 (or C-6) reacts with the keto group at C-2 to form a **furanose** (or pyranose) ring containing a hemiketal linkage (Fig. 7-5). D-Fructose readily forms the furanose ring (Fig. 7-7); the more common anomer of this sugar in combined forms or in derivatives is β-D-fructofuranose.



α-D-Glucopyranose
β-D-Glucopyranose

FIGURE 7-6 Formation of the two cyclic forms of D-glucose. Reaction between the aldehyde group at C-1 and the hydroxyl group at C-5 forms a hemiacetal linkage, producing either of two stereoisomers, the α and β anomers, which differ only in the stereochemistry around the hemiacetal carbon. This reaction is reversible. The interconversion of α and β anomers is called mutarotation.

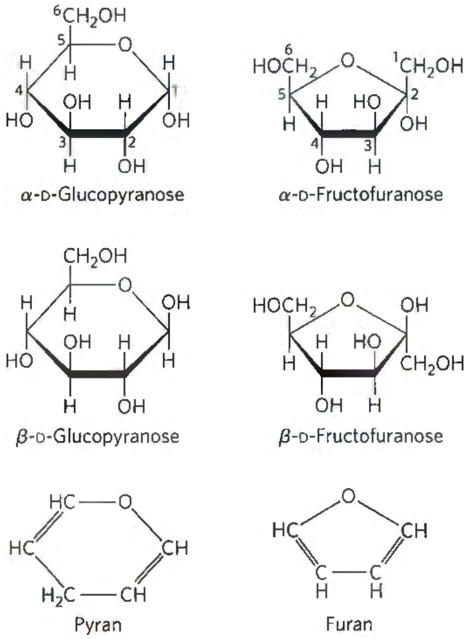
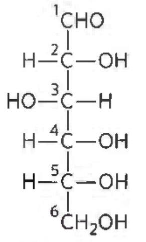


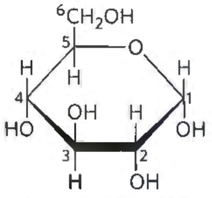
FIGURE 7-7 Pyranoses and furanoses. The pyranose forms of D-glucose and the furanose forms of D-fructose are shown here as Haworth perspective formulas. The edges of the ring nearest the reader are represented by bold lines. Hydroxyl groups below the plane of the ring in these Haworth perspectives would appear at the right side of a Fischer projection (compare with Fig. 7-6). Pyran and furan are shown for comparison.

Cyclic sugar structures are more accurately represented in **Haworth perspective formulas** than in the Fischer projections commonly used for linear sugar structures. In Haworth perspectives, the six-membered ring is tilted to make its plane almost perpendicular to that of the paper, with the bonds closest to the reader drawn thicker than those farther away, as in Figure 7-7.

KEY CONVENTION To convert the Fischer projection formula of any linear D-hexose to a Haworth perspective formula showing the molecule's cyclic structure, draw the six-membered ring (five carbons, and one oxygen at the upper right), number the carbons in a clockwise direction beginning with the anomeric carbon, then place the hydroxyl groups. If a hydroxyl group is to the right in the Fischer projection, it is placed pointing down (i.e., below the plane of the ring) in the Haworth perspective; if it is to the left in the Fischer projection, it is placed pointing up (i.e., above the plane) in the Haworth perspective. The terminal —CH_2OH group projects upward for the D enantiomer, downward for the L enantiomer. The hydroxyl on the anomeric carbon can point up or down. When the anomeric hydroxyl of a D-hexose is on the same side of the ring as C-6, the structure is by definition β; when it is on the opposite side from C-6, the structure is α.



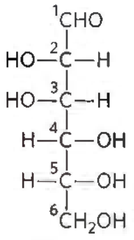
D-Glucose
Fischer projection



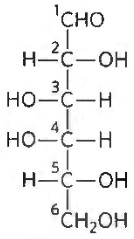
α-D-Glucopyranose
Haworth perspective

WORKED EXAMPLE 7-1 *Conversion of Fischer Projection to Haworth Perspective Formulas*

Draw the Haworth perspective formulas for D-mannose and D-galactose.



D-Mannose



D-Galactose

SOLUTION: Pyranoses are six-membered rings, so start with six-membered Haworth structures with the oxygen atom at the top right. Number the carbon atoms clockwise, starting with the aldose carbon. For mannose, place the hydroxyls on C-2, C-3, and C-4 above, above, and below the ring, respectively (because in the Fischer projection they are on the left, left, and right sides of the mannose backbone). For D-galactose, the hydroxyls are oriented below, above, and above the ring for C-2, C-3, and C-4, respectively. The hydroxyl at C-1 can point either up or down; there are two possible configurations, α and β, at this carbon.

WORKED EXAMPLE 7-2 *Drawing Haworth Perspective Formulas of Sugar Isomers*

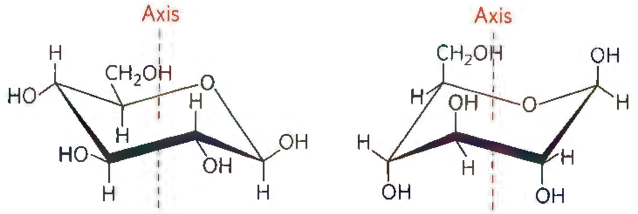
Draw the Haworth perspective formulas for α-D-mannose and β-L-galactose.

SOLUTION: The Haworth perspective formula of D-mannose from Worked Example 7-1 can have the hydroxyl group at C-1 pointing either up or down. According to the Key Convention, for the α form, the C-1 hydroxyl is pointing down when C-6 is up, as it is in D-mannose.

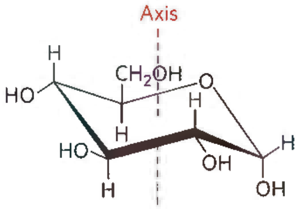
For β-L-galactose, use the Fischer representation of D-galactose (see Worked Example 7-1) to draw the correct Fischer representation of L-galactose, which is its mirror image: the hydroxyls at C-2, C-3, C-4, and C-5 are on the left, right, right, and left sides, respectively. Now draw the Haworth perspective, a six-membered ring in which the —OH groups on C-2, C-3, and C-4 are oriented up, down, and down, respectively, because in the Fischer representation they are on the left, right, and right sides. Because it is the β form, the —OH on the anomeric carbon points down (same side as C-5).

The α and β anomers of D-glucose interconvert in aqueous solution by a process called **mutarotation**, in which one ring form (say, the α anomer) opens briefly into the linear form, then closes again to produce the β anomer (Fig. 7-6). Thus, a solution of β-D-glucose and a solution of α-D-glucose eventually form identical equilibrium mixtures having identical optical properties. This mixture consists of about one-third α-D-glucose, two-thirds β-D-glucose, and very small amounts of the linear form and the five-membered ring (glucofuranose) form.

Haworth perspective formulas like those in Figure 7-7 are commonly used to show the stereochemistry of ring forms of monosaccharides. However, the six-membered pyranose ring is not planar, as Haworth perspectives suggest, but tends to assume either of two "chair" conformations (Fig. 7-8). Recall from Chapter 1 that P5 two *conformations* of a molecule are interconvertible without the breakage of covalent bonds, whereas two *configurations* can be interconverted only by breaking a covalent bond. To interconvert α and β configurations, the bond involving the ring oxygen atom has to be broken, but interconversion of the two chair forms (which are *conformers*) does not require bond breakage and does not change configurations at any of the ring carbons. The specific three-dimensional structures of the monosaccharide units are important in determining the biological properties and functions of some polysaccharides, as we shall see.



Two possible chair forms of β-D-glucopyranose
(a)



α-D-Glucopyranose
(b)

FIGURE 7-8 Conformational formulas of pyranoses. (a) Two chair forms of the pyranose ring of β-D-glucopyranose. Two *conformers* such as these are not readily interconvertible; an input of about 46 kJ of energy per mole of sugar is required to force the interconversion of chair forms. Another conformation, the "boat" (not shown), is seen only in derivatives with very bulky substituents. (b) The preferred chair conformation of α-D-glucopyranose.

Organisms Contain a Variety of Hexose Derivatives

In addition to simple hexoses such as glucose, galactose, and mannose, there are many sugar derivatives in which a hydroxyl group in the parent compound is replaced with another substituent, or a carbon atom is oxidized to a carboxyl group (**Fig. 7-9**). In glucosamine, galactosamine, and mannosamine, the hydroxyl at C-2 of the parent compound is replaced with an amino group.

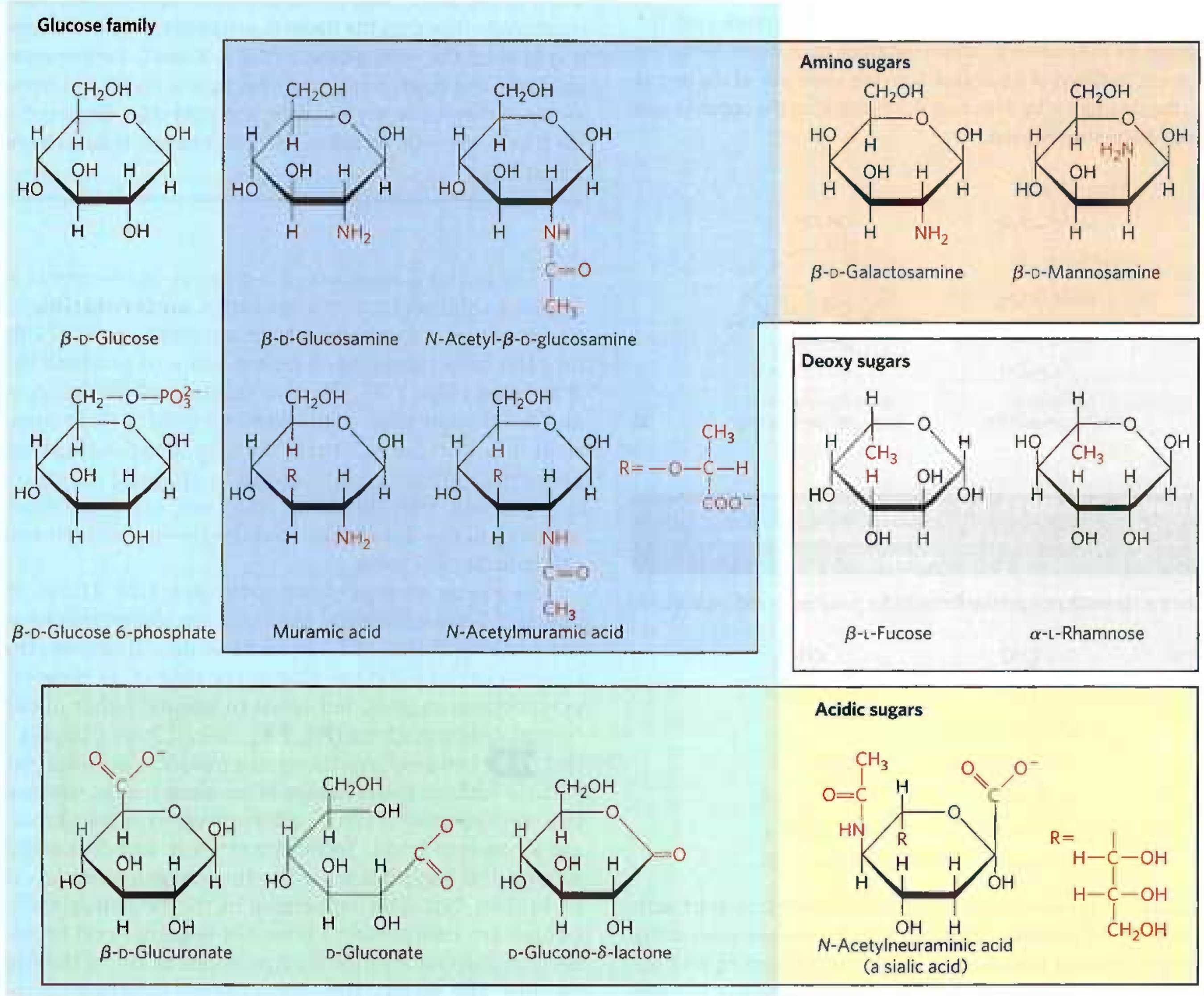

Glucose family
β-D-Glucose
β-D-Glucosamine
N-Acetyl-β-D-glucosamine
β-D-Glucose 6-phosphate
Muramic acid
N-Acetylmuramic acid
Amino sugars
β-D-Galactosamine
β-D-Mannosamine
Deoxy sugars
β-L-Fucose
α-L-Rhamnose
Acidic sugars
β-D-Glucuronate
D-Gluconate
D-Glucono-δ-lactone
N-Acetylneuraminic acid
(a sialic acid)

FIGURE 7-9 Some hexose derivatives important in biology. In amino sugars, an —NH_2 group replaces one of the —OH groups in the parent hexose. Substitution of —H for —OH produces a deoxy sugar; note that the deoxy sugars shown here occur in nature as the L isomers. The acidic sugars contain a carboxylate group, which confers a negative charge at neutral pH. D-Glucono-δ-lactone results from formation of an ester linkage between the C-1 carboxylate group and the C-5 (also known as the δ carbon) hydroxyl group of D-gluconate.

The amino group is commonly condensed with acetic acid, as in *N*-acetylglucosamine. This glucosamine derivative is part of many structural polymers, including those of the bacterial cell wall. Substitution of a hydrogen for the hydroxyl group at C-6 of L-galactose or L-mannose produces L-fucose or L-rhamnose, respectively. L-Fucose is found in the complex oligosaccharide components of glycoproteins and glycolipids; L-rhamnose is found in plant polysaccharides.

Oxidation of the carbonyl (aldehyde) carbon of glucose to the carboxyl level produces gluconic acid, used in medicine as an innocuous counterion when administering positively charged drugs (such as quinine) or ions (such as Ca^{2+}). Other aldoses yield other **aldonic acids**. Oxidation of the carbon at the other end of the carbon chain—C-6 of glucose, galactose, or mannose—forms the corresponding **uronic acid**: glucuronic, galacturonic, or mannuronic acid. Both aldonic acids and uronic acids form stable intramolecular esters called lactones (Fig. 7-9, lower left). The sialic acids are a family of sugars with the same nine-carbon backbone. One of them, *N*-acetylneuraminic acid (often referred to simply as "sialic acid"), is a derivative of *N*-acetylmannosamine that occurs in many glycoproteins and glycolipids on animal cell surfaces, providing sites of recognition by other cells or extracellular carbohydrate-binding proteins. The carboxylic acid groups of the acidic sugar derivatives are ionized at pH 7, and the compounds are therefore correctly named as the carboxylates—glucuronate, galacturonate, and so forth.

In the synthesis and metabolism of carbohydrates, the intermediates are very often not the sugars themselves but their phosphorylated derivatives. Condensation of phosphoric acid with one of the hydroxyl groups of a sugar forms a phosphate ester, as in glucose 6-phosphate (Fig. 7-9), the first metabolite in the pathway by which most organisms oxidize glucose for energy. Sugar phosphates are relatively stable at neutral pH and bear a negative charge. One effect of sugar phosphorylation within cells is to trap the sugar inside the cell; most cells do not have plasma membrane transporters for phosphorylated sugars. Phosphorylation also activates sugars for subsequent chemical transformation. Several important phosphorylated derivatives of sugars are components of nucleotides (discussed in the next chapter).

Sugars That Are, or Can Form, Aldehydes Are Reducing Sugars

Free aldehyde groups in sugars undergo a characteristic redox reaction with Cu^{2+} under alkaline conditions. As the sugar is oxidized from aldehyde to carboxylic acid, Cu^{2+} is reduced to Cu^{+}, which forms a brick-red precipitate. This reaction defines **reducing sugars**, which include, for example, glucose, galactose, mannose, ribose, and glyceraldehyde. The reaction occurs only with a *free* aldose; but because cyclic aldoses are in equilibrium with their linear forms, which do have free aldehyde groups (Fig. 7-6), all aldose monosaccharides are reducing sugars. Ketoses that can rearrange (tautomerize) to form aldehydes are also reducing sugars; fructose and ribulose are reducing sugars, for example.

The metabolism of glucose in people with diabetes can be monitored by measuring urinary glucose with a simple qualitative assay for reducing sugar, or by measuring quantitatively the nonenzymatic reaction between glucose and the hemoglobin in blood (**Box 7-2**).

Disaccharides (such as maltose, lactose, and sucrose) consist of two monosaccharides joined covalently by an ***O*-glycosidic bond**, which is formed when a hydroxyl group of one sugar molecule, typically in its cyclic form, reacts with the anomeric carbon of the other (**Fig. 7-10**). This reaction represents the formation of an acetal from a hemiacetal (such as glucopyranose) and an alcohol (a hydroxyl group of the second sugar molecule) (Fig. 7-5), and the resulting compound is called a glycoside. Glycosidic bonds are readily hydrolyzed by acid but resist cleavage by base. Thus disaccharides can be hydrolyzed to yield their free monosaccharide components by boiling with dilute acid.

When the anomeric carbon is involved in a glycosidic bond, the easy interconversion of linear and cyclic forms shown in Figure 7-6 is prevented. Formation of a glycosidic bond therefore renders a sugar nonreducing. In describing disaccharides or polysaccharides, the end of a chain with a free anomeric carbon (one not involved in a glycosidic bond) is called the **reducing end**.

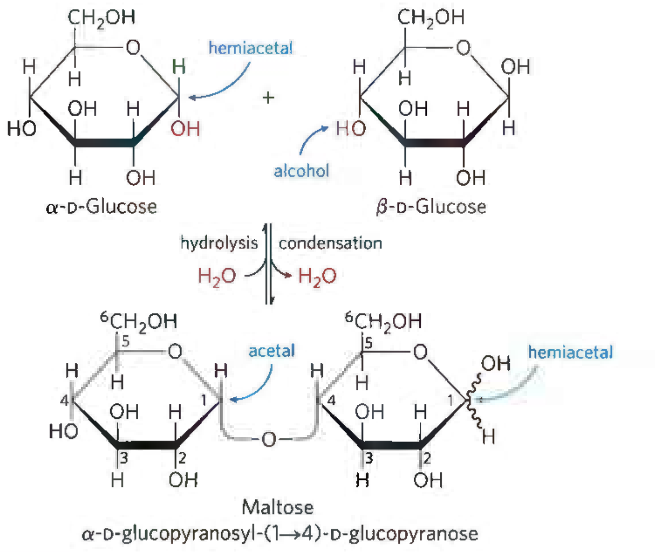


FIGURE 7-10 Formation of maltose. A disaccharide is formed from two monosaccharides (here, two molecules of D-glucose) when an —OH (alcohol) of one monosaccharide molecule (right) condenses with the intramolecular hemiacetal of the other (left), with elimination of H_2O and formation of a glycosidic bond. The reversal of this reaction is hydrolysis—attack by H_2O on the glycosidic bond. The maltose molecule, shown here, retains a reducing hemiacetal at the C-1 not involved in the glycosidic bond. Because mutarotation interconverts the α and β forms of the hemiacetal, the bonds at this position are sometimes depicted with wavy lines to indicate that the structure may be either α or β.

BOX 7-2 **MEDICINE**

Blood Glucose Measurements in the Diagnosis and Treatment of Diabetes

Glucose is the principal fuel for the brain. When the amount of glucose reaching the brain is too low, the consequences can be dire: lethargy, coma, permanent brain damage, and death. Complex hormonal mechanisms have evolved to ensure that the concentration of glucose in the blood remains high enough (about 5 mM) to satisfy the brain's needs—but not too high, because elevated blood glucose can also have serious physiological consequences.

Individuals with insulin-dependent diabetes mellitus do not produce sufficient insulin, the hormone that normally serves to reduce blood glucose concentration. If the diabetes is untreated, blood glucose levels may rise to several-fold higher than normal. These high glucose levels are believed to be at least one cause of the serious long-term consequences of untreated diabetes—kidney failure, cardiovascular disease, blindness, and impaired wound healing—so one goal of therapy is to provide just enough insulin (by injection) to keep blood glucose levels near normal. To maintain the correct balance of exercise, diet, and insulin, individuals with diabetes must measure their blood glucose concentration several times a day, and adjust the amount of insulin injected appropriately.

The concentrations of glucose in blood can be determined by a simple assay for reducing sugar. A single drop of blood is added to a test strip containing the enzyme glucose oxidase, which catalyzes the reaction:

$$\text{D-Glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

A second enzyme, a peroxidase, catalyzes reaction of the H_2O_2 with a colorless compound to create a colored product, which is quantified with a simple photometer that reads out the blood glucose concentration.

Because blood glucose levels change with the timing of meals and exercise, single-time measurements do not reflect the *average* blood glucose over hours and days, so dangerous increases may go undetected. The average glucose concentration can be assessed by looking at its effect on hemoglobin, the oxygen-carrying protein in erythrocytes (p. 153). Transporters in the erythrocyte membrane equilibrate intracellular and plasma glucose concentrations, so hemoglobin is constantly exposed to glucose at whatever concentration is present in the blood. A series of relatively slow *nonenzymatic* reactions (Fig. 1) occurs between glucose and primary amino groups in hemoglobin (either the amino-terminal Val or the ε-amino groups of Lys residues) (Fig. 2). The rate of this process is proportional to the concentration of glucose, so the reaction can be used to estimate the average blood glucose level over weeks. The amount of glycated hemoglobin (GHB) present at any time reflects the average blood glucose concentration over the circulating lifetime of the erythrocyte (about 120 days), although the concentration in the two weeks before the test is the most important in setting the level of GHB.

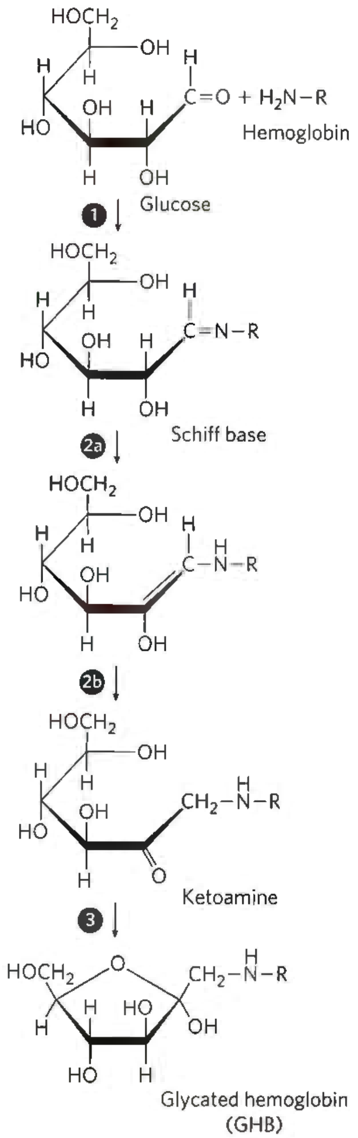


FIGURE 1 The nonenzymatic reaction of glucose with a primary amino group in hemoglobin begins with ① formation of a Schiff base, which ② undergoes a rearrangement to generate a stable product; ③ this ketoamine can further cyclize to yield GHB.

The disaccharide maltose (Fig. 7-10) contains two D-glucose residues joined by a glycosidic linkage between C-1 (the anomeric carbon) of one glucose residue and C-4 of the other. Because the disaccharide retains a free anomeric carbon (C-1 of the glucose residue on the right in Fig. 7-10), maltose is a reducing sugar. The configuration of the anomeric carbon atom in the glycosidic linkage is α. The glucose residue with the free anomeric carbon is capable of existing in α- and β-pyranose forms.

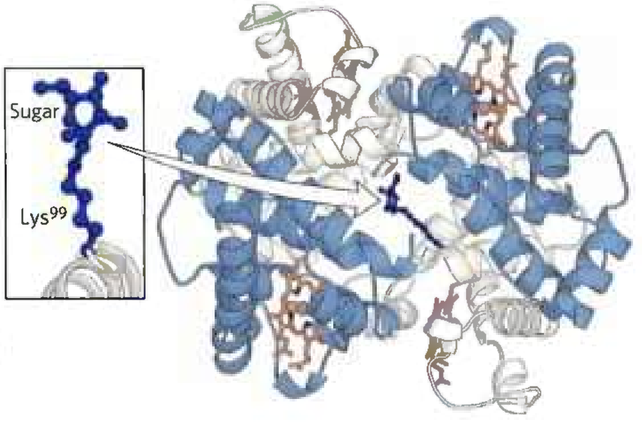


FIGURE 2 Glycated hemoglobin, showing the hemes (red) and the glycated Lys^{99} residue (blue). [Data from PDB ID 3B75, N. T. Saraswathi et al., 2008.]

Analyte ID	Percent	Time (min)	Area
Injection	0.0	0.11	17,682
A1a	0.4	0.30	8,051
A1b	1.0	0.45	19,267
A1c	5.9	1.10	110,946
A0	92.6	1.56	1,727,669
		Total area	1,883,615

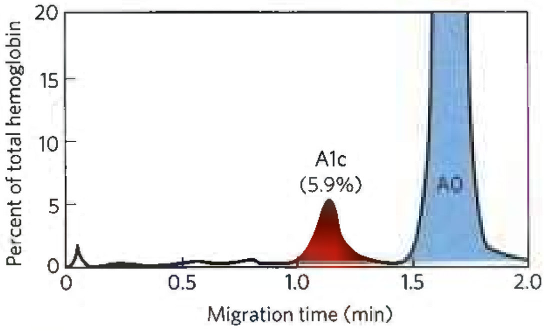


FIGURE 3 Pattern of hemoglobin (detected by its absorption at 415 nm) after electrophoretic separation of nonglycated (A0) and monoglycated (A1c) forms in a thin glass capillary. Integration of the area under the peaks allows calculation of the amount of GHB (HbA1c) as a percentage of total hemoglobin. Shown here is the profile of an individual with a normal level of HbA1c (5.9%).

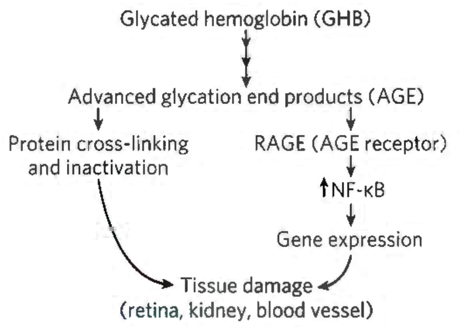

Glycated hemoglobin (GHB)
Advanced glycation end products (AGE)
Protein cross-linking and inactivation
RAGE (AGE receptor)
↑NF-κB
Gene expression
Tissue damage
(retina, kidney, blood vessel)

FIGURE 4 Pathways from glycated hemoglobin to the tissue damage associated with diabetes.

The extent of **hemoglobin glycation** (so named to distinguish it from glycosylation, the *enzymatic* transfer of glucose to a protein) is measured clinically by extracting hemoglobin from a small sample of blood and separating GHB from unmodified hemoglobin electrophoretically (Fig. 3), taking advantage of the charge difference resulting from modification of the amino group(s). Normal values of the monoglycated hemoglobin referred to as HbA1c are about 5% of total hemoglobin (corresponding to an average blood glucose level of 120 mg/100 mL). In people with untreated diabetes, however, this value may be as high as 13%, indicating an average blood glucose level of about 300 mg/100 mL—dangerously high. One criterion for success in an individual program of insulin therapy (the timing, frequency, and amount of insulin injected) is maintaining HbA1c values at about 7%.

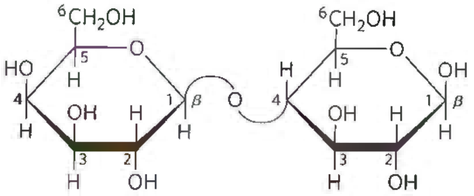
Glycated hemoglobin undergoes a series of rearrangements, oxidations, and dehydrations of the carbohydrate moiety to produce a heterogeneous mixture of *advanced glycation end products* (AGE), such as ε-*N*-carboxymethyllysine and methylglyoxal. AGE are believed to be responsible for at least some of the pathology of diabetes. AGE can leave the erythrocyte and form covalent cross-links between proteins, interfering with normal protein function (Fig. 4). The accumulation of relatively high concentrations of AGE in people with diabetes may, by cross-linking critical proteins, cause the damage to the kidneys, retinas, and cardiovascular system that characterizes the disease. Some AGE also can act through membrane receptors for AGE (RAGE), triggering intracellular responses that include activation of a transcription factor (NF-κB) and consequent changes in gene expression. This pathogenic process is a potential target for drug action.

KEY CONVENTION To name reducing disaccharides such as maltose unambiguously, and especially to name more complex oligosaccharides, several rules are followed. By convention, the name describes the compound written with its nonreducing end to the left, and we can "build up" the name in the following order. (1) Give the configuration (α or β) at the anomeric carbon joining the first monosaccharide unit (on the left) to the second. (2) Name the nonreducing residue; to distinguish five- and six-membered ring structures, insert "furano" or "pyrano" into the name. (3) Indicate in parentheses the two carbon atoms joined by

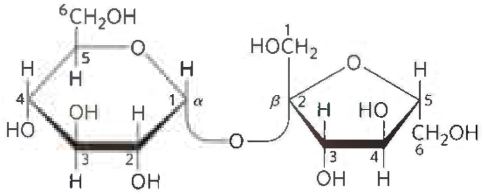
TABLE 7-1 Symbols and Abbreviations for Common Monosaccharides and Some of Their Derivatives

Abequose	Abe	Glucuronic acid	◆ GlcA
Arabinose	Ara	Galactosamine	□ GalN
Fructose	Fru	Glucosamine	◪ GlcN
Fucose	▲ Fuc	*N*-Acetylgalactosamine	□ GalNAc
Galactose	○ Gal	*N*-Acetylglucosamine	■ GlcNAc
Glucose	● Glc	Iduronic acid	◇ IdoA
Mannose	● Man	Muramic acid	Mur
Rhamnose	Rha	*N*-Acetylmuramic acid	Mur2Ac
Ribose	Rib	*N*-Acetylneuraminic acid (a sialic acid)	◆ Neu5Ac
Xylose	★ Xyl		

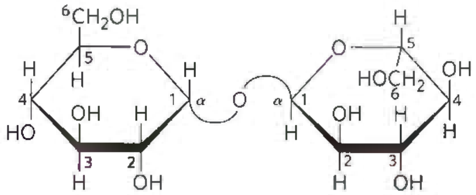
Note: In a commonly used convention, hexoses are represented as circles, *N*-acetylhexosamines as squares, and hexosamines as squares divided diagonally. All sugars with the "gluco" configuration are blue, those with the "galacto" configuration are yellow, and "manno" sugars are green. Other substituents can be added as needed: sulfate (S), phosphate (P), *O*-acetyl (OAc), or *O*-methyl (OMe).



Lactose (β form)
β-D-galactopyranosyl-(1⟶4)-β-D-glucopyranose
Gal(β1⟶4)Glc



Sucrose
β-D-fructofuranosyl α-D-glucopyranoside
Fru(2β↔α1)Glc ≡ Glc(α1↔2β)Fru



Trehalose
α-D-glucopyranosyl α-D-glucopyranoside
Glc(α1↔1α)Glc

FIGURE 7-11 Three common disaccharides. Like maltose in Figure 7-10, these disaccharides are shown as Haworth perspectives. The common name, full systematic name, and abbreviation are given. Formal nomenclature for sucrose names glucose as the parent glycoside, although it is typically depicted as shown, with glucose on the left. The two abbreviated symbols shown for sucrose are equivalent (≡).

the glycosidic bond, with an arrow connecting the two numbers; for example, (1⟶4) shows that C-1 of the first-named sugar residue is joined to C-4 of the second. (4) Name the second residue. If there is a third residue, describe the second glycosidic bond by the same conventions. (To shorten the description of complex polysaccharides, three-letter abbreviations or colored symbols for the monosaccharides are often used, as given in **Table 7-1**.) Following this convention for naming oligosaccharides, maltose is α-D-glucopyranosyl-(1⟶4)-D-glucopyranose. Because most sugars encountered in this book are the D enantiomers and the pyranose form of hexoses predominates, we generally use a shortened version of the formal name of such compounds, giving the configuration of the anomeric carbon and naming the carbons joined by the glycosidic bond. In this abbreviated nomenclature, maltose is Glc(α1⟶4)Glc. ■

The disaccharide lactose (**Fig. 7-11**), which yields D-galactose and D-glucose on hydrolysis, occurs naturally in milk and gives milk its sweetness. The anomeric carbon of the glucose residue is available for oxidation, and thus lactose is a reducing disaccharide. The reducing end of this disaccharide, which by convention is drawn on the right, is glucose, and the disaccharide is named as a derivative of glucose. Its abbreviated name is Gal(β1⟶4)Glc, with the linkage shown in parentheses; the anomeric carbon of galactose is β, and C-1 of Gal is linked to C-4 of Glc.

The enzyme lactase—absent in lactose-intolerant individuals—begins the digestive process in the small intestine by splitting the (β1⟶4) bond of lactose into monosaccharides, which can be absorbed from the small intestine. Lactose, like other disaccharides, is not absorbed from the small intestine, and in lactose-intolerant individuals the undigested lactose passes into the large intestine. Here, the increased osmolarity due to dissolved lactose opposes the absorption of water from the intestine into the bloodstream, causing watery, loose stools. In addition, fermentation of the lactose by intestinal bacteria produces large volumes of CO_2, which leads to the bloating, cramps, and gas associated with lactose intolerance.

Sucrose (table sugar) is a disaccharide of glucose and fructose. It is formed by plants but not by animals. In contrast to maltose and lactose, sucrose contains no free anomeric carbon atom; the anomeric carbons of both monosaccharide units are involved in the glycosidic bond (Fig. 7-11). Sucrose is therefore a nonreducing sugar, and its stability—its resistance to oxidation—makes it a suitable molecule for the storage and transport of energy in plants. In the abbreviated nomenclature, a double-headed arrow connects the symbols specifying the anomeric carbons and their configurations. Thus the abbreviated name of sucrose is either Glc(α1↔2β)Fru or Fru(β2↔1α)Glc. Sucrose is a major intermediate product of photosynthesis; in many plants it is the principal form in which sugar is transported from the leaves to other parts of the plant body.

Trehalose, Glc(α1↔1α)Glc (Fig. 7-11) — a disaccharide of D-glucose that, like sucrose, is a nonreducing sugar — is a major constituent of the circulating fluid (hemolymph) of insects. It serves as an energy-storage compound, and it serves as antifreeze in some invertebrate organisms.

SUMMARY 7.1 *Monosaccharides and Disaccharides*

- Sugars (saccharides) contain an aldehyde or ketone group and two or more hydroxyl groups.
- Monosaccharides generally contain several chiral carbons and therefore exist in a variety of stereochemical forms, which may be represented on paper as Fischer projections. Epimers are sugars that differ in configuration at only one carbon atom.
- Monosaccharides with at least six carbons commonly form cyclic structures, in which the aldehyde or ketone group is joined to a hydroxyl group of the same molecule. The cyclic structure can be represented as a Haworth perspective formula. The carbon atom originally found in the aldehyde or ketone group (the anomeric carbon) can assume either of two configurations, α and β, which are interconvertible by mutarotation. In the linear form of the monosaccharide, which is in equilibrium with the cyclic forms, the anomeric carbon is easily oxidized, making the compound a reducing sugar.
- Naturally occurring hexoses include some that have —NH_2 at C-2 (amino sugars), often with the amino group acetylated. Oxidation of the carbonyl carbon of glucose and other aldoses yields aldonic acids (gluconic acid); oxidation at C-6 produces uronic acids (glucuronate). Some sugar intermediates are phosphate esters (for example, glucose 6-phosphate).
- A hydroxyl group of one monosaccharide can add to the anomeric carbon of a second monosaccharide to form an acetal called a glycoside. Oligosaccharides are short polymers of several different monosaccharides joined by glycosidic bonds. At one end of the chain, the reducing end, is a monosaccharide residue with its anomeric carbon not involved in a glycosidic bond, and therefore available to be oxidized.
- Disaccharides and oligosaccharides are named as derivatives of the sugar at the reducing end. Their names provide the order of monosaccharide units, the configuration at each anomeric carbon, and the carbon atoms involved in the glycosidic linkage(s).

7.2 Polysaccharides

Most carbohydrates found in nature occur as polysaccharides, polymers of medium to high molecular weight (M_r > 20,000). **P2** Polysaccharides, also called **glycans**, differ from each other in the identity of their recurring monosaccharide units, in the length of their chains, in the types of bonds linking the units, and in the degree of branching. **Homopolysaccharides** contain only a single monomeric sugar species; **heteropolysaccharides** contain two or more kinds of monomers (**Fig. 7-12**). Some homopolysaccharides serve as storage forms of monosaccharides that are used as fuels; starch and glycogen are homopolysaccharides of this type. Other homopolysaccharides (cellulose and chitin, for example) serve as structural elements in plant cell walls and animal exoskeletons. Heteropolysaccharides provide extracellular support for organisms of all kingdoms. For example, the rigid layer of the bacterial cell envelope (the peptidoglycan) is composed in part of a heteropolysaccharide built from two alternating monosaccharide units (see Fig. 6-32). In animal tissues, the extracellular space is occupied by several types of heteropolysaccharides, which form a matrix that holds individual cells together and provides protection, shape, and support to cells, tissues, and organs.

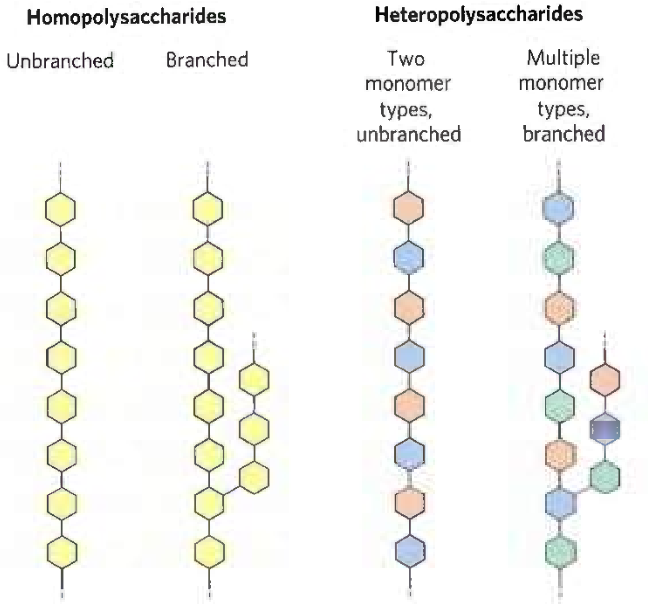

Homopolysaccharides
Unbranched
Branched
Heteropolysaccharides
Two monomer types, unbranched
Multiple monomer types, branched

FIGURE 7-12 Homopolysaccharides and heteropolysaccharides. Polysaccharides may be composed of one, two, or several different monosaccharides, in straight or branched chains of varying length.

P4 Unlike proteins, polysaccharides generally do not have defined lengths or molecular weights. This difference is a consequence of the mechanisms of assembly of the two types of polymer. As we shall see in Chapter 27, proteins are synthesized on a template (messenger RNA) of defined sequence and length, by enzymes that follow the template exactly. For polysaccharide synthesis there is no template; rather, the program for polysaccharide synthesis is intrinsic to the enzymes that catalyze the polymerization of the monomeric units, and there is no specific stopping point in the synthetic process; the products thus vary in length.

Some Homopolysaccharides Are Storage Forms of Fuel

The most important storage polysaccharides are starch in plant cells and glycogen in animal cells. Both polysaccharides occur intracellularly as large clusters or granules. Starch and glycogen molecules are heavily hydrated, because they have many exposed hydroxyl groups available to hydrogen-bond with water. Most plant cells have the ability to form starch, and starch storage is especially abundant in tubers (underground stems), such as potatoes, and in seeds.

Starch contains two types of glucose polymer, amylose and amylopectin (**Fig. 7-13**). Amylose consists of long, unbranched chains of D-glucose residues connected by (α1→4) linkages (as in maltose). Such chains vary in molecular weight from a few thousand to more than a million. Amylopectin is even larger (M_r up to 200 million) but unlike amylose is highly branched. The glycosidic linkages joining successive glucose residues in amylopectin chains are (α1→4); the branch points (occurring every 24 to 30 residues) are (α1→6) linkages.

Glycogen is the main storage polysaccharide of animal cells. Like amylopectin, glycogen is a polymer of (α1→4)-linked glucose subunits, with (α1→6)-linked branches, but glycogen is more extensively branched (on average, a branch every 8 to 12 residues) and more compact than starch. Glycogen is especially abundant in the liver, where it may constitute as much as 7% of the wet weight; it is also present in skeletal muscle. In hepatocytes, glycogen is found in large α granules (see Fig. 15-1), which are clusters of smaller β granules, each of which is a single, highly branched glycogen molecule with an average molecular weight of several million. The large α glycogen granules also contain, in tightly bound form, the enzymes responsible for the synthesis and degradation of glycogen (see Fig. 15-16).

Because each branch of glycogen ends with a nonreducing sugar unit, a glycogen molecule with n branches has $n + 1$ nonreducing ends, but only one reducing end. When glycogen is used as an energy source, glucose units are removed one at a time from the nonreducing ends. Degradative enzymes that act only at nonreducing ends can work simultaneously on the many branches, speeding the conversion of the polymer to monosaccharides.

Why not store glucose in its monomeric form? P3 Hepatocytes in the fed state store glycogen equivalent to a glucose concentration of 0.4 M. The actual concentration of glycogen, which contributes little to the osmolarity of the cytosol, is about 0.01 μM. If the cytosol contained 0.4 M glucose, the osmolarity would be threateningly elevated, leading to osmotic entry of water that might rupture the cell (see Fig. 2-12).

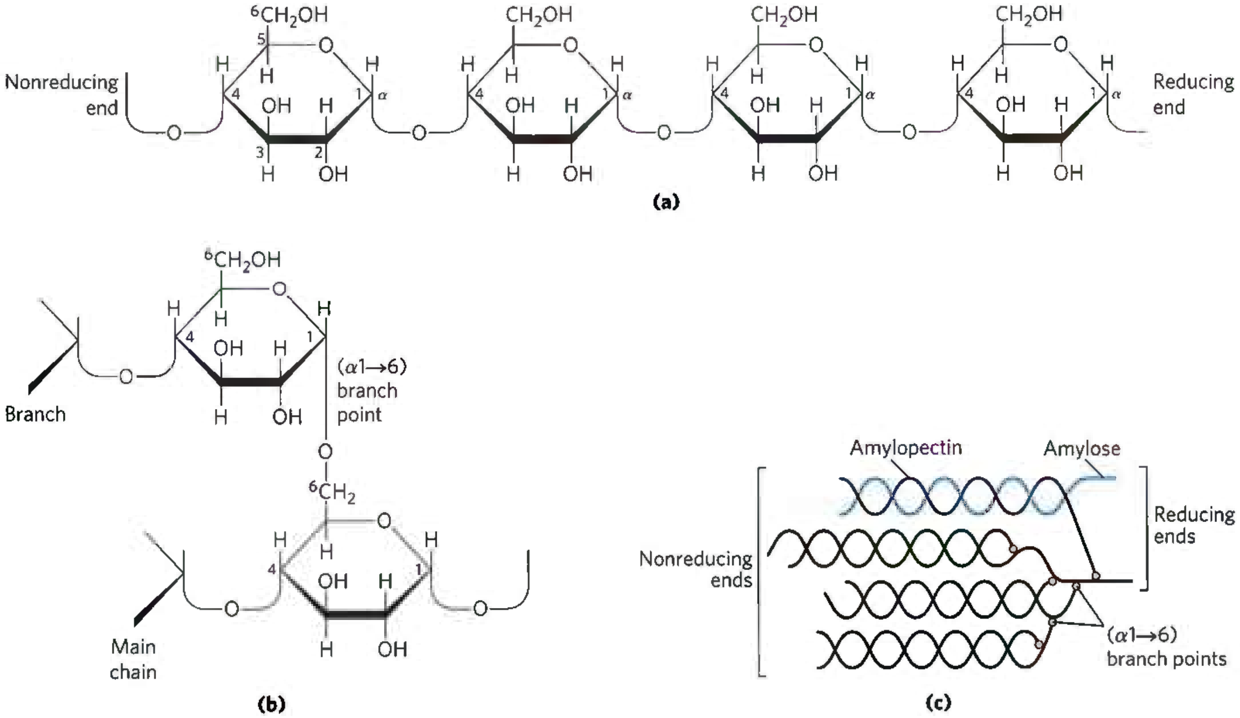


FIGURE 7-13 Starch and glycogen. (a) A short segment of a linear polymer of D-glucose residues in (α1→4) linkage, shown here as Haworth perspectives. This is the basic structure of amylose of starch (in plants) and glycogen (in animals). A single chain of amylose can contain several thousand glucose residues. (b) An (α1→6) branch point of amylopectin or glycogen. (c) A cluster of amylose and amylopectin like that believed to occur in starch granules. Strands of amylopectin (black) form double-helical structures with each other or with amylose strands (blue). Amylopectin has frequent (α1→6) branch points (red). Glucose residues at the nonreducing ends of the outer branches are removed enzymatically during the mobilization of starch for energy production. Glycogen has a similar structure but is more highly branched and more compact.

Some Homopolysaccharides Serve Structural Roles

Cellulose, a tough, fibrous, water-insoluble substance, is found in the cell walls of plants, particularly in stalks, stems, trunks, and all the woody portions of the plant body. Cellulose constitutes much of the mass of wood, and cotton is almost pure cellulose. Like amylose, the cellulose molecule is a linear, unbranched homopolysaccharide, consisting of 10,000 to 15,000 D-glucose units. But there is a very important difference: **P5** in cellulose the glucose residues have the β configuration (**Fig. 7-14**), whereas in amylose the glucose is in the α configuration. The glucose residues in cellulose are linked by (β1→4) glycosidic bonds, in contrast to the (α1→4) bonds of amylose. This difference causes individual molecules of cellulose and amylose to fold differently in space, giving them very different macroscopic structures and physical properties.

The tough, fibrous nature of cellulose makes it useful in such commercial products as cardboard and insulation material, and it is a major constituent of cotton and linen fabrics. Cellulose is also the starting material for the commercial production of cellophane, rayon, and lyocell.

Chitin is a linear homopolysaccharide composed of *N*-acetylglucosamine residues in (β1→4) linkage (**Fig. 7-15**). The only chemical difference from cellulose is the replacement of the hydroxyl group at C-2 with an acetylated amino group, which makes chitin more hydrophobic and water-resistant than cellulose. Chitin forms extended fibers similar to those of cellulose, and like cellulose cannot be digested by vertebrates. Chitin is the principal component of the hard exoskeletons of nearly a million species of arthropods—insects, lobsters, and crabs, for example—and is probably the second most abundant polysaccharide, next to cellulose, in nature; an estimated 1 billion tons of chitin are produced in the biosphere each year.

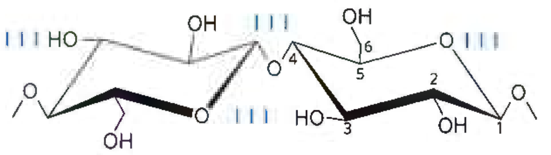


FIGURE 7-14 Cellulose. Two units of a cellulose chain; the D-glucose residues are in (β1→4) linkage. The rigid chair structures can rotate relative to one another.

Steric Factors and Hydrogen Bonding Influence Homopolysaccharide Folding

P5 The folding of polysaccharides in three dimensions follows the same principles as those governing polypeptide structure: subunits with a more-or-less rigid structure dictated by covalent bonds form three-dimensional macromolecular structures that are stabilized by weak interactions within or between molecules, such as hydrogen bonds, interactions due to the hydrophobic effect, van der Waals interactions, and, for polymers with charged subunits, electrostatic interactions. Because polysaccharides have so many hydroxyl groups, hydrogen bonding has an especially important influence on their structure. Glycogen, starch, cellulose, and chitin are composed of pyranoside (six-membered ring) subunits, as are the oligosaccharides of glycoproteins and glycolipids, to be discussed later. Such molecules can be represented as a series of rigid pyranose rings connected by an oxygen atom bridging two carbon atoms (the glycosidic bond). There is, in principle, free rotation about both C—O bonds linking the residues, but as in polypeptides (see Figs 4-2, 4-8), rotation about each bond is limited by steric hindrance by substituents. The three-dimensional structures of these molecules can be described in terms of the dihedral angles, ϕ and ψ, about the glycosidic bond (**Fig. 7-16**). The bulkiness of the pyranose ring and its substituents, along with electronic effects at the anomeric carbon, place constraints on the angles ϕ and ψ; thus, certain conformations are much more stable than others.

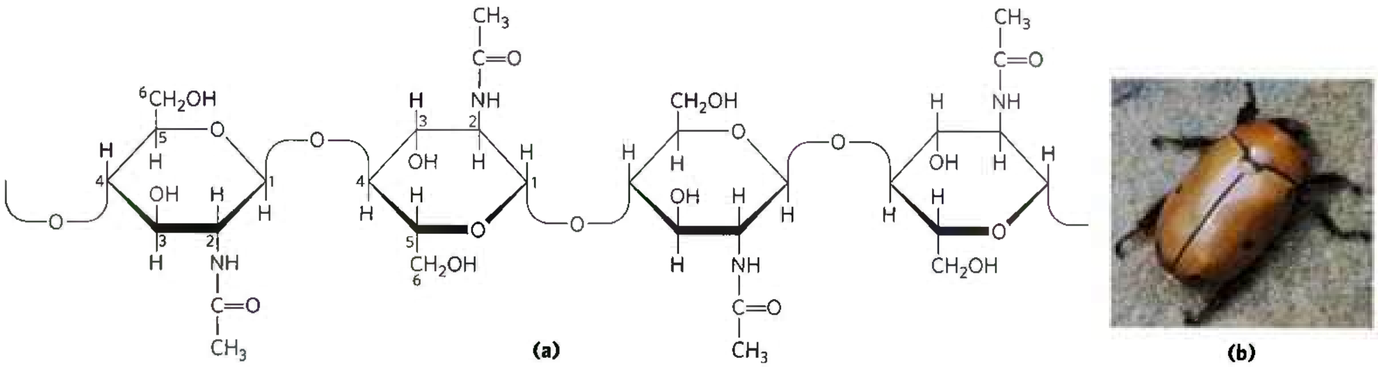
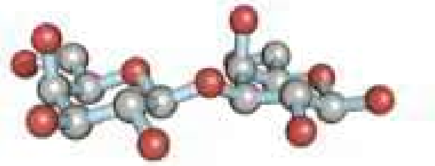
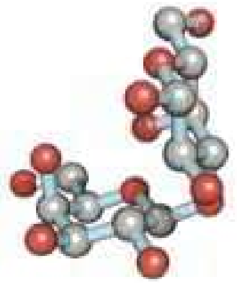


FIGURE 7-15 Chitin. (a) A short segment of chitin, a homopolymer of *N*-acetyl-D-glucosamine units in (β1→4) linkage. (b) A spotted June beetle (*Pelidnota punctata*), showing its surface armor (exoskeleton) of chitin. [(b) Paul Whitten/Science Source.]



Low-energy conformation is extended and maximizes H-bonding.



High-energy conformation is sterically hindered.

FIGURE 7-16 Different energetic conformations of a disaccharide. The torsion angles ϕ (phi) and ψ (psi), which define the spatial relationship between adjacent rings, can in principle have any value from 0° to 360°. In fact, some torsion angles give conformations that maximize hydrogen bonding and minimize energy, whereas others give conformations that are sterically hindered, as we can see in two conformers of the disaccharide Gal(β1→3)Gal at opposite energetic extremes.

P5 The most stable three-dimensional structure for the (α1→4)-linked chains of starch and glycogen is a tightly coiled helix (**Fig. 7-17**), stabilized by interchain hydrogen bonds. The average plane of each residue along the amylose chain forms a 60° angle with the average plane of the preceding residue, so the helical structure has six residues per turn. For amylose, the core of the helix is of precisely the right dimensions to accommodate iodine as complex ions (I_3^- and I_5^-). This interaction gives an intensely blue product, making it a common qualitative test for amylose.

P5 For cellulose, the most stable conformation is that in which each chair is turned 180° relative to its neighbors, yielding a straight, extended chain. All —OH groups are available for hydrogen bonding with neighboring chains. With several chains lying side by side, a stabilizing network of interchain and intrachain hydrogen bonds produces straight, stable supramolecular fibers of great tensile strength (**Fig. 7-18**). This property of cellulose has made it useful to civilizations for millennia. Many manufactured products, including papyrus, paper, cardboard, rayon, insulating tiles, and a variety of other useful materials, are derived from cellulose. The water content of these materials is low because extensive interchain hydrogen bonding between cellulose molecules satisfies their capacity for hydrogen-bond formation.

Peptidoglycan Reinforces the Bacterial Cell Wall

The rigid component of bacterial cell walls (peptidoglycan) is a heteropolymer of alternating (β1→4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues (see Fig. 6-32). The linear polymers lie side by side in the cell wall, cross-linked by short peptides, the exact structure of which depends on the bacterial species. The peptide cross-links weld the polysaccharide chains into a strong sheath (peptidoglycan) that envelops the entire cell and prevents cellular swelling and lysis due to the osmotic entry of water.

The enzyme lysozyme kills bacteria by hydrolyzing the (β1→4) glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid. The enzyme is found in human tears, where it is presumably a defense against bacterial infections of the eye, and in hen's eggs, where it prevents bacterial infection of the developing embryo. Penicillin and related antibiotics kill bacteria by preventing synthesis of the peptidoglycan cross-links, leaving the cell wall too weak to resist osmotic lysis (p. 211).

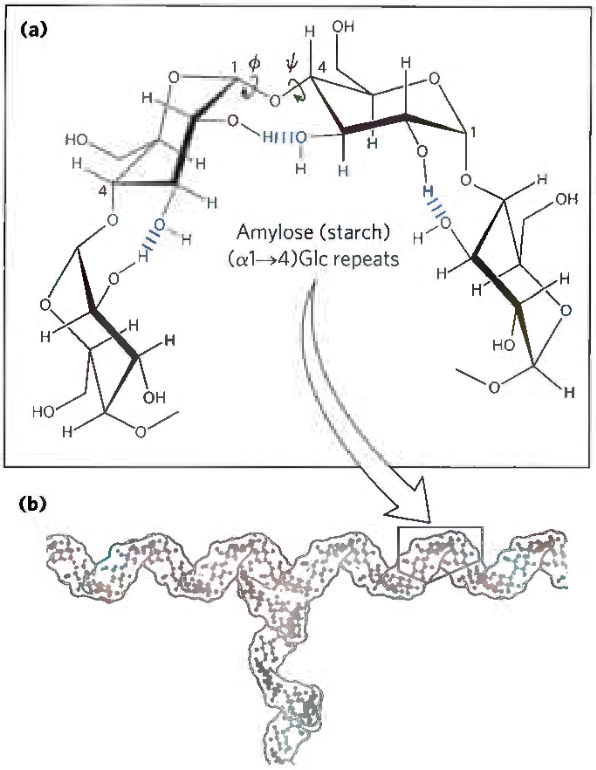


FIGURE 7-17 Helical structure of starch (amylose). (a) Four glucose units of starch (amylose), showing both the free rotation possible around the ψ and ϕ angles of the (α1→4) glycosidic bonds and the hydrogen bonding possible between adjacent rings. In this most stable conformation, with adjacent rigid chairs at 60° to one another, the polysaccharide chain is curved. (b) A model of a segment of amylopectin (branched amylose). The conformation of (α1→4) linkages in amylose, amylopectin, and glycogen causes these polymers to assume tightly coiled helical structures. [(b) Data from www.biotopics.co.uk/jsmol/amylopectin.html.]

Glycosaminoglycans Are Heteropolysaccharides of the Extracellular Matrix

The extracellular space in the tissues of multicellular animals is filled with a gel-like material, the **extracellular matrix (ECM)**, which holds the cells together and

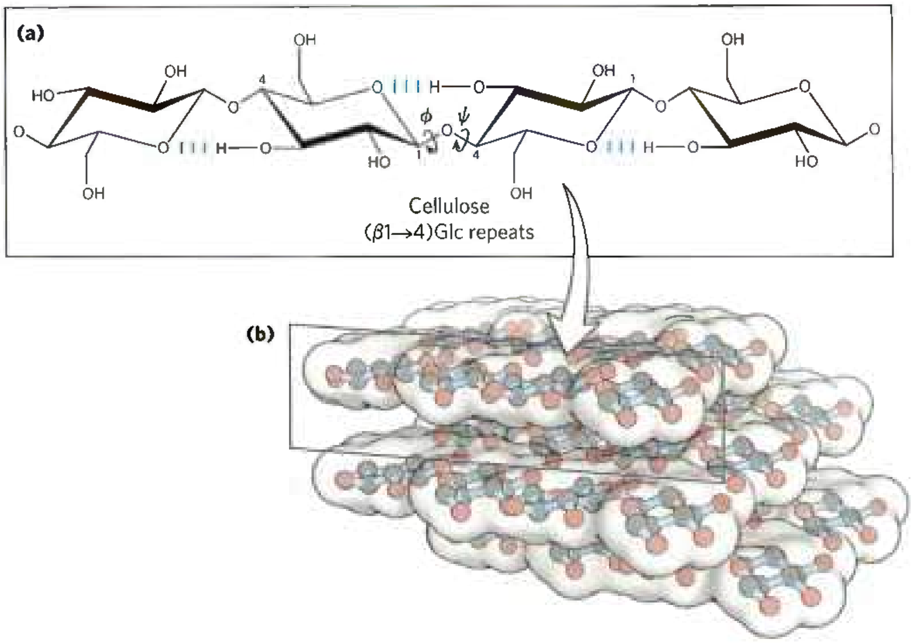


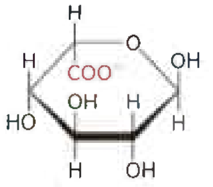
FIGURE 7-18 The linear structure of cellulose chains. (a) Four glucose units of cellulose. In the most stable conformation of cellulose, the (β1→4) glycosidic linkages put the adjacent rigid chairs at 180° relative to each other. (b) A model of straight, unbranched cellulose segments, held together in layers stabilized by interchain hydrogen bonds. [(b) Data from Cornell, B. 2016. Sugar Polymers. Available at: http://ib.bioninja.com.au. (Accessed 11 March 2020).]

provides a porous pathway for the diffusion of nutrients and oxygen to individual cells. The ECM is composed of an interlocking meshwork of heteropolysaccharides (also called ground substance) and fibrous proteins such as fibrillar collagens, elastins, and fibronectins. The basement membrane is a specialized ECM that underlies epithelial cells; it consists of specialized collagens, laminins, and heteropolysaccharides.

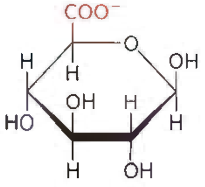
These heteropolysaccharides, the **glycosaminoglycans**, are a family of linear polymers composed of repeating disaccharide units (**Fig. 7-19**). They are unique to animals and bacteria and are not found in plants. One of the two monosaccharides is always either *N*-acetylglucosamine or *N*-acetylgalactosamine; the other is in most cases a uronic acid, usually D-glucuronic or L-iduronic acid. Some glycosaminoglycans contain esterified sulfate groups. **P2** The combination of sulfate groups and the carboxylate groups of the uronic acid residues gives glycosaminoglycans a very high density of negative charge. To minimize the repulsive forces among neighboring charged groups, these molecules assume an extended conformation in solution, forming a rodlike helix in which the negatively charged carboxylate groups occur on alternate sides of the helix (as shown for heparin in Fig. 7-19). The extended rod form also provides maximum separation between the negatively charged sulfate groups. The specific patterns of sulfated and nonsulfated sugar residues in glycosaminoglycans allow specific recognition by a variety of protein ligands that bind electrostatically to these molecules. The sulfated glycosaminoglycans are attached to extracellular proteins to form proteoglycans (Section 7.3).

The glycosaminoglycan **hyaluronan** (hyaluronic acid) contains alternating residues of D-glucuronic acid and *N*-acetylglucosamine (Fig. 7-19). With up to 50,000 repeats of the basic disaccharide unit, hyaluronan has a molecular weight of several million; it forms clear, highly viscous, noncompressible solutions that serve as lubricants in the synovial fluid of joints and give the vitreous humor of the vertebrate eye its jellylike consistency (the Greek *hyalos* means "glass"; hyaluronan can have a glassy or translucent appearance). Hyaluronan is also a component of the ECM of cartilage and tendons, to which it contributes tensile strength and elasticity as a result of its strong noncovalent interactions with other components of the matrix. Hyaluronidase, an enzyme secreted by some pathogenic bacteria, can hydrolyze the glycosidic linkages of hyaluronan, rendering tissues more susceptible to bacterial invasion. In many animal species, a similar enzyme in sperm hydrolyzes the outer glycosaminoglycan coat around an ovum, allowing sperm penetration.

Other glycosaminoglycans differ from hyaluronan in three respects: they are generally much shorter polymers, they are covalently linked to specific proteins (proteoglycans), and one or both monomeric units differ from those of hyaluronan. **Chondroitin sulfate** (Greek *chondros*, "cartilage") contributes to the tensile strength of cartilage, tendons, ligaments, heart valves, and the walls of the aorta. **Dermatan sulfate** (Greek *derma*, "skin") contributes to the pliability of skin and is also present in blood vessels and heart valves. In this polymer, many of the glucuronate residues present in chondroitin sulfate are replaced by their C-5 epimer, L-iduronate (IdoA).



α-L-Iduronate
(IdoA)



β-D-Glucuronate
(GlcA)

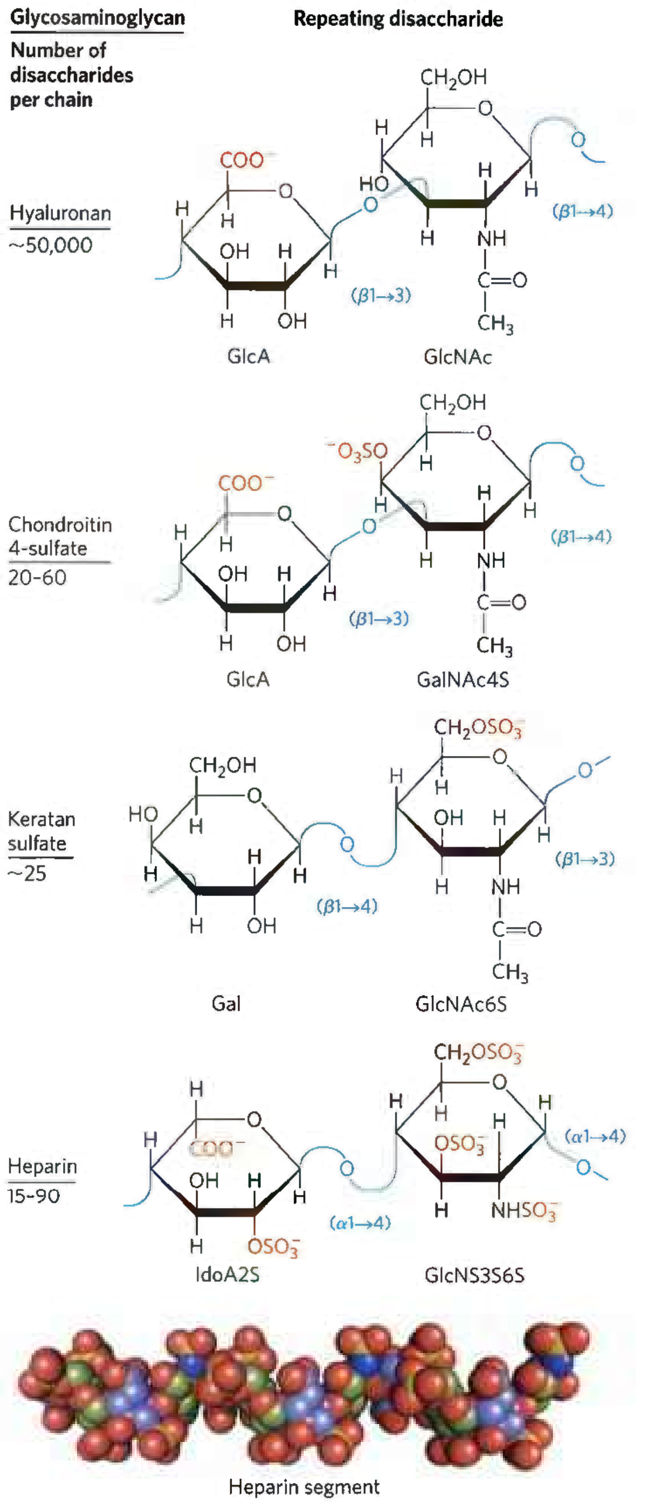


FIGURE 7-19 Repeating units of some common glycosaminoglycans of extracellular matrix. The molecules are copolymers of alternating uronic acid and amino sugar residues (keratan sulfate is the exception), with sulfate esters in any of several positions, except in hyaluronan. The ionized carboxylate and sulfate groups give these polymers their characteristic high negative charge. Therapeutic heparin contains primarily iduronic acid (IdoA) and a smaller proportion of glucuronic acid (not shown) and is generally highly sulfated and heterogeneous in length. The space-filling model shows a heparin segment as its structure in solution, as determined by NMR spectroscopy. Carbons in iduronic acid sulfate are colored blue; those in glucosamine sulfate are green. Oxygen and sulfur are shown in red and yellow, respectively. Hydrogen atoms are not shown (for clarity). [Data for molecular model from PDB ID 1HPN, B. Mulloy et al., *Biochem. J.* 293:849, 1993.]

Keratan sulfates (Greek *keras*, "horn") have no uronic acid, and their sulfate content is variable. They are present in cornea, cartilage, bone, and a variety of horny structures formed from dead cells: horn, hair, hoofs, nails, and claws. **Heparan sulfate** (Greek *hēpar*, "liver"; it was originally isolated from dog liver) contains variable, nonrandom arrangements of sulfated and nonsulfated sugars. The exact sequence of sulfated residues gives the molecule the ability to interact specifically with a large number of proteins, including growth factors and ECM components, as well as various enzymes and factors present in plasma. Heparin is a highly sulfated, intracellular form of heparan sulfate produced primarily by mast cells (a type of leukocyte, or immune cell). Its physiological role is not yet clear, but purified heparin is used as a therapeutic agent to inhibit coagulation of blood through its capacity to bind the protease inhibitor antithrombin (see Fig. 7-24).

Table 7-2 summarizes the composition, properties, roles, and occurrence of the polysaccharides described in Section 7.2.

SUMMARY 7.2 *Polysaccharides*

- Homopolysaccharides contain only a single monomeric sugar species; heteropolysaccharides contain two or more kinds of monomers.
- The homopolysaccharides starch and glycogen are storage fuels in plant, animal, and bacterial cells. They consist of D-glucose units with (α1→4) linkages, and both contain some branches.
- The homopolysaccharides cellulose, chitin, and dextran serve structural roles. Cellulose, composed of (β1→4)-linked D-glucose residues, lends strength and rigidity to plant cell walls. Chitin, a polymer of (β1→4)-linked *N*-acetylglucosamine, strengthens the exoskeletons of arthropods.
- Homopolysaccharides assume stable conformations dictated by weak interactions. The chair form of the pyranose ring is essentially rigid, so the conformation of the polymers is determined by rotation about the C—O bonds of the glycosidic linkage. Starch and glycogen form helical structures with intrachain hydrogen bonding; cellulose and chitin form long, straight strands that interact with neighboring strands.
- Bacterial cell walls are strengthened by peptidoglycan in which the repeating disaccharide is GlcNAc(β1→4)Mur2Ac.
- Glycosaminoglycans are extracellular heteropolysaccharides in which one of the two monosaccharide units is a uronic acid (keratan sulfate is an exception) and the other is an *N*-acetylated amino sugar. The high density of negative charge on these molecules forces them to assume extended conformations. These polymers (hyaluronan, chondroitin sulfate, dermatan sulfate, and

TABLE 7-2 Structures and Roles of Some Polysaccharides

Polymer	Type[a]	Repeating unit[b]	Size (number of monosaccharide units)	Roles/significance
Starch				Energy storage: in plants
Amylose	Homo-	$(\alpha 1\rightarrow 4)$Glc, linear	50–5,000	
Amylopectin	Homo-	$(\alpha 1\rightarrow 4)$Glc, with $(\alpha 1\rightarrow 6)$Glc branches every 24–30 residues	Up to 10^6	
Glycogen	Homo-	$(\alpha 1\rightarrow 4)$Glc, with $(\alpha 1\rightarrow 6)$Glc branches every 8–12 residues	Up to 50,000	Energy storage: in bacteria and animal cells
Cellulose	Homo-	$(\beta 1\rightarrow 4)$Glc	Up to 15,000	Structural: in plants, gives rigidity and strength to cell walls
Chitin	Homo-	$(\beta 1\rightarrow 4)$GlcNAc	Very large	Structural: in insects, spiders, crustaceans, gives rigidity and strength to exoskeletons
Dextran	Homo-	$(\alpha 1\rightarrow 6)$Glc, with $(\alpha 1\rightarrow 3)$ branches	Wide range	Structural: in bacteria, extracellular adhesive
Peptidoglycan	Hetero-; peptides attached	4)Mur2Ac$(\beta 1\rightarrow 4)$ GlcNAc$(\beta 1$	Very large	Structural: in bacteria, gives rigidity and strength to cell envelope
Hyaluronan (a glycosaminoglycan)	Hetero-; acidic	4)GlcA$(\beta 1\rightarrow 3)$ GlcNAc$(\beta 1$	Up to 100,000	Structural: in vertebrates, extracellular matrix of skin and connective tissue; viscosity and lubrication in joints

[a] Each polymer is classified as a homopolysaccharide (homo-) or heteropolysaccharide (hetero-).

[b] The abbreviated names for the peptidoglycan, agarose, and hyaluronan repeating units indicate that the polymer contains repeats of this disaccharide unit. For example, in peptidoglycan, the GlcNAc of one disaccharide unit is $(\beta 1\rightarrow 4)$-linked to the first residue of the next disaccharide unit.

keratan sulfate) provide viscosity, adhesiveness, and tensile strength to the extracellular matrix.

7.3 Glycoconjugates: Proteoglycans, Glycoproteins, and Glycolipids

In addition to their important roles as fuel stores (starch, glycogen, dextran) and as structural materials (cellulose, chitin, peptidoglycans), polysaccharides and oligosaccharides are information carriers. Some provide communication between cells and their extracellular surroundings; others label proteins for transport to and localization in specific organelles, or for destruction when the protein is malformed or superfluous; and others serve as recognition sites for extracellular signal molecules (growth factors, for example) or extracellular parasites (bacteria or viruses). On almost every eukaryotic cell, specific oligosaccharide chains attached to components of the plasma membrane form a carbohydrate layer (the glycocalyx), several nanometers thick, that serves as an information-rich surface that the cell shows to its surroundings. These oligosaccharides are central players in cell-cell recognition and adhesion, cell migration during development, blood clotting, the immune response, wound healing, and other cellular processes. In most of these cases, the informational carbohydrate is covalently joined to a protein or a lipid to form a **glycoconjugate**, which is the biologically active molecule (**Fig. 7-20**).

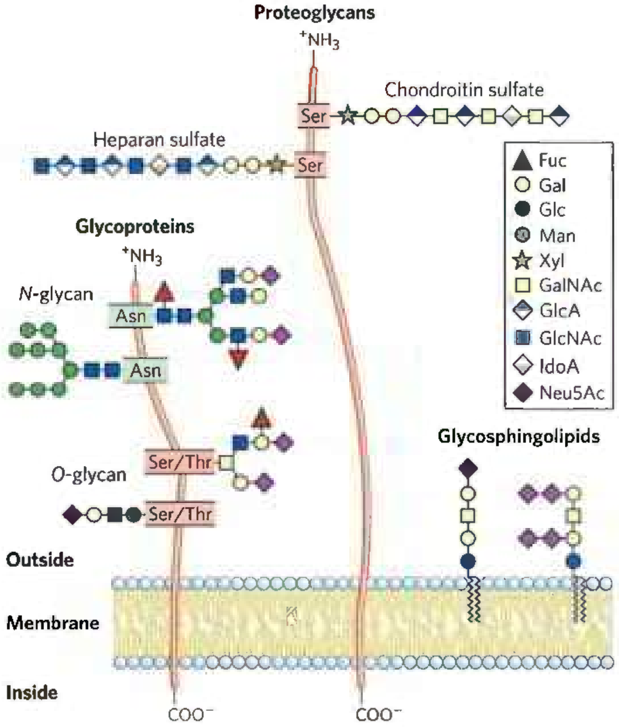


FIGURE 7-20 Glycoconjugates. The structures of some typical proteoglycans, glycoproteins, and glycosphingolipids described in the text.

Proteoglycans are macromolecules of the cell surface or ECM in which one or more sulfated glycosaminoglycan chains are joined covalently to a membrane protein or a secreted protein. The glycosaminoglycan chain can bind to extracellular proteins through electrostatic interactions between the protein and the negatively charged sugar moieties on the proteoglycan. Proteoglycans are major components of all extracellular matrices.

Glycoproteins have one or several oligosaccharides of varying complexity joined covalently to a protein. They are usually found on the outer face of the plasma membrane (as part of the glycocalyx), in the ECM, and in the blood. Inside cells, they are found in specific organelles such as Golgi complexes (where the oligosaccharide moieties are added to the proteins), secretory granules, and lysosomes. **P6** The oligosaccharide portions of glycoproteins are very heterogeneous and, like glycosaminoglycans, are rich in information, forming highly specific sites for recognition and high-affinity binding by carbohydrate-binding proteins called lectins. Some cytosolic and nuclear proteins can be glycosylated as well.

Glycolipids are plasma membrane components in which the hydrophilic head groups are oligosaccharides. **Glycosphingolipids** are a class of glycolipids with a specific backbone structure that will be considered in more detail in Chapter 10. They have complex oligosaccharide components that act as specific sites for recognition by lectins, as they do in glycoproteins. Neurons are rich in glycosphingolipids, which act in nerve conduction and myelin formation. Glycosphingolipids also play a role in signal transduction in cells.

Proteoglycans Are Glycosaminoglycan-Containing Macromolecules of the Cell Surface and Extracellular Matrix

Mammalian cells can produce dozens of types of proteoglycans. Many are secreted into the ECM, where they act as tissue organizers and influence cellular activities, such as growth factor activation and adhesion. The basic proteoglycan unit consists of a "core protein" with covalently attached glycosaminoglycan(s). The point of attachment is a Ser residue, to which the glycosaminoglycan is joined through a tetrasaccharide linker (**Fig. 7-21**).

Some proteoglycans are integral membrane proteins (see Fig. 11-9). For example, the basal lamina, a specialized layer of the ECM that separates two organized groups of cells, contains a family of core proteins (M_r 20,000 to 40,000), each with several covalently attached heparan sulfate chains. There are two major families of membrane heparan sulfate proteoglycans. **Syndecans** have a single transmembrane domain and an extracellular domain bearing three to five chains of heparan sulfate and, in some cases, chondroitin sulfate (**Fig. 7-22a**). **Glypicans** are attached to the membrane by a GPI anchor, a *g*lycosylated derivative of the membrane lipid *p*hosphatidyl*i*nositol (see Fig. 11-16). Both syndecans and glypicans can be shed into the extracellular space. A protease in the ECM that cuts proteins close to the membrane surface releases syndecan ectodomains (domains outside the plasma membrane), and a phospholipase that breaks the connection to the GPI anchor releases glypicans. These mechanisms provide a way for a cell to change its surface features quickly. Shedding is highly regulated and is activated in proliferating cells, such as cancer cells. Proteoglycan shedding

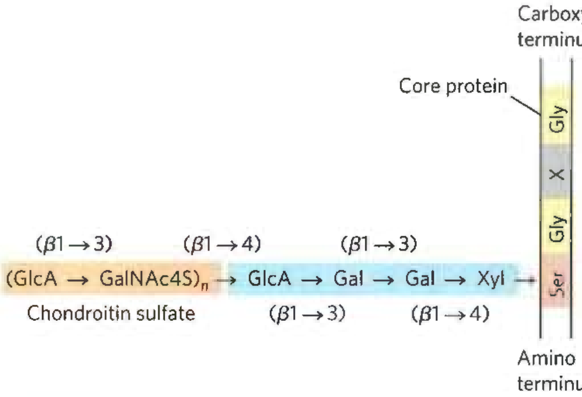


FIGURE 7-21 Proteoglycan structure, showing the tetrasaccharide bridge. A typical tetrasaccharide linker (blue) connects a glycosaminoglycan — in this case, chondroitin sulfate (orange) — to a Ser residue in the core protein. The xylose residue at the reducing end of the linker is joined by its anomeric carbon to the hydroxyl of the Ser residue.

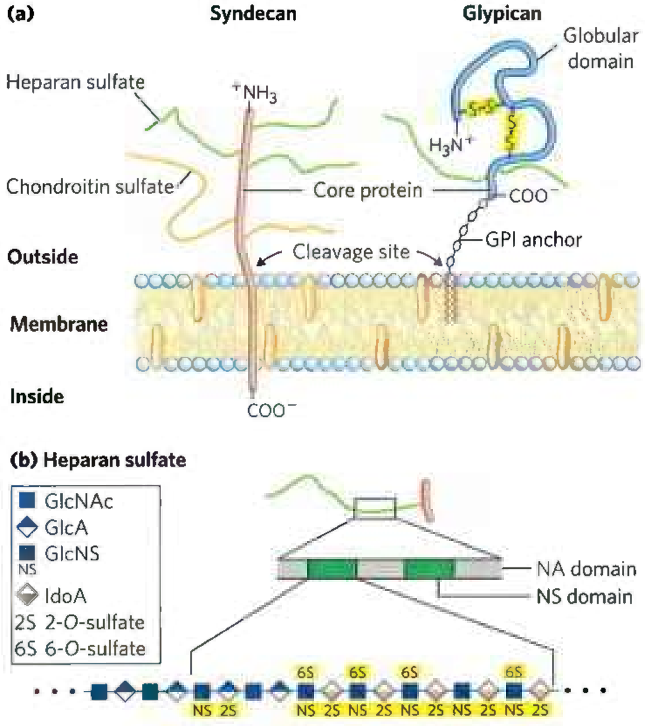


FIGURE 7-22 Two families of membrane proteoglycans. (a) Syndecans and glypicans can be shed from the membrane by enzymatic cleavage near the outer membrane surface. (b) Along a heparan sulfate chain, regions rich in sulfated sugars, the NS domains (green), alternate with regions with chiefly unmodified residues of GlcNAc and GlcA, the NA domains (gray). One of the NS domains is shown in more detail, revealing a high density of modified residues: GlcNS (*N*-sulfoglucosamine) and both GlcA and IdoA, with a sulfate ester at C-2. [(a) Information from U. Häcker et al., *Nature Rev. Mol. Cell Biol.* 6:530, 2005. (b) Information from J. Turnbull et al., *Trends Cell Biol.* 11:75, 2001.]

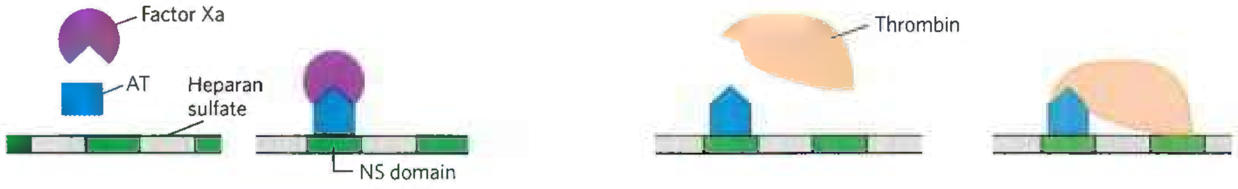
is involved in cell-cell recognition and adhesion, and in the proliferation and differentiation of cells. Numerous chondroitin sulfate and dermatan sulfate proteoglycans also exist, some as membrane-bound entities, others as secreted products in the ECM.

The glycosaminoglycan chains in proteoglycans can bind to a variety of extracellular ligands and thereby modulate the ligands' interaction with specific receptors of the cell surface. Detailed studies of heparan sulfate demonstrate a domain structure that is not random; some domains (typically three to eight disaccharide units long) differ from neighboring domains in sequence and in ability to bind to specific proteins. Highly sulfated domains (called NS domains) alternate with domains having unmodified GlcNAc and GlcA residues (*N*-acetylated, or NA, domains) (Fig. 7-22b). The exact pattern of sulfation in the NS domain depends on the particular proteoglycan; given the number of possible modifications of the GlcNAc–IdoA (iduronic acid) dimer, at least 32 different disaccharide units are possible. Furthermore, the same core protein can display different heparan sulfate structures when synthesized in different cell types.

Heparan sulfate molecules with precisely organized NS domains bind specifically to extracellular proteins and signaling molecules to alter their activities. The change in activity may result from a conformational change in the protein that is induced by the binding (**Fig. 7-23a**), or it may be due to the ability of adjacent domains of heparan sulfate to bind to two different proteins, bringing them into close proximity and enhancing protein-protein interactions (Fig. 7-23b). A third general mechanism of action is the binding of extracellular signal molecules (growth factors, for example) to heparan sulfate, which increases their local concentrations and enhances their interaction with growth factor receptors on the cell surface; in this case, the heparan sulfate acts as a coreceptor (Fig. 7-23c). For example, fibroblast growth factor (FGF), an extracellular protein signal that stimulates cell division, first binds to heparan sulfate moieties of syndecan molecules in the target cell's plasma membrane. Syndecan presents FGF to the FGF plasma membrane receptor, and only then can FGF interact productively with its receptor to trigger cell division. Finally, in another type of mechanism, the NS domains interact — electrostatically and otherwise — with a variety of soluble molecules outside the cell, maintaining high local concentrations at the cell surface (Fig. 7-23d).

The protease thrombin, essential to blood coagulation (see Fig. 6-44), is inhibited by another blood protein, antithrombin, which prevents premature blood clotting. Antithrombin does not bind to or inhibit thrombin in the absence of heparan sulfate. In the presence of heparan sulfate or heparin, the binding affinity of thrombin for antithrombin increases 2,000-fold, and thrombin is strongly inhibited. When thrombin and antithrombin are crystallized in the presence of a short (16 residue) segment of

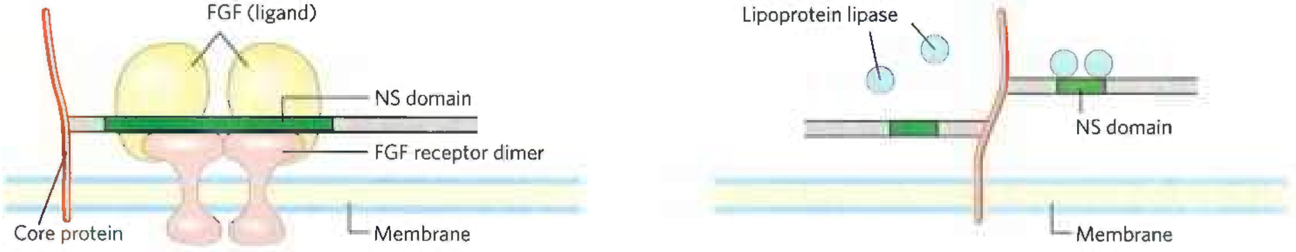
(a) Conformational activation **(b) Enhanced protein-protein interaction**



A conformational change induced in the protein antithrombin (AT) on binding a specific pentasaccharide NS domain allows its interaction with blood clotting factor Xa, preventing clotting.

Binding of AT and thrombin to two adjacent NS domains brings the two proteins into close proximity, favoring their interaction, which inhibits blood clotting.

(c) Coreceptor for extracellular ligands **(d) Cell surface localization/concentration**



NS domains interact with both the fibroblast growth factor (FGF) and its receptor, bringing the oligomeric complex together and increasing the effectiveness of a low concentration of FGF.

The high density of negative charges in heparan sulfate attracts positively charged lipoprotein lipase molecules and holds them by electrostatic and sequence-specific interactions with NS domains.

FIGURE 7-23 Four types of protein interactions with NS domains of heparan sulfate. [Information from J. Turnbull et al., *Trends Cell Biol.* 11:75, 2001.]

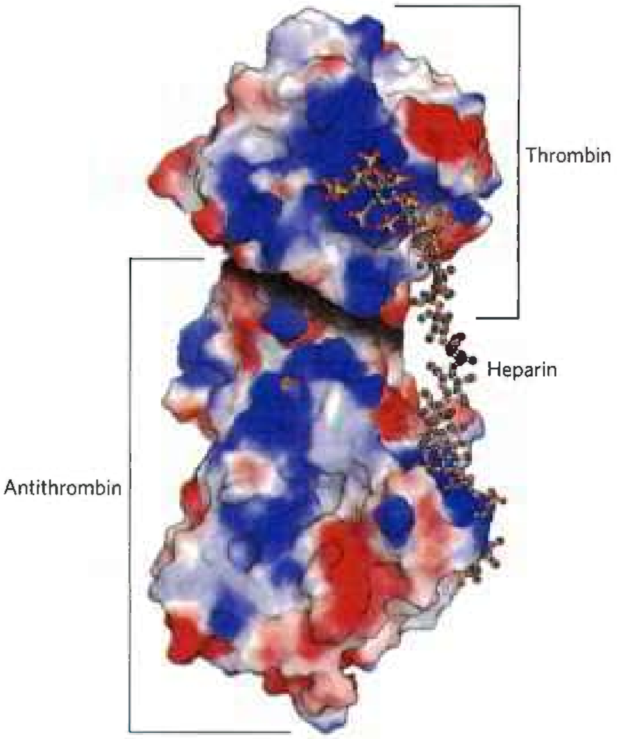


FIGURE 7-24 Molecular basis for heparan sulfate enhancement of the binding of thrombin to antithrombin. In this crystal structure of thrombin, antithrombin, and a 16 residue heparan sulfate–like polymer, all crystallized together, the binding sites for heparan sulfate in both proteins are rich in Arg and Lys residues. These positively charged regions, shown in blue in this electrostatic representation of the proteins, allow strong electrostatic interaction with multiple negatively charged sulfates and carboxylates of the heparan sulfate. Consequently, the affinity of antithrombin for thrombin is three orders of magnitude greater in the presence of heparan sulfate than in its absence. [Data from PDB ID 1TB6, W. Li et al., *Nat. Struct. Mol. Biol.* 11:857, 2004.]

heparan sulfate, the negatively charged heparan sulfate mimic is seen to bridge positively charged regions of the two proteins, causing an allosteric change that inhibits thrombin's protease activity (**Fig. 7-24**). The binding sites for heparan sulfate and heparin in both proteins are rich in Arg and Lys residues; the amino acids' positive charges interact electrostatically with the sulfates of the glycosaminoglycans. Antithrombin also inhibits two other blood coagulation proteins (factors IXa and Xa) in a heparan sulfate–dependent process.

The importance of correctly synthesizing sulfated domains in heparan sulfate is demonstrated in mutant ("knockout") mice lacking the enzyme that sulfates the C-2 hydroxyl of iduronate (IdoA). These animals are born without kidneys and with severe developmental abnormalities of the skeleton and eyes. Other studies demonstrate that membrane proteoglycans are important in the liver for clearing lipoproteins from the blood. Finally, there is growing evidence that proteoglycans containing heparan sulfate and chondroitin sulfate provide directional cues for axon outgrowth, influencing the path taken by developing axons in the nervous system.

The functional importance of proteoglycans and the glycosaminoglycans associated with them can also be seen in the effects of mutations that block the synthesis or degradation of these polymers in humans (**Box 7-3**).

Some proteoglycans can form **proteoglycan aggregates**, enormous supramolecular assemblies of many core proteins all bound to a single molecule of hyaluronan (also called hyaluronate). Aggrecan core protein (M_r ~250,000) has multiple chains of chondroitin sulfate and keratan sulfate, joined to Ser residues in the core protein through trisaccharide linkers, to give an aggrecan monomer of M_r ~2×10^6. When a hundred or more of these "decorated" core proteins bind a single, extended molecule of hyaluronan (**Fig. 7-25**), the resulting proteoglycan aggregate (M_r >2×10^8) and its associated water of hydration occupy a volume about equal to that of a bacterial cell! Aggrecan interacts strongly with collagen in the ECM of cartilage, contributing to the development, tensile strength, and resilience of this connective tissue.

Interwoven with these enormous extracellular proteoglycans are fibrous matrix proteins such as collagen, elastin, and fibronectin, forming a cross-linked meshwork that gives the whole ECM strength and resilience. Some of these proteins are multiadhesive, a single protein having binding sites for several different matrix molecules. **Fibronectin**, for example, has separate domains that bind fibrin, heparan sulfate, and collagen. Fibronectin and a number of other proteins in the extracellular matrix contain the conserved RGD sequence (Arg–Gly–Asp), through which they bind to a family of proteins called **integrins**. Integrins mediate signaling between the cell interior and the network of molecules in the ECM. The overall picture of cell-matrix

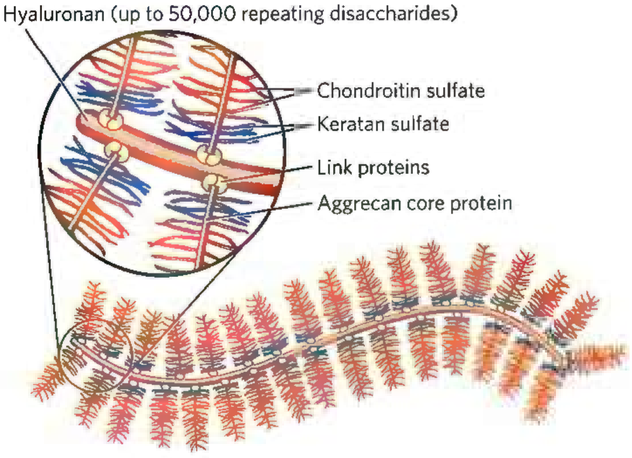


FIGURE 7-25 Proteoglycan aggregate of the extracellular matrix. Schematic drawing of a proteoglycan with many aggrecan molecules. One very long molecule of hyaluronan is associated noncovalently with about 100 molecules of the core protein aggrecan. Each aggrecan molecule contains many covalently bound chondroitin sulfate and keratan sulfate chains. Link proteins at the junction between each core protein and the hyaluronan backbone mediate the core protein–hyaluronan interaction.

BOX 7-3 MEDICINE

Defects in the Synthesis or Degradation of Sulfated Glycosaminoglycans Can Lead to Serious Human Disease

Glycosaminoglycan synthesis requires enzymes that activate monomeric sugars, transport them across membranes, condense the activated sugars into polysaccharides, and add sulfates. Mutations in any of these enzymes in humans can lead to structural defects in the glycosaminoglycan (or in the proteoglycans formed from them). The result can be any of a wide variety of defects in cell signaling, cell proliferation, tissue morphogenesis, or interactions with growth factors (Fig. 1). For example, failure to extend the disaccharide unit GlcNAc-GlcA leads to a bone abnormality in which multiple, large bone spurs develop (Fig. 2).

When the defect occurs in degradative enzymes, the accumulation of incompletely degraded glycosaminoglycans can produce diseases ranging from moderate, as in Scheie syndrome, with joint stiffening but normal intelligence and life span, to severe, as in Hurler syndrome, with enlarged internal organs, heart disease, dwarfism, intellectual disability, and early death. Glycosaminoglycans were formerly called mucopolysaccharides, and diseases caused by genetic defects in their breakdown are often still called mucopolysaccharidoses.

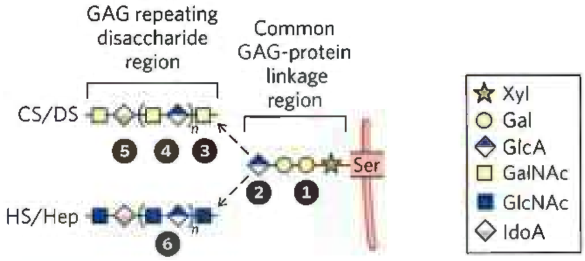

GAG repeating disaccharide region
Common GAG-protein linkage region
CS/DS
HS/Hep
n
n
Ser
⑤ ④ ③
② ①
⑥
Xyl
Gal
GlcA
GalNAc
GlcNAc
IdoA

FIGURE 1 A segment of proteoglycan showing the normal structure of the glycosaminoglycans (GAGs) chondroitin sulfate or dermatan sulfate (CS/DS) (top) and heparan sulfate or heparin (HS/Hep) (bottom), attached through the linkage region to a Ser residue in the core protein. When a specific biosynthetic enzyme is absent because of a mutation, the numbered elements cannot be added to the growing oligosaccharide, and the product is truncated. The dysfunctional GAGs result in several types of human disease, depending on the site of truncation: ① progeroid-type Ehlers-Danlos syndrome—with hyperextensible joints, fragile skin, and premature aging; ② short stature or frequent joint dislocations; ③ neuropathy (nerve damage); ④ skeletal defects; ⑤ bipolar disorder or diaphragmatic hernia; ⑥ and bone deformations in the form of large bone spurs.

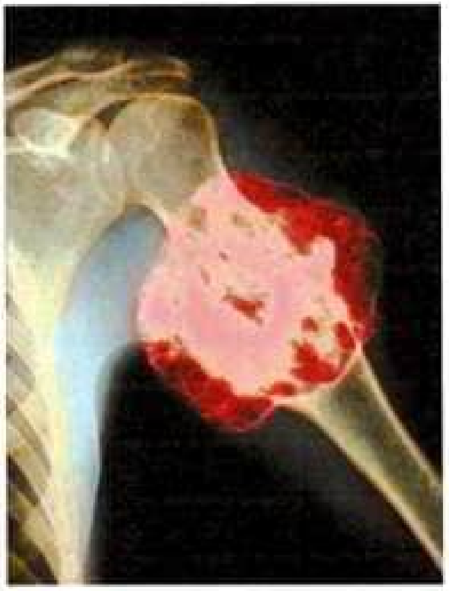


FIGURE 2 Bone deformation characteristic of multiple hereditary exostoses, a disease resulting from a genetic inability to add the GlcNAc-GlcA disaccharide to the growing heparan sulfate or heparin chain (see ⑥ in Fig. 1). The extra bone growth is artificially colored red in this x-ray of the humerus (upper arm bone). [CNRI/Science Photo Library/Science Source.]

interactions that emerges (**Fig. 7-26**) shows an array of interactions between cellular and extracellular molecules. These interactions serve not merely to anchor cells to the ECM, providing the strength and elasticity of skin and joints. **P7** They also provide paths that direct the migration of cells in developing tissue and serve to convey information in both directions across the plasma membrane.

Glycoproteins Have Covalently Attached Oligosaccharides

Glycoproteins are carbohydrate-protein conjugates in which the glycans are branched and are much smaller and more structurally diverse than the huge glycosaminoglycans of proteoglycans. The carbohydrate is attached at its anomeric carbon through a glycosidic link to the —OH of a Ser or Thr residue (*O*-linked), or through an *N*-glycosyl link to the amide nitrogen of an Asn residue (*N*-linked) (**Fig. 7-27**). ***N*-glycosyl bonds** join the anomeric carbon of a sugar to a nitrogen atom in glycoproteins and nucleotides (see Fig. 8-1). Some glycoproteins have a single oligosaccharide chain, but many have more than one; the carbohydrate may constitute from 1% to 70% of the glycoprotein by mass. About half of all proteins of mammals are glycosylated.

As we shall see in Chapter 11, the external surface of the plasma membrane has many membrane glycoproteins with arrays of covalently attached oligosaccharides of varying complexity. **Mucins** are secreted or membrane glycoproteins that can contain large numbers of *O*-linked oligosaccharide chains. Mucins are present in most secretions; they are what gives mucus its characteristic slipperiness. Many of the proteins secreted by eukaryotic

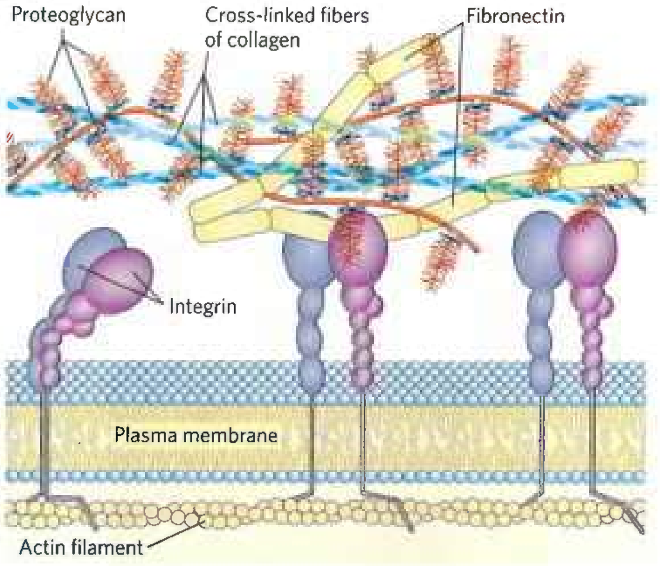


FIGURE 7-26 Interactions between cells and the extracellular matrix. The association between cells and the proteoglycan of the extracellular matrix is mediated by a membrane protein (integrin) and by an extracellular protein (fibronectin, with its RGD motif) with binding sites for both integrin and the proteoglycan. Note the close association of collagen fibers with the fibronectin and proteoglycan.

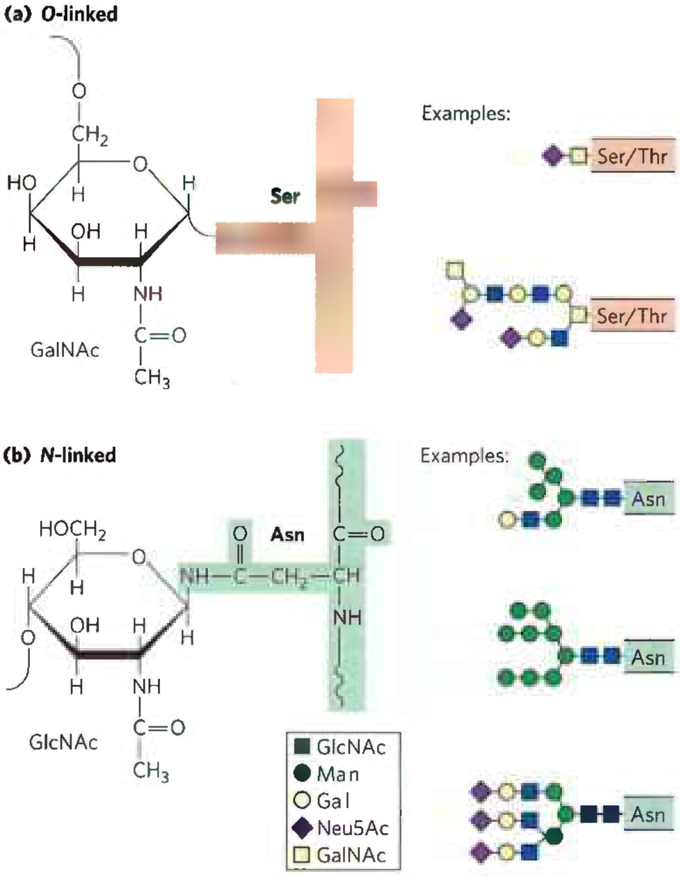


FIGURE 7-27 Oligosaccharide linkages in glycoproteins. (a) *O*-linked oligosaccharides have a glycosidic bond to the hydroxyl group of Ser or Thr residues (light red), illustrated here with GalNAc as the sugar at the reducing end of the oligosaccharide. One simple chain and one complex chain are shown. (b) *N*-linked oligosaccharides have an *N*-glycosyl bond to the amide nitrogen of an Asn residue (green), illustrated here with GlcNAc as the terminal sugar. Three common types of oligosaccharide chains that are *N*-linked in glycoproteins are shown. A complete description of oligosaccharide structure requires specification of the position and stereochemistry (α or β) of each glycosidic linkage.

cells are glycoproteins, including most of the proteins of blood. For example, immunoglobulins (antibodies) and certain hormones, such as follicle-stimulating hormone, luteinizing hormone, and thyroid-stimulating hormone, are glycoproteins. Some of the proteins secreted by the pancreas are glycosylated, as are most of the proteins contained in lysosomes. Many milk proteins, including the major whey protein α-lactalbumin, are glycosylated.

Glycomics is the systematic characterization of all carbohydrate components of a given cell or tissue, including those attached to proteins and to lipids. For glycoproteins, this also means determining which proteins are glycosylated and where in the amino acid sequence each oligosaccharide is attached. This is a challenging undertaking, but worthwhile because of the potential insights it offers into normal patterns of glycosylation and the ways in which they are altered during development or in genetic diseases or cancer. Current methods of characterizing the entire carbohydrate complement of cells depend heavily on sophisticated application of nuclear magnetic resonance and mass spectrometry.

The structures of a large number of *O*- and *N*-linked oligosaccharides from a variety of glycoproteins are known; Figures 7-20 and 7-27 show a few typical examples. In Chapter 27, we consider the mechanisms by which specific proteins acquire specific oligosaccharide moieties.

What are the biological advantages of adding oligosaccharides to proteins? The very hydrophilic carbohydrate clusters alter the polarity and solubility of the proteins with which they are conjugated. **P7** Oligosaccharide chains that are attached to newly synthesized proteins in the endoplasmic reticulum (ER) and elaborated in the Golgi complex serve as destination labels and also act in protein quality control, targeting misfolded proteins for degradation (see Figs. 27-38, 27-39). When numerous negatively charged oligosaccharide chains are clustered in a single region of a protein, the charge repulsion among them favors the formation of an extended, rodlike structure in that region. The bulkiness and negative charge of oligosaccharide chains also protect some proteins from attack by proteolytic enzymes.

Beyond these global physical effects on protein structure, there are also more specific biological effects of oligosaccharide chains in glycoproteins (Section 7.4). The importance of normal protein glycosylation is clear from at least 40 different genetic disorders of glycosylation having been found in humans, all causing severely defective physical or mental development; some of these disorders are fatal.

Glycolipids and Lipopolysaccharides Are Membrane Components

Glycoproteins are not the only cellular components that bear complex oligosaccharide chains; some lipids, too, have covalently bound oligosaccharides. **Gangliosides** are membrane lipids of eukaryotic cells in which the polar head group, the part of the lipid that forms the outer surface of the membrane, is a complex oligosaccharide containing a sialic acid (Fig. 7-9) and other monosaccharide residues. Some of the oligosaccharide moieties of gangliosides, such as those that determine human blood groups (see Fig. 10-13), are identical with those found in certain glycoproteins, which therefore also contribute to blood group type. Like the oligosaccharide moieties of glycoproteins, those of membrane lipids are generally, perhaps always, found on the outer face of the plasma membrane.

Lipopolysaccharides are the dominant surface feature of the outer membrane of gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*. These molecules are prime targets of the antibodies produced by the vertebrate immune system in response to bacterial infection and are therefore important determinants of the serotype of bacterial strains. (Serotypes are strains that are distinguished on the basis of antigenic properties.) The lipopolysaccharides of *S. typhimurium* contain six fatty acids bound to two glucosamine residues, one of which is the point of attachment for a complex oligosaccharide (**Fig. 7-28**). *E. coli* has similar but unique lipopolysaccharides. The lipid A portion of the lipopolysaccharides of some bacteria is called endotoxin; its toxicity to humans and other animals is responsible for the dangerously lowered blood pressure that occurs in toxic shock syndrome resulting from gram-negative bacterial infections. ■

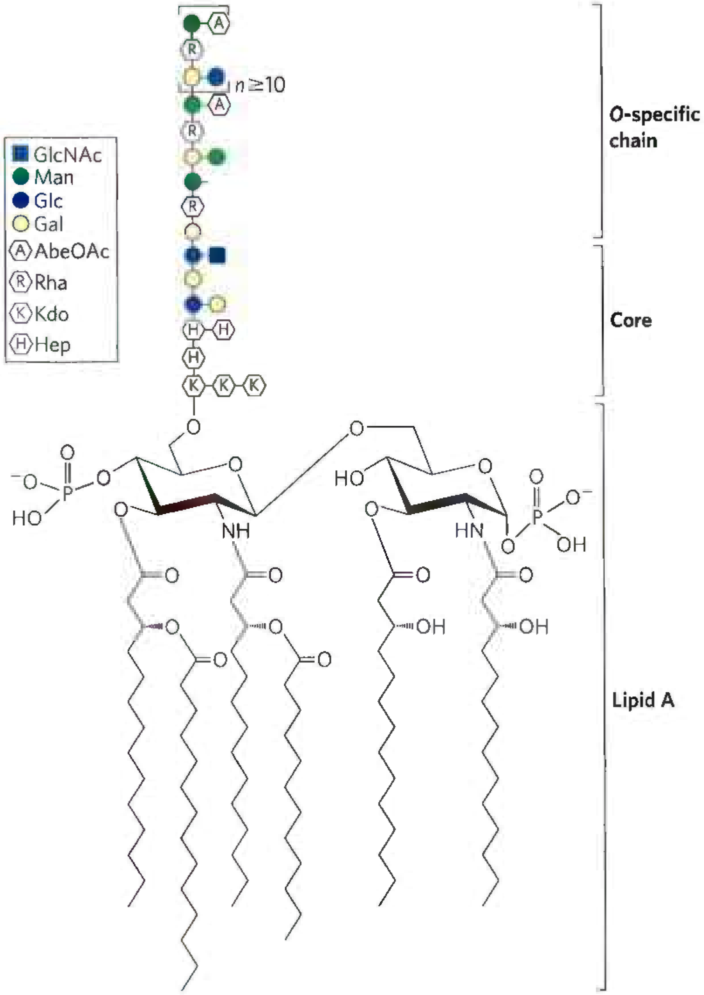


FIGURE 7-28 Bacterial lipopolysaccharides. Schematic diagram of the lipopolysaccharide of the outer membrane of *S. typhimurium*. Kdo is 3-deoxy-D-*manno*-octulosonic acid (previously called ketodeoxyoctonic acid); Hep is L-*glycero*-D-*manno*-heptose; AbeOAc is abequose (a 3,6-dideoxyhexose) acetylated on one of its hydroxyls. Different bacterial species have subtly different lipopolysaccharide structures, but they have in common a lipid A region, composed of six fatty acid residues and two phosphorylated glucosamines, a core oligosaccharide, and an "O-specific" chain, which is the principal determinant of the serotype (immunological reactivity) of the bacterium. The outer membranes of the gram-negative bacteria *S. typhimurium* and *E. coli* contain so many lipopolysaccharide molecules that the cell surface is almost completely covered with O-specific chains.

SUMMARY 7.3 *Glycoconjugates: Proteoglycans, Glycoproteins, and Glycolipids*

- Proteoglycans are huge molecules in which one or more large glycans, called sulfated glycosaminoglycans (heparan sulfate, chondroitin sulfate, dermatan sulfate, or keratan sulfate), are covalently attached to a core protein. Bound to the outside of the plasma membrane through a peptide or lipid, proteoglycans provide points of adhesion, recognition, and information transfer between cells, or between a cell and the extracellular matrix. In the extracellular matrix, enormous proteoglycan aggregates form, bound to a long hyaluronan molecule.
- Glycoproteins contain oligosaccharides covalently linked to Asn, Ser, or Thr residues. The oligosaccharides are typically branched and smaller than glycosaminoglycans. Many cell surface or extracellular proteins are glycoproteins, as are most secreted proteins. The covalently attached oligosaccharides influence the folding and stability of the proteins, provide critical information about the targeting of newly synthesized proteins, and allow specific recognition by other proteins.
- Glycolipids and glycosphingolipids in plants and animals and lipopolysaccharides in bacteria are components of the cell envelope, with covalently attached oligosaccharide chains exposed on the cell's outer surface.

7.4 Carbohydrates as Informational Molecules: The Sugar Code

Glycobiology, the study of the structure and function of glycoconjugates, is one of the most active and exciting areas of biochemistry and cell biology. The challenge is to understand how P6 cells use specific oligosaccharides to encode important information about intracellular targeting of proteins, cell-cell interactions, cell differentiation and tissue development, and extracellular signals. Our discussion uses just a few examples to illustrate the diversity of structure and the range of biological activity of the glycoconjugates. In Chapter 20 we discuss the biosynthesis of polysaccharides, and in Chapter 27, the assembly of oligosaccharide chains on glycoproteins.

Oligosaccharide Structures Are Information-Dense

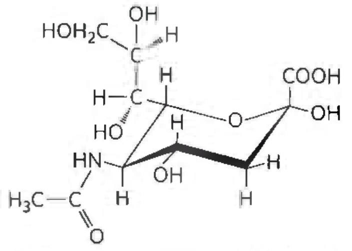
Improved methods for the analysis of oligosaccharide and polysaccharide structure have revealed remarkable complexity and diversity in the oligosaccharides of glycoproteins and glycolipids. Consider the oligosaccharide chains in Figure 7-27, typical of those found in many glycoproteins. The most complex of those shown contains 14 monosaccharide residues of four different kinds, variously linked as $(1\rightarrow2)$, $(1\rightarrow3)$, $(1\rightarrow4)$, $(1\rightarrow6)$, $(2\rightarrow3)$, and $(2\rightarrow6)$, some with the α and some with the β configuration. Branched structures, not found in nucleic acids or proteins, are common in oligosaccharides. With the reasonable assumption that 20 different monosaccharide subunits are available for construction of oligosaccharides, we can calculate that many billions of different hexameric oligosaccharides are possible; this compares with $6.4 \times 10^7\,(20^6)$ different hexapeptides possible for the 20 common amino acids, and 4,096 (4^6) different hexanucleotides for the four nucleotide subunits. If we also allow for variations in oligosaccharides resulting from sulfation of one or more residues, the number of possible oligosaccharides increases by two orders of magnitude. In reality, only a subset of possible combinations is found, given the restrictions imposed by the biosynthetic enzymes and the availability of precursors. Nevertheless, the enormously rich structural information in glycans does not merely rival but far surpasses that of nucleic acids in the density of information contained in a molecule of modest size. Each of the oligosaccharides represented in Figures 7-20 and 7-27 presents a unique, three-dimensional face—a word in the sugar code—readable by the proteins that interact with it.

Lectins Are Proteins That Read the Sugar Code and Mediate Many Biological Processes

P6 **Lectins** bind carbohydrates with high specificity and with moderate to high affinity. These proteins serve in a wide variety of cell-cell recognition, signaling, and adhesion processes and in intracellular targeting of newly synthesized proteins. Plant lectins, abundant in seeds, probably serve as deterrents to insects and other predators. In the laboratory, purified plant lectins are useful reagents for detecting and separating glycans and glycoproteins with different oligosaccharide moieties. Here we discuss just a few examples of the roles of lectins in animal cells.

Some peptide hormones that circulate in the blood have oligosaccharide moieties that strongly influence their circulatory half-life. Luteinizing hormone and thyrotropin (polypeptide hormones produced in the pituitary) have N-linked oligosaccharides that end with the disaccharide GalNAc4S($\beta1\rightarrow4$)GlcNAc, which is recognized by a lectin (receptor) of hepatocytes. (GalNAc4S is N-acetylgalactosamine sulfated on the —OH group at C-4.) Receptor-hormone interaction mediates the uptake and destruction of luteinizing hormone and thyrotropin, reducing their concentration in the blood. Thus the blood levels of these hormones undergo a periodic rise (due to pulsatile secretion by the pituitary) and fall (due to constant destruction by hepatocytes).

Residues of Neu5Ac (a sialic acid) situated at the ends of the oligosaccharide chains of many plasma glycoproteins (Fig. 7-20) protect these proteins from uptake and degradation in the liver. For example, ceruloplasmin, a copper-containing serum glycoprotein, has several oligosaccharide chains ending in Neu5Ac. The mechanism that removes sialic acid residues from serum glycoproteins is unclear. It may be due to the activity of the enzyme neuraminidase (also called sialidase) produced by invading organisms or to a steady, slow release of the residues by extracellular enzymes. The plasma membrane of hepatocytes has lectin molecules (asialoglycoprotein receptors; "asialo-" indicating "without sialic acid") that specifically bind oligosaccharide chains with galactose residues no longer "protected" by a terminal Neu5Ac residue. Receptor-ceruloplasmin interaction triggers endocytosis and destruction of the ceruloplasmin.



N-Acetylneuraminic acid (Neu5Ac)
(a sialic acid)

A similar mechanism is apparently responsible for removing "old" erythrocytes from the mammalian bloodstream. Newly synthesized erythrocytes have several membrane glycoproteins with oligosaccharide chains that end in Neu5Ac. In the laboratory, when the sialic acid residues are removed by withdrawing a sample of blood from experimental animals, treating it with neuraminidase in vitro, and reintroducing it into the circulation, the treated erythrocytes disappear from the bloodstream within a few hours; erythrocytes with intact oligosaccharides (withdrawn and reintroduced without neuraminidase treatment) continue to circulate for days.

Selectins are a family of plasma membrane lectins that mediate cell-cell recognition and adhesion in a wide range of cellular processes. One such process is the movement of immune cells (leukocytes) through the capillary wall, from blood to tissues, at sites of infection or inflammation (**Fig. 7-29**). At an infection site, P-selectin on the surface of capillary endothelial cells interacts with a specific oligosaccharide of the surface glycoproteins of circulating leukocytes. This interaction slows the leukocytes as they roll along the endothelial lining of the capillaries. A second interaction, between integrin molecules in the leukocyte plasma membrane and an adhesion protein on the endothelial cell surface, now stops the leukocyte and allows it to move through the capillary wall into the infected tissues to initiate the immune attack. Two other selectins participate in this "lymphocyte homing": E-selectin on the endothelial cell and L-selectin on the leukocyte bind their cognate oligosaccharides on the leukocyte and endothelial cell, respectively. Several of the selectins essential to lymphocyte homing bind specifically to the tetrasaccharide Neu5Ac-(α2→3)-D-Gal-(β1→4)(α-L-Fuc-[1→4])-D-GlcNAc, called sialyl Lewis x or sialyl Le^{x}.

Human selectins mediate the inflammatory responses in rheumatoid arthritis, asthma, psoriasis, multiple sclerosis, and the rejection of transplanted organs, and thus there is great interest in developing drugs that inhibit selectin-mediated cell adhesion. Many carcinomas express sialyl Lewis x, which, when shed into the circulation, facilitates tumor cell survival and metastasis. Carbohydrate derivatives that mimic the sialyl Lewis x portion of sialoglycoproteins and compete for specific selectin-binding sites, or that alter the biosynthesis of the oligosaccharide, might prove effective as selectin-specific drugs for treating chronic inflammation or metastatic disease.

Several animal viruses, including the influenza virus, attach to their host cells through interactions with oligosaccharides displayed on the host cell surface. The lectin of the influenza virus, known as the HA (hemagglutinin) protein, is essential for viral entry and infection. After the virus has entered a host cell and has been replicated, the newly synthesized viral particles bud out of the cell, wrapped in a portion of its plasma membrane. A viral sialidase (neuraminidase) trims the terminal sialic acid residue from the host cell's oligosaccharides, releasing the viral particles from their interaction with the cell and preventing their aggregation with one another. Another round of infection can now begin. The antiviral drugs oseltamivir (Tamiflu) and zanamivir (Relenza) are used clinically in the treatment of influenza. These drugs are sugar analogs; they inhibit the viral sialidase by competing with the host cell's oligosaccharides for binding (**Fig. 7-30**). This prevents the release of viruses from the infected cell by sialidase, and also causes viral particles to aggregate, both of which block another cycle of infection.

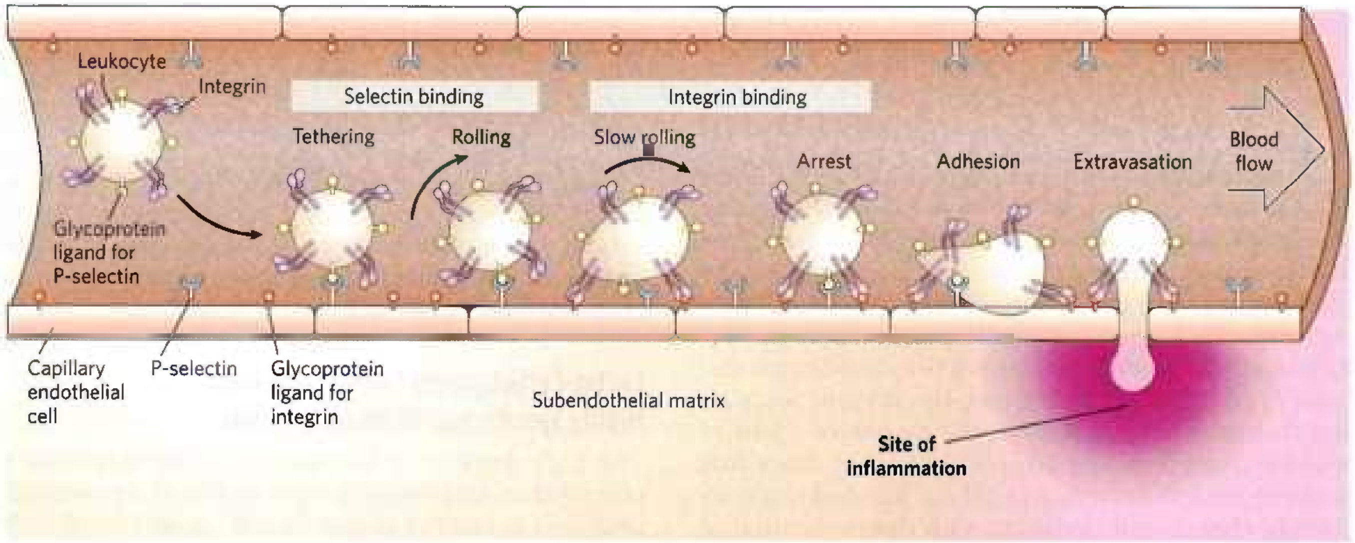


FIGURE 7-29 Role of lectin-ligand interactions in leukocyte movement to the site of an infection or injury. A leukocyte circulating through a capillary is slowed by transient interactions between P-selectin molecules in the plasma membrane of the capillary endothelial cells and glycoprotein ligands for P-selectin on the leukocyte surface. As the leukocyte interacts with successive P-selectin molecules, it rolls along the capillary surface. Near a site of inflammation, stronger interactions between integrin in the leukocyte surface and its ligand in the capillary surface lead to tight adhesion. The leukocyte stops rolling and, under the influence of signals from the site of inflammation (such as sialyl Lewis x), escapes through the capillary wall as it moves toward the region of inflammation.

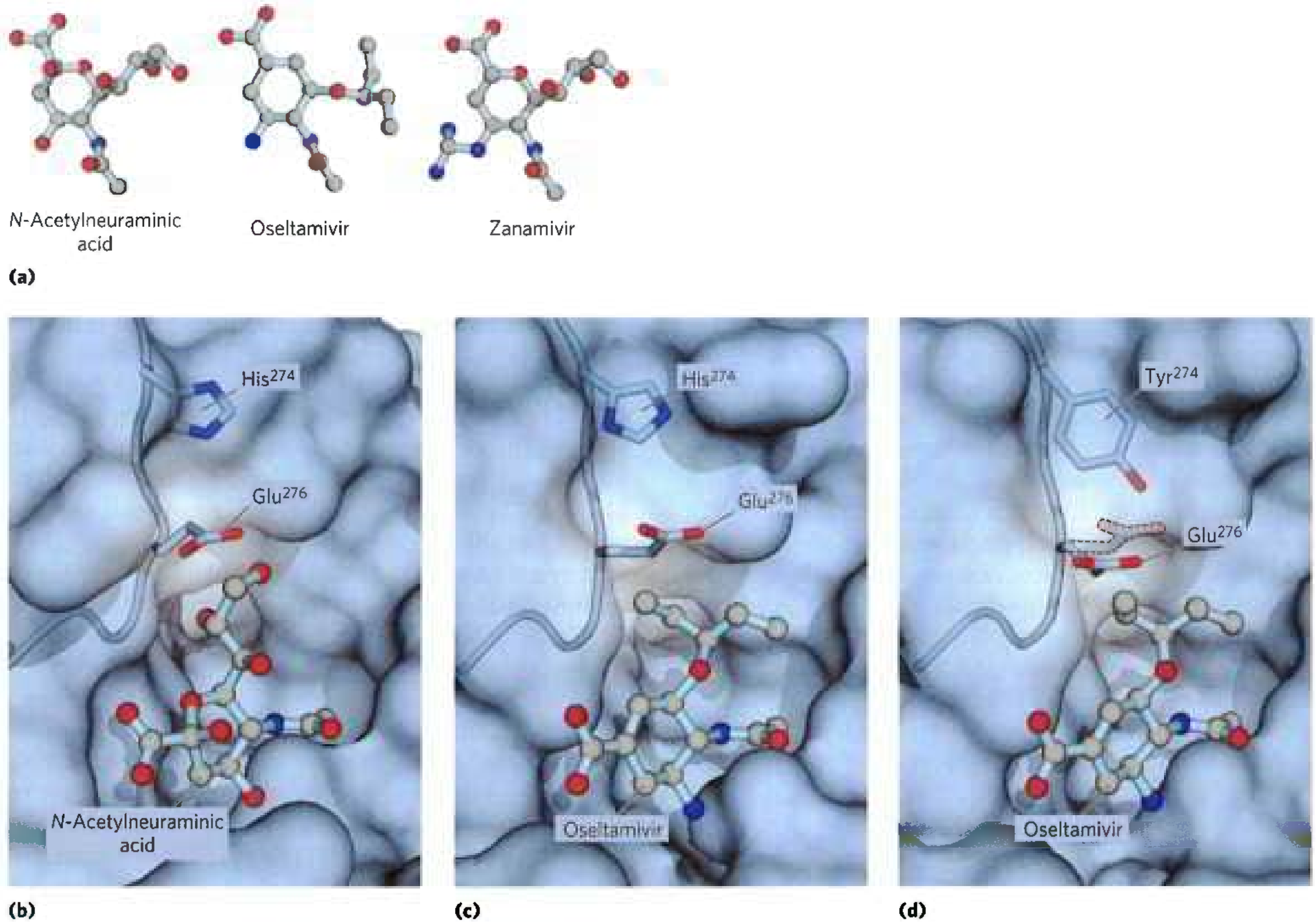


FIGURE 7-30 Binding site on influenza neuraminidase for *N*-acetylneuraminic acid and an antiviral drug. (a) The normal binding ligand for influenza neuraminidase is a sialic acid, *N*-acetylneuraminic acid. The drugs oseltamivir and zanamivir occupy the same site on the enzyme, competitively inhibiting it and blocking viral release from the host cell. (b) The normal interaction with *N*-acetylneuraminic acid in the binding site. (c) Oseltamivir can fit into this site by pushing a nearby Glu residue out of the way. (d) A mutation in the influenza virus's gene for neuraminidase replaces a His near this Glu residue with the larger side chain of a Tyr. Now, oseltamivir is not as effective at pushing the Glu out of its way, and the drug binds much less well to the binding site, making the mutant virus effectively resistant to oseltamivir. [Data from (b) PDB ID 2BAT, J. N. Varghese et al., *Proteins* 14:327, 1992; (c) PDB ID 2HU4, R. J. Russell et al., *Nature* 443:45, 2006; (d) PDB ID 3CL0, P. J. Collins et al., *Nature* 453:1258, 2008.]

Some of the most devastating of the human parasitic diseases, widespread in much of the developing world, are caused by eukaryotic microorganisms that display unusual surface oligosaccharides, which in some cases are known to be protective for the parasites. These organisms include the trypanosomes, responsible for African sleeping sickness (see Box 6-1) and Chagas disease; *Plasmodium falciparum*, the malaria parasite; and *Entamoeba histolytica*, the causative agent of amoebic dysentery. The prospect of finding drugs that interfere with the synthesis of these unusual oligosaccharide chains, and therefore with the replication of the parasites, has inspired much recent work on the biosynthetic pathways of these oligosaccharides. ■

Lectins also act intracellularly, in sorting proteins for transportation to specific cellular compartments (see Chapter 27). For example, P7 an oligosaccharide containing mannose 6-phosphate, recognized by a lectin, acts as a molecular "ZIP code" that tags newly synthesized proteins in the Golgi complex for transfer to the lysosome (see Fig. 27-41).

Lectin-Carbohydrate Interactions Are Highly Specific and Often Multivalent

The high density of information in the structure of oligosaccharides provides a sugar code with an essentially unlimited number of unique "words" small enough to be read by a single protein. In their carbohydrate-binding sites, lectins have a subtle molecular complementarity that allows interaction only with their correct carbohydrate binding partners. Often a divalent metal ion such as Ca^{2+} or Mn^{2+}

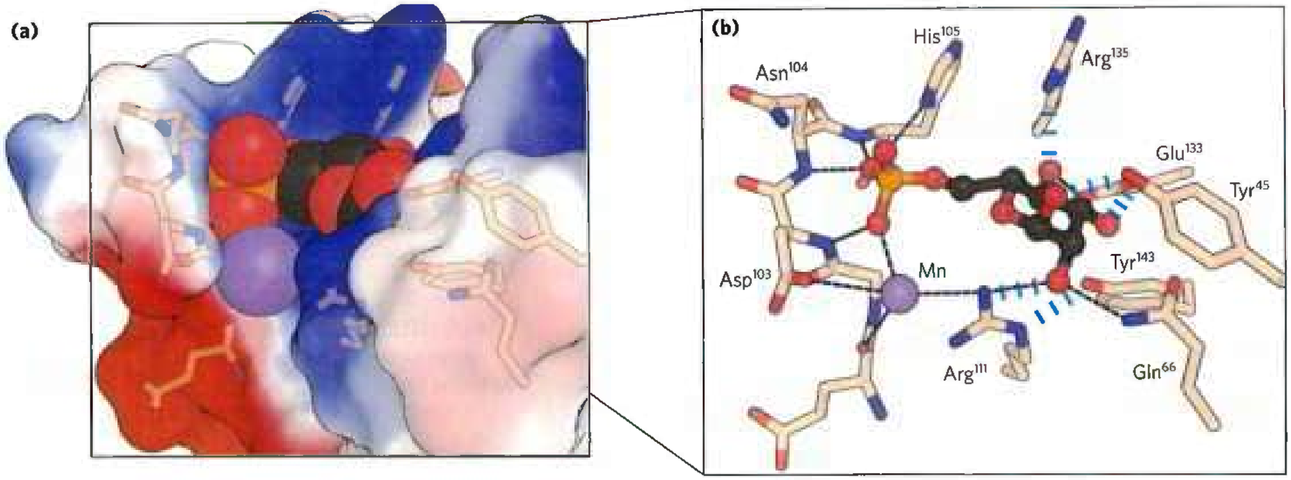


FIGURE 7-31 Details of a lectin-carbohydrate interaction. (a) Structure of the bovine mannose 6-phosphate receptor complexed with mannose 6-phosphate. The protein is represented as a surface contour image, showing the surface as predominantly negatively charged (red) or positively charged (blue). Mannose 6-phosphate is shown as a space-filling model; a manganese ion is shown as a violet sphere. (b) An enlarged view of the binding site. Mannose 6-phosphate is hydrogen-bonded to Arg^{111} and coordinated with the manganese ion (shown smaller than its van der Waals radius, for clarity). Each hydroxyl group of mannose is hydrogen-bonded to the protein. The His^{105} hydrogen-bonded to a phosphate oxygen of mannose 6-phosphate may be the residue that, when protonated at low pH, causes the receptor to release mannose 6-phosphate into the lysosome. [Data from PDB ID 1M6P, D. L. Roberts et al., *Cell* 93:639, 1998.]

is part of the binding site. Lectin-ligand interactions can have extraordinarily high specificity. The affinity between an oligosaccharide and an individual carbohydrate binding domain (CBD) of a lectin is sometimes modest (micromolar to millimolar K_d values), but the effective affinity is often greatly increased by lectin multivalency, in which a single lectin molecule has multiple CBDs. In a cluster of oligosaccharides—as is commonly found on a membrane surface, for example—each oligosaccharide can engage one of the lectin's CBDs, strengthening the interaction. When cells express multiple lectin receptors, the avidity of the interaction can be very high, enabling highly cooperative events such as cell attachment and rolling (Fig. 7-29).

X-ray crystallographic studies of the structure of the lectin that is the receptor for mannose 6-phosphate reveal details that explain the specificity of the binding and the role of a divalent cation in the lectin-sugar interaction (**Fig. 7-31**). When the protein tagged with mannose 6-phosphate reaches the lysosome (which has a lower internal pH than the Golgi complex), the receptor loses its affinity for mannose 6-phosphate and the tagged protein is released into the lysosomal matrix.

In addition to such highly specific interactions, there are more general interactions that contribute to the binding of many carbohydrates to their lectins. For example, many sugars have a more polar side and a less polar side (**Fig. 7-32**); the more polar side hydrogen-bonds with the lectin, while the less polar side undergoes interactions with nonpolar amino acid residues through the hydrophobic effect. The sum of all these interactions produces high-affinity binding and high specificity of lectins for their carbohydrate ligands. This represents a kind of information transfer that is clearly central in many processes within and between cells. **Figure 7-33** summarizes some of the biological interactions mediated by the sugar code.

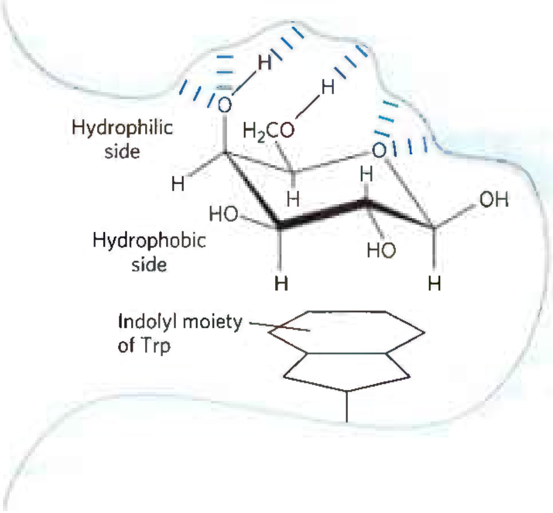


FIGURE 7-32 Interactions of sugar residues due to the hydrophobic effect. Sugar units such as galactose have a more polar side (the top of the chair as shown here, with the ring oxygen and several hydroxyls), available to hydrogen-bond with the lectin, and a less polar side that can interact with nonpolar side chains in the protein, such as the indole ring of Trp residues, through the hydrophobic effect. [Information from a figure provided by Dr. C.-W. von der Lieth, Heidelberg; H.-J. Gabius, *Naturwissenschaften* 87:108, 2000, Fig. 6.]

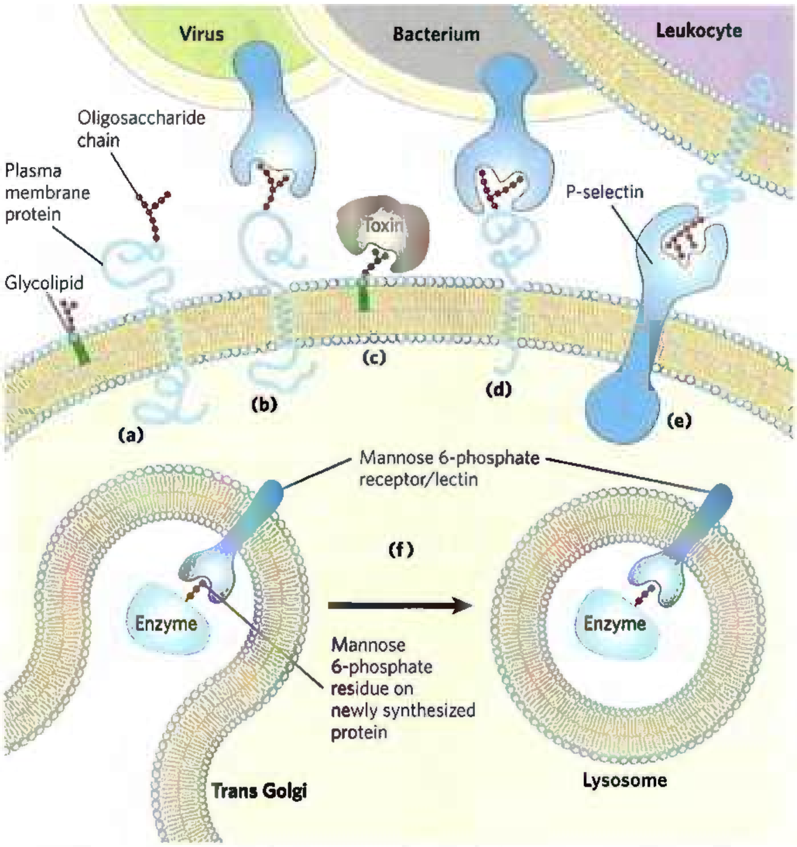


FIGURE 7-33 Role of oligosaccharides in recognition events at the cell surface and in the endomembrane system. (a) Oligosaccharides with unique structures (represented as strings of red hexagons) are components of a variety of glycoproteins or glycolipids on the outer surface of plasma membranes. Their oligosaccharide moieties are bound by extracellular lectins with high specificity and affinity. (b) Viruses that infect animal cells, such as the influenza virus, bind to cell surface glycoproteins as the first step in infection. (c) Bacterial toxins, such as the cholera and pertussis toxins, bind to a surface glycolipid before entering a cell. (d) Some bacteria adhere to and then colonize or infect animal cells. (e) Selectins (lectins) in the plasma membrane of certain cells mediate cell-cell interactions, such as those of leukocytes with the endothelial cells of the capillary wall at an infection site. (f) The mannose 6-phosphate receptor/lectin of the trans Golgi complex binds to the oligosaccharide of lysosomal enzymes, targeting them for transfer into the lysosome. [Information from N. Sharon and H. Lis, *Sci. Am.* 268 (January):82, 1993.]

SUMMARY 7.4 *Carbohydrates as Informational Molecules: The Sugar Code*

- Monosaccharides can be assembled into an almost limitless variety of oligosaccharides, which differ in the stereochemistry and position of glycosidic bonds, the type and orientation of substituent groups, and the number and type of branches. Glycans are far more information-dense than nucleic acids or proteins.
- The sugar specificity of many plant lectins makes them powerful laboratory reagents in glycobiology. Receptor/lectins in the surface of hepatocytes recognize glycoproteins that have lost their terminal sialic acid residue and mediate the normal uptake (by hepatocytes) and destruction of blood cells and of certain circulating glycoprotein hormones.
- Bacterial and viral pathogens and some eukaryotic parasites adhere to their animal cell targets through binding of lectins on the pathogens to oligosaccharides on the target cell surface, or vice versa. Interactions are often polyvalent.
- Structural studies of lectin-sugar complexes show the detailed complementarity between the two molecules, which accounts for the strength and specificity of lectin interactions with carbohydrates.

7.5 Working with Carbohydrates

Oligosaccharide analysis is complicated by the fact that, unlike nucleic acids and proteins, oligosaccharides can be branched and are joined by a variety of linkages. The high charge density of many oligosaccharides and polysaccharides, and the relative lability of the sulfate esters in glycosaminoglycans, present further difficulties.

For simple, linear polymers such as amylose, the positions of the glycosidic bonds are determined by the classical method of exhaustive methylation: treating the intact polysaccharide with methyl iodide in a strongly basic medium to convert all free hydroxyls to acid-stable methyl ethers, then hydrolyzing the methylated polysaccharide in acid. The only free hydroxyls in the monosaccharide derivatives so produced are those that were involved in glycosidic bonds. To determine the sequence of monosaccharide residues, including any branches that are present, exoglycosidases of known specificity are used to remove residues one at a time from the nonreducing end(s). The known specificity of these exoglycosidases often allows deduction of the position and stereochemistry of the linkages.

For analysis of the oligosaccharide moieties of glycoproteins and glycolipids, the oligosaccharides are released by purified enzymes—glycosidases that specifically cleave *O*- or *N*-linked oligosaccharides, or lipases that remove lipid head groups. Alternatively, *O*-linked glycans can be released from glycoproteins by treatment with hydrazine.

The resulting mixtures of carbohydrates are resolved into their individual components by a variety of methods (**Fig. 7-34**), including the same techniques used in protein and amino acid separation: fractional precipitation by solvents, and ion-exchange and size-exclusion chromatography (see Fig. 3-17). Highly purified lectins, attached covalently to an insoluble support, can be used in affinity chromatography of carbohydrates.

Hydrolysis of oligosaccharides and polysaccharides in strong acid yields a mixture of monosaccharides, which can be identified and quantified by chromatographic techniques to yield the overall composition of the polymer.

Oligosaccharide analysis relies heavily on mass spectrometry (see Figs 3-28 and 3-29) and high-resolution NMR spectroscopy (see Fig. 4-31). NMR analysis alone, especially for oligosaccharides of moderate size, can yield much information about sequence, linkage position, and anomeric carbon configuration. Automated procedures and commercial instruments are used for the routine determination of oligosaccharide structure, but the sequencing of branched oligosaccharides joined by more than one type of bond remains a far more formidable task than determining the linear sequences of proteins and nucleic acids.

Another important tool in working with carbohydrates is chemical synthesis, which has proved to be a powerful approach to understanding the biological functions of glycosaminoglycans and oligosaccharides. The chemistry involved in such syntheses is difficult, but carbohydrate chemists can now synthesize short segments of almost any glycosaminoglycan, with correct stereochemistry, chain length, and sulfation pattern, and oligosaccharides significantly more complex than those

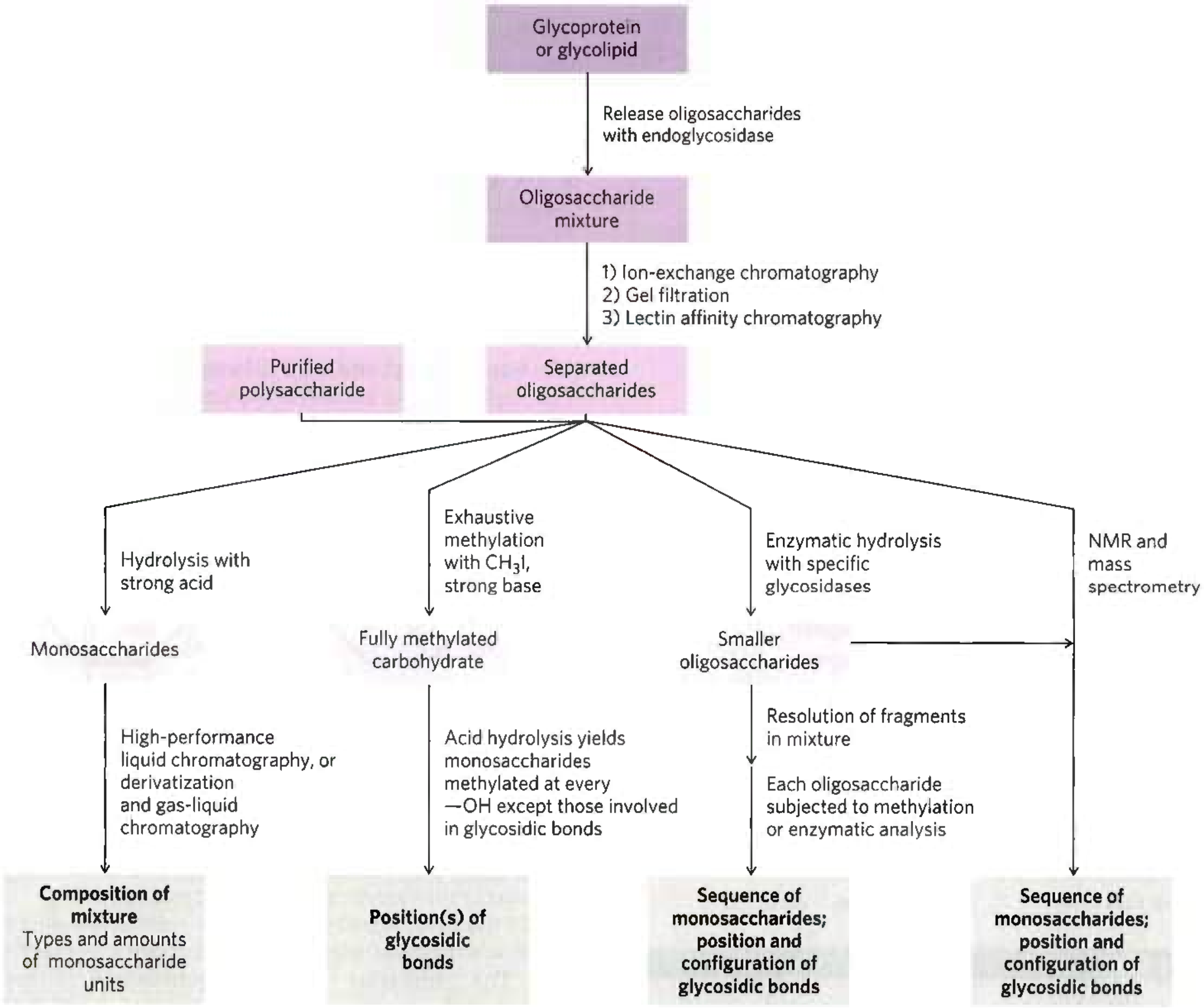

Glycoprotein or glycolipid
Release oligosaccharides with endoglycosidase
Oligosaccharide mixture
1) Ion-exchange chromatography
2) Gel filtration
3) Lectin affinity chromatography
Purified polysaccharide
Separated oligosaccharides
Hydrolysis with strong acid
Exhaustive methylation with CH₃I, strong base
Enzymatic hydrolysis with specific glycosidases
NMR and mass spectrometry
Monosaccharides
Fully methylated carbohydrate
Smaller oligosaccharides
High-performance liquid chromatography, or derivatization and gas-liquid chromatography
Acid hydrolysis yields monosaccharides methylated at every —OH except those involved in glycosidic bonds
Resolution of fragments in mixture
Each oligosaccharide subjected to methylation or enzymatic analysis
Composition of mixture
Types and amounts of monosaccharide units
Position(s) of glycosidic bonds
Sequence of monosaccharides; position and configuration of glycosidic bonds
Sequence of monosaccharides; position and configuration of glycosidic bonds

FIGURE 7-34 Methods of carbohydrate analysis. A carbohydrate purified in the first stage of the analysis often requires all four analytical routes for its complete characterization.

shown in Figure 7-27. Solid-phase oligosaccharide synthesis is based on the same principles (and has the same advantages) as peptide synthesis (see Fig. 3-30), but requires a set of tools unique to carbohydrate chemistry: blocking groups and activating groups that allow the synthesis of glycosidic linkages with the correct hydroxyl group. Synthetic approaches of this type currently represent an area of great interest, because it is difficult to purify defined oligosaccharides in adequate quantities from natural sources.

SUMMARY 7.5 *Working with Carbohydrates*

- Establishing the complete structure of oligosaccharides and polysaccharides is a more complex problem than protein and nucleic acid analysis. Traditional chemical and enzymatic approaches, as well as mass spectrometry and high-resolution NMR spectroscopy, applicable to small samples of carbohydrate, yield essential information about sequence, configuration at anomeric and other carbons, and positions of glycosidic bonds. Solid-phase synthetic methods yield defined oligosaccharides that are of great value in exploring lectin-oligosaccharide interactions and may prove clinically useful.

KEY TERMS

Terms in bold are defined in the glossary.

carbohydrate	229
monosaccharide	229
oligosaccharide	229
disaccharide	229
polysaccharide	229
aldose	230
ketose	230
enantiomer	232
Fischer projection formulas	232
epimers	232
hemiacetal	234
hemiketal	234
anomers	234
anomeric carbon	234
pyranose	234
furanose	234
Haworth perspective formulas	235
mutarotation	235
reducing sugar	237
***O*-glycosidic bonds**	237
reducing end	237
hemoglobin glycation	239
glycan	241
homopolysaccharide	241
heteropolysaccharide	241
starch	242
glycogen	242
cellulose	243
extracellular matrix (ECM)	244
glycosaminoglycan	245
hyaluronan	245
chondroitin sulfate	245
heparan sulfate	246
glyconjugate	247
proteoglycan	247
glycoprotein	248
glycolipid	248
syndecan	248
glypican	248
glycomics	252
ganglioside	253
lectin	254
selectins	255

PROBLEMS

1. **Sugar Alcohols** In the monosaccharide derivatives known as sugar alcohols, the carbonyl oxygen is reduced to a hydroxyl group. For example, D-glyceraldehyde can be reduced to glycerol. Why can the sugar alcohol glycerol no longer be designated D or L?

2. **Recognizing Epimers** Using Figure 7-3, identify the epimers of **(a)** D-allose, **(b)** D-gulose, and **(c)** D-ribose at C-2, C-3, and C-4.

3. **Configuration and Conformation** Which bond(s) in α-D-glucose must be broken to change its configuration to β-D-glucose? Which bond(s) must be broken to convert D-glucose to D-mannose? Which bond(s) must be broken to convert one "chair" form of D-glucose to the other?

4. **Sugar Structures** Compare and contrast the structural features of each pair: **(a)** Cellulose and glycogen **(b)** D-Glucose and D-fructose **(c)** Maltose and sucrose

5. **Haworth Structures** Draw the Haworth perspective formulas for α-D-mannose and β-L-galactose.

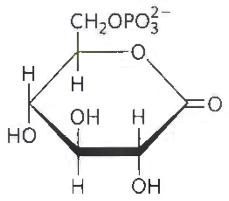
6. **Reducing Sugars** Draw the structural formula for α-D-glucosyl-(1→6)-D-mannosamine, and circle the part of this structure that makes the compound a reducing sugar.

7. **Hemoglobin Glycation** The measurement of glycated hemoglobin (the HbA1c level) to monitor the regulation of blood glucose (Box 7-2) is not reliable in individuals with certain conditions or diseases. Can you suggest an explanation for a lower-than-normal HbA1c level in a patient who definitely is not diabetic?

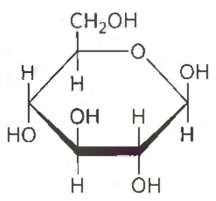
8. **Hemiacetal and Glycosidic Linkages** Explain the difference between a hemiacetal and a glycoside.

9. **A Taste of Honey** The sweetness of honey gradually decreases at a high temperature. Also, high-fructose corn syrup (a commercial product in which much of the glucose in corn syrup is converted to fructose) is used for sweetening *cold* drinks but not *hot* drinks. What chemical property of fructose could account for both of these observations?

10. **Gluconolactone and Glucose Oxidation States** The cyclic glucose derivative 6-phosphogluconolactone is an intermediate in the pentose phosphate pathway (discussed in Chapter 14). Compare the oxidation state of C-1 for the cyclic form of both gluconolactone and β-D-glucose.



6-Phosphogluconolactone



β-D-Glucose

11. **Invertase "Inverts" Sucrose** As sweet as sucrose is, an equimolar mixture of its constituent monosaccharides, D-glucose and D-fructose, is sweeter. Besides enhancing sweetness, fructose has hygroscopic properties that improve the texture of foods, reducing crystallization and increasing moisture.

In the food industry, hydrolyzed sucrose is called invert sugar, and the yeast enzyme that hydrolyzes it is called invertase. The hydrolysis reaction is generally monitored by measuring the specific rotation of the solution, which is positive (+66.4°) for sucrose but becomes negative (inverts) as more D-glucose (specific rotation = +52.7°) and D-fructose (specific rotation = −92°) form.

From what you know about the chemistry of the glycosidic bond, how would you hydrolyze sucrose to invert sugar nonenzymatically in a home kitchen?

12. Manufacture of Liquid-Filled Chocolates The manufacture of chocolates containing a liquid center is an interesting application of enzyme engineering. The flavored liquid center consists largely of an aqueous solution of sugars rich in fructose to provide sweetness. The technical dilemma is the following: the chocolate coating must be prepared by pouring hot, melted chocolate over a solid (or almost solid) core, yet the final product must have a liquid, fructose-rich center. Suggest a way to solve this problem. (Hint: Sucrose is much less soluble than a mixture of glucose and fructose.)

13. Anomers of Sucrose? Lactose exists in two anomeric forms, but no anomeric forms of sucrose have been reported. Why?

14. Gentiobiose Gentiobiose (D-Glc(β1→6)D-Glc) is a disaccharide found in some plant glycosides. Draw the Haworth structure of gentiobiose based on its abbreviated name. Is it a reducing sugar? Does it undergo mutarotation?

15. Identifying Reducing Sugars Is *N*-acetyl-β-D-glucosamine (Fig. 7-9) a reducing sugar? What about D-gluconate? Is the disaccharide GlcN(α1→1α)Glc a reducing sugar?

16. Physical Properties of Cellulose and Glycogen The almost pure cellulose obtained from the seed threads of *Gossypium* (cotton) is tough, fibrous, and completely insoluble in water. In contrast, glycogen obtained from muscle or liver disperses readily in hot water to make a turbid solution. Despite their markedly different physical properties, both substances are (1→4)-linked D-glucose polymers of comparable molecular weight. What structural features of these two polysaccharides underlie their different physical properties? Suggest possible biological advantages of their respective properties.

17. Dimensions of a Polysaccharide Compare the dimensions of a molecule of cellulose and a molecule of amylose, each of M_r 200,000.

18. Growth Rate of Bamboo The stems of bamboo, a tropical grass, can grow at the phenomenal rate of 0.3 m/day under optimal conditions. Given that the stems are composed almost entirely of cellulose fibers oriented in the direction of growth, calculate the number of sugar residues per second that must be added enzymatically to growing cellulose chains to account for the growth rate. Each D-glucose unit contributes ~0.5 nm to the length of a cellulose molecule.

19. Glycoproteins versus Proteoglycans Which characteristics describe glycoproteins and which describe proteoglycans?

(a) Exclusively located at the cell surface and in the extracellular matrix
(b) May contain *N*-linked glycosidic bonds
(c) Found in Golgi complexes, secretory granules, and lysosomes
(d) Include the heparan sulfate family
(e) Sulfated glycosaminoglycan chains can only be covalently linked to a Ser residue
(f) Form highly specific sites for recognition and high-affinity binding by lectins

20. Relative Stability of Two Conformers Explain why the two structures shown in Figure 7-16 are so different in energy (stability). Hint: See Figure 1-21.

21. Volume of Chondroitin Sulfate in Solution One critical function of chondroitin sulfate is to act as a lubricant in skeletal joints by creating a gel-like medium that is resilient to friction and shock. This function seems to be related to a distinctive property of chondroitin sulfate: the volume occupied by the molecule is much greater in solution than in the dehydrated solid. Why is the volume so much larger in solution?

22. Heparin Interactions Heparin, a highly negatively charged glycosaminoglycan, is used clinically as an anticoagulant. It acts by binding several plasma proteins, including antithrombin III, an inhibitor of blood clotting. The 1:1 binding of heparin to antithrombin III seems to cause a conformational change in the protein that greatly increases its ability to inhibit clotting. What amino acid residues of antithrombin III are likely to interact with heparin?

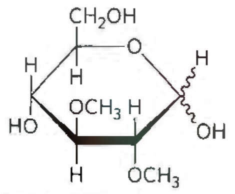
23. Permutations of a Trisaccharide Three different hexoses (A, B, and C) can be combined to form a large number of trisaccharides. What structural features of trisaccharides allow so many permutations and combinations?

24. Effect of Sialic Acid on SDS Polyacrylamide Gel Electrophoresis Suppose you have four forms of a protein, all with identical amino acid sequence but containing zero, one, two, or three oligosaccharide chains, each ending in a single sialic acid residue. Draw the gel pattern you would expect when a mixture of these four glycoproteins is subjected to SDS polyacrylamide gel electrophoresis (see Fig. 3-18) and stained for protein. Identify any bands in your drawing.

25. Information Content of Oligosaccharides The carbohydrate portion of some glycoproteins may serve as a cellular recognition site. To perform this function, the oligosaccharide(s) must have the potential to exist in a large variety of forms. Which can produce a greater variety of structures: oligopeptides composed of five different amino acid residues, or oligosaccharides composed of five different monosaccharide residues? Explain.

26. Castor Bean Toxin The seed of the castor bean (*Ricinus communis*) contains large amounts of ricin, a poison that is deadly to animals, including humans. One of the two subunits of this toxin is a lectin that binds terminal *N*-acetylgalactosamine residues on glycoproteins on the surface of eukaryotic cells, allowing the other subunit to enter the cell and kill it by preventing proteins from being made. Suggest a possible antidote to prevent or reverse ricin-mediated entry of the toxin.

27. Determination of the Extent of Branching in Amylopectin A biochemist wants to determine the amount of branching in amylopectin, defined by the number of (α1→6) glycosidic bonds present. First, she treats the sample with methyl iodide, a methylating agent that replaces the hydrogen of every sugar hydroxyl with a methyl group, converting —OH to —OCH_3. She then hydrolyzes all the glycosidic bonds in the treated sample in aqueous acid and measures the amount of 2,3-di-*O*-methylglucose formed.



2,3-Di-*O*-methylglucose

In this representation of 2,3-di-*O*-methylglucose, the wavy bonds at C-1 indicate that the structure represents both anomers (α and β).

(a) Explain the basis of this procedure for determining the number of $(\alpha 1\rightarrow 6)$ branch points in amylopectin. What happens to the unbranched glucose residues in amylopectin during the methylation and hydrolysis procedure?

(b) A 258 mg sample of amylopectin that was treated by the methylation and hydrolysis procedure described yielded 12.4 mg of 2,3-di-*O*-methylglucose. Determine what percentage of the glucose residues in the amylopectin contained an $(\alpha 1\rightarrow 6)$ branch. (Assume that the average molecular weight of a glucose residue in amylopectin is 162 g/mol and the molecular weight of 2,3-di-*O*-methylglucose is 208 g/mol.)

DATA ANALYSIS PROBLEM

28. Determining the Structure of ABO Blood Group Antigens The human ABO blood group system was discovered in 1901, and in 1924 this trait was shown to be inherited at a single gene locus with three alleles. In 1960, W. T. J. Morgan published a paper summarizing what was known at that time about the structure of the ABO antigen molecules. When the paper was published, the complete structures of the A, B, and O antigens were not yet known; this paper is an example of what scientific knowledge looks like "in the making."

In any attempt to determine the structure of an unknown biological compound, researchers must deal with two fundamental problems: (1) If you don't know what *it* is, how do you know if *it* is pure? (2) If you don't know what *it* is, how do you know that your extraction and purification conditions have not changed *its* structure? Morgan addressed problem 1 through several methods. One method is described in his paper as observing "constant analytical values after fractional solubility tests" (p. 312). In this case, "analytical values" are measurements of chemical composition, melting point, and so forth.

(a) Based on your understanding of chemical techniques, what could Morgan mean by "fractional solubility tests"?

(b) Why would the analytical values obtained from fractional solubility tests of a *pure* substance be constant, and those of an *impure* substance not be constant?

Morgan addressed problem 2 by using an assay to measure the immunological activity of the substance present in different samples.

(c) Why was it important for Morgan's studies, and especially for addressing problem 2, that this activity assay be quantitative (measuring a level of activity) rather than simply qualitative (measuring only the presence or absence of a substance)?

The structure of the blood group antigens is shown in Figure 10-13. In his paper, Morgan listed several properties of the three antigens, A, B, and O, that were known at that time (p. 314):

1. Type B antigen has a higher content of galactose than A or O.
2. Type A antigen contains more total amino sugars than B or O.
3. The glucosamine:galactosamine ratio for the A antigen is roughly 1:2; for B, it is roughly 2:5.

(d) Which of these findings is (are) consistent with the known structures of the blood group antigens?

(e) How do you explain the discrepancies between Morgan's data and the known structures?

In later work, Morgan and his colleagues used a clever technique to obtain structural information about the blood group antigens. Enzymes had been found that would specifically degrade the antigens. However, these were available only as crude enzyme preparations, perhaps containing more than one enzyme of unknown specificity. Degradation of the blood type antigens by these crude enzymes could be inhibited by the addition of particular sugar molecules to the reaction. Only sugars found in the blood type antigens would cause this inhibition. One enzyme preparation, isolated from the protozoan *Trichomonas foetus*, would degrade all three antigens and was inhibited by the addition of particular sugars. The results of these studies are summarized in the table below, showing the percentage of substrate remaining unchanged when the *T. foetus* enzyme acted on the blood group antigens in the presence of sugars.

	Unchanged substrate (%)		
Sugar added	**A antigen**	**B antigen**	**O antigen**
Control—no sugar	3	1	1
L-Fucose	3	1	100
D-Fucose	3	1	1
L-Galactose	3	1	3
D-Galactose	6	100	1
N-Acetylglucosamine	3	1	1
N-Acetylgalactosamine	100	6	1

For the O antigen, a comparison of the control and L-fucose results shows that L-fucose inhibits the degradation of the antigen. This is an example of product inhibition, in which an excess of reaction product shifts the equilibrium of the reaction, preventing further breakdown of substrate.

(f) Although the O antigen contains galactose, *N*-acetylglucosamine, and *N*-acetylgalactosamine, none of these sugars inhibited the degradation of this antigen. Based on these data, is the enzyme preparation from *T. foetus* an endoglycosidase or an exoglycosidase? (Endoglycosidases cut bonds between interior residues; exoglycosidases remove one residue at a time from the end of a polymer.) Explain your reasoning.

(g) Fucose is also present in the A and B antigens. Based on the structure of these antigens, why does fucose fail to prevent their degradation by the *T. foetus* enzyme? What structure would be produced?

(h) Which of the results in (f) and (g) are consistent with the structures shown in Figure 10-13? Explain your reasoning.

Reference

Morgan, W.T.J. 1960. The Croonian Lecture: a contribution to human biochemical genetics; the chemical basis of blood-group specificity. *Proc. R. Soc. Lond. B Biol. Sci.* 151:308–347.

Chapter 8

NUCLEOTIDES AND NUCLEIC ACIDS

8.1 Some Basic Definitions and Conventions *263*

8.2 Nucleic Acid Structure *269*

8.3 Nucleic Acid Chemistry *278*

8.4 Other Functions of Nucleotides *294*

Nucleotides have a variety of roles in cellular metabolism. They are the energy currency in metabolic transactions, the essential chemical links in the response of cells to hormones and other extracellular stimuli, and the structural components of an array of enzyme cofactors and metabolic intermediates. And, last but certainly not least, they are the constituents of nucleic acids: **deoxyribonucleic acid (DNA)** and **ribonucleic acid (RNA)**, the molecular repositories of genetic information. The structure of every protein, and ultimately of every biomolecule and cellular component, is a product of information programmed into the nucleotide sequence of cellular (or viral) nucleic acids. The ability to store and transmit genetic information from one generation to the next is a fundamental condition for life.

The discussion in this chapter reinforces or introduces five principles:

P1 **Nucleic acids are both repositories and functional expressions of biological information.** Biological information is one of the required conditions for life, a blueprint for each species transmitted from one generation to the next. RNA can be a functional expression of that information, directing the synthesis of proteins or in some cases acting directly as a signal or a reaction catalyst.

P2 **The transmission of biological information relies on molecular complementarity.** Chromosomes are the largest molecules in any cell. They are polymers composed of a small set of common nucleotides, with information embedded in the nucleotide sequence. The common nucleotides in RNA and DNA are organized so that two strands of nucleic acid can maintain a complementary and uniform structure over vast molecular distances. This extended potential for both variable sequence and complementarity, and thus information storage and transmission, is a property shared by no other class of biological molecule.

P3 **Biological information is subject to natural damage and change.** DNA damage is a constant, and it results in occasional mutation—the raw material for evolution.

P4 **Biological information can be accessed, interpreted, and altered in the laboratory.** The information embedded in nucleic acids is of singular importance to biochemistry and molecular biology. The techniques for sequencing, synthesizing, and altering nucleic acids are continually advancing.

P5 **Nucleoside triphosphates occupy a central role in cellular metabolism, serving as an energy currency and as important regulatory signals.** ATP is the ultimate product of catabolic pathways, providing fuel for anabolic pathways.

This chapter provides an overview of the chemical nature of the nucleotides and nucleic acids found in most cells, as well as the tools used to study them. A more detailed examination of the function of nucleic acids is the focus of Part III of this text.

8.1 Some Basic Definitions and Conventions

The amino acid sequence of every protein in a cell, and the nucleotide sequence of every RNA, is specified by a nucleotide sequence in the cell's DNA. A segment of a DNA molecule that contains the information required for the synthesis of a functional biological product, whether protein or RNA, is referred to as a **gene**. A cell typically has many thousands of genes, and DNA molecules, not surprisingly, tend to be very large. **P1** The storage of biological information and the transmission of that information from one generation to the next are the only known functions of DNA.

RNAs have a broader range of functions, and several classes are found in cells. **Ribosomal RNAs (rRNAs)** are components of ribosomes, the complexes that carry out the synthesis of proteins. **Messenger RNAs (mRNAs)** are intermediaries, carrying information for the synthesis of a protein from one or a few genes to a ribosome. **Transfer RNAs (tRNAs)** are adapter molecules that faithfully translate the information in mRNA into a specific sequence of amino acids. In addition to these major classes, there are many RNAs (**noncoding** or **ncRNAs**) with a wide variety of special functions, described in depth in Part III.

Nucleotides and Nucleic Acids Have Characteristic Bases and Pentoses

A **nucleotide** has three characteristic components: (1) a nitrogenous (nitrogen-containing) base, (2) a pentose, and (3) one or more phosphates (**Fig. 8-1**). The molecule without a phosphate group is called a **nucleoside**. The nitrogenous bases are derivatives of two parent compounds, **pyrimidine** and **purine**. The bases and pentoses of the common nucleotides are heterocyclic compounds.

KEY CONVENTION The carbon atoms and nitrogen atoms in the parent structures are conventionally numbered to facilitate the naming and identification of the many derivative compounds. The convention for the pentose ring follows rules outlined in Chapter 7, but in the pentoses of nucleotides and nucleosides the carbon numbers are given a prime (′) designation to distinguish them from the numbered atoms of the nitrogenous bases. ■

The base of a nucleotide is joined covalently (at N-1 of pyrimidines and N-9 of purines) in an *N*-*β*-glycosyl bond to the 1′ carbon of the pentose, and the phosphate is esterified to the 5′ carbon. The *N*-*β*-glycosyl bond is formed by removal of the elements of water (a hydroxyl group from the pentose and hydrogen from the base), as in *O*-glycosidic bond formation.

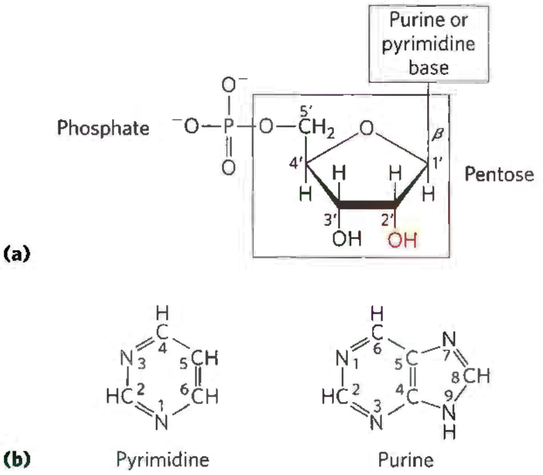


FIGURE 8-1 Structure of nucleotides. (a) General structure showing the numbering convention for the pentose ring. This is a ribonucleotide. In deoxyribonucleotides the —OH group on the 2′ carbon (in red) is replaced with —H. (b) The parent compounds of the pyrimidine and purine bases of nucleotides and nucleic acids, showing the numbering conventions.

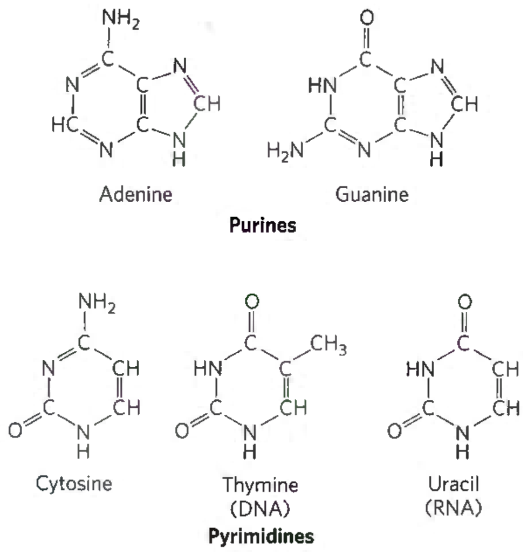


FIGURE 8-2 Major purine and pyrimidine bases of nucleic acids. Some of the common names of these bases reflect the circumstances of their discovery. Guanine, for example, was first isolated from guano (bird manure), and thymine was first isolated from thymus tissue.

Both DNA and RNA contain two major purine bases, **adenine** (A) and **guanine** (G), and two major pyrimidines. In both DNA and RNA, one of the pyrimidines is **cytosine** (C), but the second common pyrimidine is not the same in both: it is **thymine** (T) in DNA and **uracil** (U) in RNA. Only occasionally does thymine occur in RNA or uracil in DNA. The structures of the five major bases are shown in **Figure 8-2**, and the nomenclature of their corresponding nucleotides and nucleosides is summarized in **Table 8-1**.

Nucleic acids have two kinds of pentoses. The recurring deoxyribonucleotide units of DNA contain 2′-deoxy-D-ribose, and the ribonucleotide units of RNA contain D-ribose. In nucleotides, both types of pentoses are in their *β*-furanose (closed five-membered ring) form (**Fig. 8-3a**). As Figure 8-3b shows, the pentose ring is not planar but occurs in one of a variety of conformations generally described as "puckered."

KEY CONVENTION Although DNA and RNA seem to have two distinguishing features — different pentoses and the presence of uracil in RNA and thymine in DNA — it is the pentoses that uniquely define the identity of a nucleic acid. If the nucleic acid contains 2′-deoxy-D-ribose, it is DNA by definition, even if it contains uracil. Similarly, if the nucleic acid contains D-ribose, it is RNA, regardless of its base composition. The presence of uracil or thymine is not a defining characteristic. ■

TABLE 8-1 Nucleotide and Nucleic Acid Nomenclature

Base	Nucleoside	Nucleotide	Nucleic acid
Purines			
Adenine	Adenosine Deoxyadenosine	Adenylate Deoxyadenylate	RNA DNA
Guanine	Guanosine Deoxyguanosine	Guanylate Deoxyguanylate	RNA DNA
Pyrimidines			
Cytosine	Cytidine Deoxycytidine	Cytidylate Deoxycytidylate	RNA DNA
Thymine	Thymidine or deoxythymidine	Thymidylate or deoxythymidylate	DNA
Uracil	Uridine	Uridylate	RNA

Note: "Nucleoside" and "nucleotide" are generic terms that include both ribo- and deoxyribo- forms. Also, ribonucleosides and ribonucleotides are here designated simply as nucleosides and nucleotides (e.g., riboadenosine as adenosine), and deoxyribonucleosides and deoxyribonucleotides as deoxynucleosides and deoxynucleotides (e.g., deoxyriboadenosine as deoxyadenosine). Both forms of naming are acceptable, but the shortened names are more commonly used. Thymine is an exception; "ribothymidine" is used to describe its unusual occurrence in RNA.

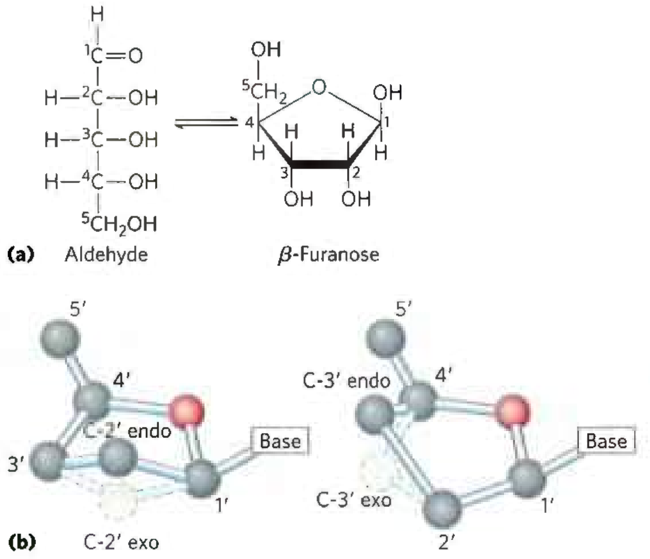


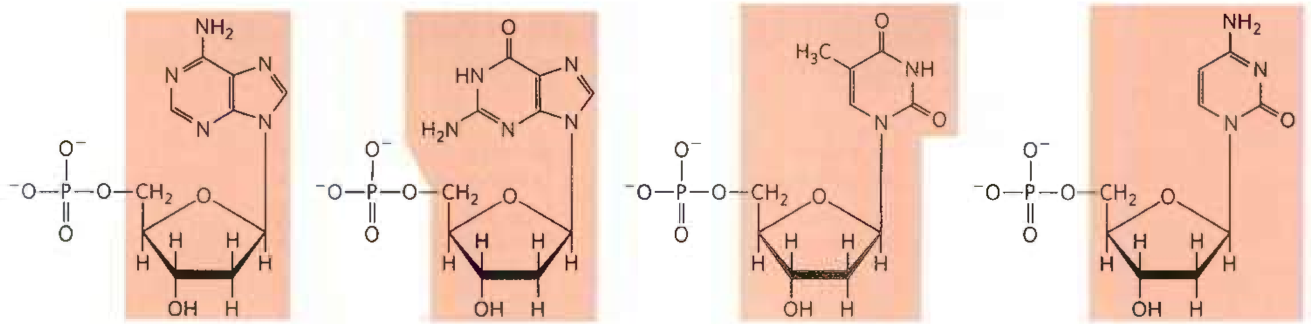
FIGURE 8-3 Conformations of ribose. (a) In solution, the straight-chain (aldehyde) and ring (β-furanose) forms of free ribose are in equilibrium. RNA contains only the ring form, β-D-ribofuranose. Deoxyribose undergoes a similar interconversion in solution, but in DNA it exists solely as β-2′-deoxy-D-ribofuranose. (b) Ribofuranose rings in nucleotides can exist in four different puckered conformations. In all cases, four of the five atoms are nearly in a single plane. The fifth atom (C-2′ or C-3′) is on either the same (endo) side or the opposite (exo) side of the plane relative to the C-5′ atom.

Figure 8-4a gives the structures and names of the four major **deoxyribonucleotides**, the structural units of DNAs, and Figure 8-4b shows the four major **ribonucleotides**, the structural units of RNAs. Deoxyribonucleotides are also referred to as deoxyribonucleoside 5′-monophosphates, deoxynucleotides, and deoxynucleoside triphosphates; ribonucleotides are also called ribonucleoside 5′-monophosphates.

Although nucleotides bearing the major purines and pyrimidines are most common, both DNA and RNA also contain some minor bases (Fig. 8-5). In DNA the most common of these are methylated forms of the major bases; in some viral DNAs, certain bases may be hydroxymethylated or glucosylated. Altered or unusual bases in DNA molecules often have roles in regulating or protecting the genetic information. Hundreds of different modified bases are also found in RNAs, especially in rRNAs and tRNAs (see Fig. 8-25 and Fig. 26-22). The modifications are usually introduced by enzymes that act after the RNA or DNA is synthesized.

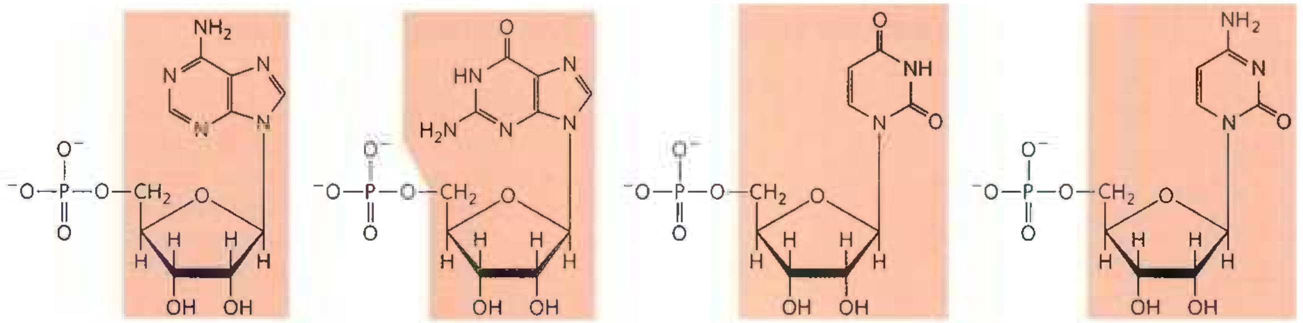
KEY CONVENTION The nomenclature for the minor bases can be confusing. Like the major bases, many minor bases have common names — hypoxanthine, for example, shown as its nucleoside inosine in Figure 8-5. When an atom in the purine ring or the pyrimidine ring is substituted, the usual convention (used here) is simply to indicate the ring position of the substituent by its number — for example, 5-methylcytosine, 7-methylguanine, and 5-hydroxymethylcytosine (shown as the nucleosides in Fig. 8-5). The element to which the substituent is attached (N, C, O) is not identified. The convention changes when the substituted atom is exocyclic (i.e., not within the ring structure), in which case the type of atom is identified, and the ring position to which it is attached is denoted with a superscript. The amino nitrogen attached to C-6 of adenine is N^6; similarly, the carbonyl oxygen and amino nitrogen at C-6 and C-2 of guanine are O^6 and N^2, respectively. Examples of this nomenclature are N^6-methyladenosine and N^2-methylguanosine (Fig. 8-5). ■

Cells also contain nucleotides with phosphate groups in positions other than on the 5′ carbon (Fig. 8-6). **Ribonucleoside 2′,3′-cyclic monophosphates** are isolatable intermediates, and **ribonucleoside 3′-monophosphates** are end products of the hydrolysis of RNA by certain ribonucleases. Other variations are adenosine 3′,5′-cyclic



Nucleotide:	Deoxyadenylate (deoxyadenosine 5′-monophosphate)	Deoxyguanylate (deoxyguanosine 5′-monophosphate)	Deoxythymidylate (deoxythymidine 5′-monophosphate)	Deoxycytidylate (deoxycytidine 5′-monophosphate)
Symbols:	A, dA, dAMP	G, dG, dGMP	T, dT, dTMP	C, dC, dCMP
Nucleoside:	Deoxyadenosine	Deoxyguanosine	Deoxythymidine	Deoxycytidine

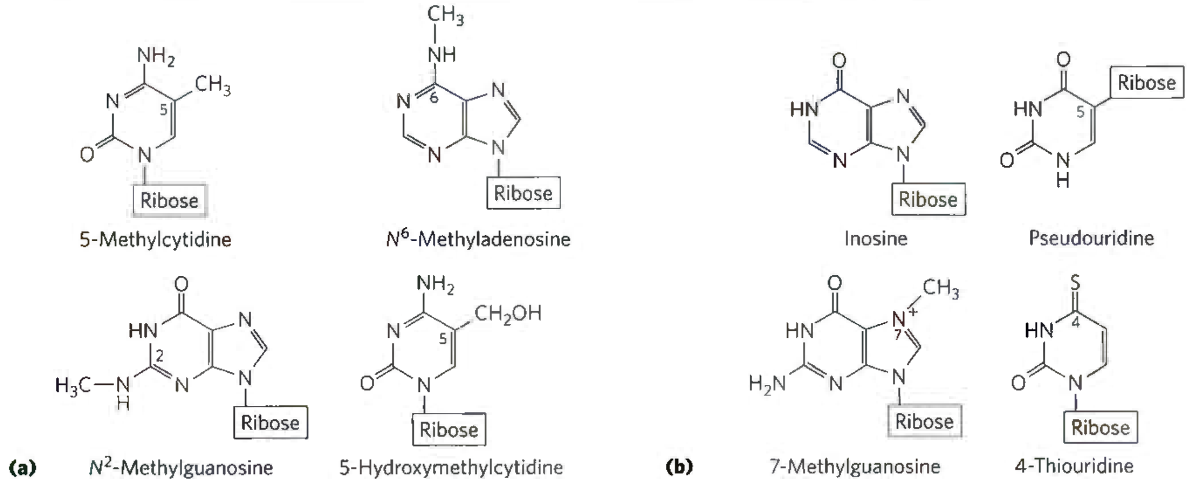
(a) Deoxyribonucleotides



Nucleotide:	Adenylate (adenosine 5′-monophosphate)	Guanylate (guanosine 5′-monophosphate)	Uridylate (uridine 5′-monophosphate)	Cytidylate (cytidine 5′-monophosphate)
Symbols:	A, AMP	G, GMP	U, UMP	C, CMP
Nucleoside:	Adenosine	Guanosine	Uridine	Cytidine

(b) Ribonucleotides

FIGURE 8-4 Deoxyribonucleotides and ribonucleotides of nucleic acids. All nucleotides are shown in their free form at pH 7.0. The nucleotide units of (a) DNA and (b) RNA are shown. For each nucleotide, the more common name is given, followed by the complete name in parentheses and symbols used to represent them. All abbreviations assume that the phosphate group is at the 5′ position. The nucleoside portion of each molecule is shaded in light red. In this and the following illustrations, the ring carbons are not shown.



5-Methylcytidine

N^6-Methyladenosine

Inosine

Pseudouridine

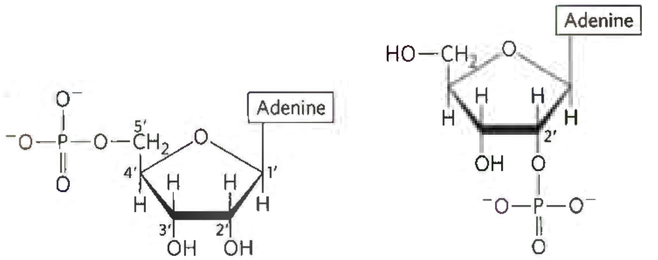
(a) N^2-Methylguanosine

5-Hydroxymethylcytidine

(b) 7-Methylguanosine

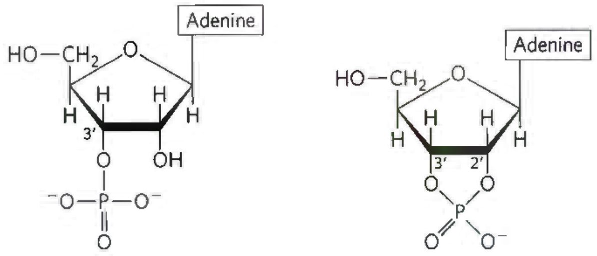
4-Thiouridine

FIGURE 8-5 Some minor purine and pyrimidine bases, shown as the nucleosides. (a) Minor bases of DNA. 5-Methylcytidine occurs in the DNA of animals and higher plants, N^6-methyladenosine in bacterial DNA, and 5-hydroxymethylcytidine in the DNA of animals and of bacteria infected with certain bacteriophages. (b) Some minor bases of tRNAs. Inosine contains the base hypoxanthine. Note that pseudouridine, like uridine, contains uracil; they are distinct in the point of attachment to the ribose — in uridine, uracil is attached through N-1, the usual attachment point for pyrimidines; in pseudouridine, uracil is attached through C-5.



Adenosine 5′-monophosphate

Adenosine 2′-monophosphate



Adenosine 3′-monophosphate

Adenosine 2′,3′-cyclic monophosphate

FIGURE 8-6 Some adenosine monophosphates. Adenosine 2′-monophosphate, 3′-monophosphate, and 2′,3′-cyclic monophosphate are formed by enzymatic and alkaline hydrolysis of RNA.

monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP), considered at the end of this chapter.

Phosphodiester Bonds Link Successive Nucleotides in Nucleic Acids

The successive nucleotides of both DNA and RNA are covalently linked through phosphate-group "bridges," in which the 5′-phosphate group of one nucleotide unit is joined to the 3′-hydroxyl group of the next nucleotide, creating a **phosphodiester linkage** (**Fig. 8-7**). Thus the covalent backbones of nucleic acids consist of alternating phosphate and pentose residues, and the nitrogenous bases may be regarded as side groups joined to the backbone at regular intervals. The backbones of both DNA and RNA are hydrophilic. The hydroxyl groups of the sugar residues form hydrogen bonds with water. The phosphate groups, with a pK_a near 0, are completely ionized and negatively charged at pH 7, and the negative charges are generally neutralized by ionic interactions with positive charges on proteins, metal ions, and polyamines.

KEY CONVENTION All the phosphodiester linkages in DNA and RNA have the same orientation along the chain (Fig. 8-7), giving each linear nucleic acid strand a specific polarity and distinct 5′ and 3′ ends. By definition, the **5′ end** lacks a nucleotide attached at the 5′ position, and the **3′ end** lacks a nucleotide attached at the 3′ position. Other groups (most often one or more phosphates) may be present on one or both ends. The 5′⟶3′ orientation of a strand of nucleic acid refers to the *ends* of the strand and the orientation of individual nucleotides, not the orientation of the individual phosphodiester bonds linking its constituent nucleotides. ■

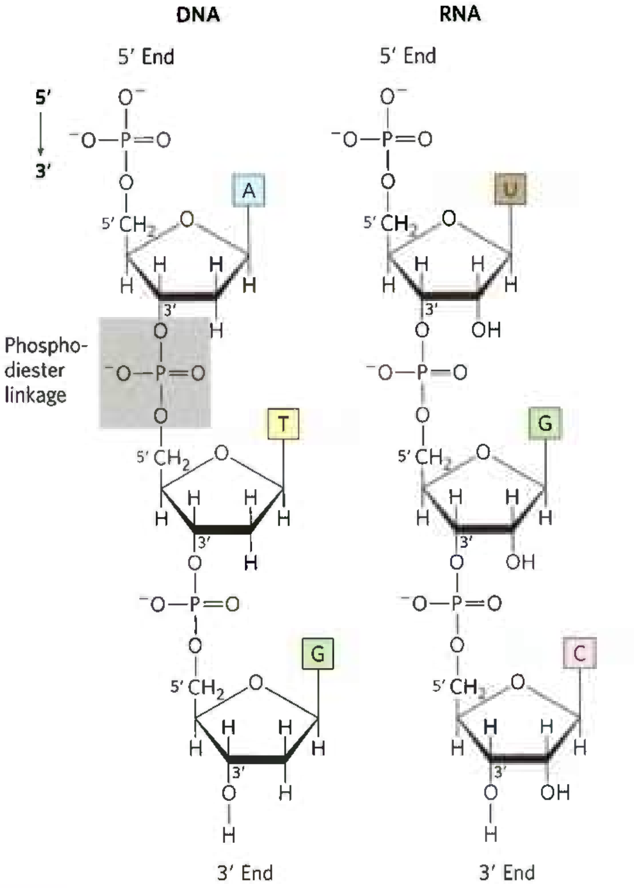
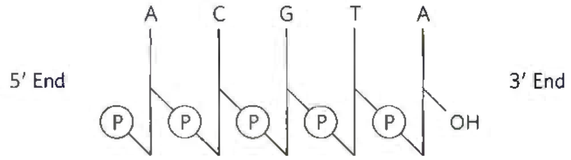


FIGURE 8-7 Phosphodiester linkages in the covalent backbone of DNA and RNA. The phosphodiester bonds (one of which is shaded in the DNA) link successive nucleotide units. The backbone of alternating pentose and phosphate groups in both types of nucleic acid is highly polar. The 5′ and 3′ ends of the macromolecule may be free or may have an attached phosphoryl group.

The covalent backbone of DNA and RNA is subject to slow, nonenzymatic hydrolysis of the phosphodiester bonds. In the test tube, RNA is hydrolyzed rapidly under alkaline conditions, but DNA is not; the 2′-hydroxyl groups in RNA (absent in DNA) are directly involved in the process. Cyclic 2′,3′-monophosphate nucleotides are the first products of the action of alkali on RNA and are rapidly hydrolyzed further to yield a mixture of 2′- and 3′-nucleoside monophosphates (**Fig. 8-8**).

The nucleotide sequences of nucleic acids can be represented schematically, as illustrated below by a segment of DNA with five nucleotide units. The phosphate groups are symbolized by Ⓟ, and each deoxyribose is symbolized by a vertical line, from C-1′ at the top to C-5′ at the bottom (but keep in mind that the sugar is always in its closed-ring β-furanose form in nucleic acids). The connecting lines between nucleotides (which pass through Ⓟ) are drawn diagonally from the middle (C-3′) of the deoxyribose of one nucleotide to the bottom (C-5′) of the next.



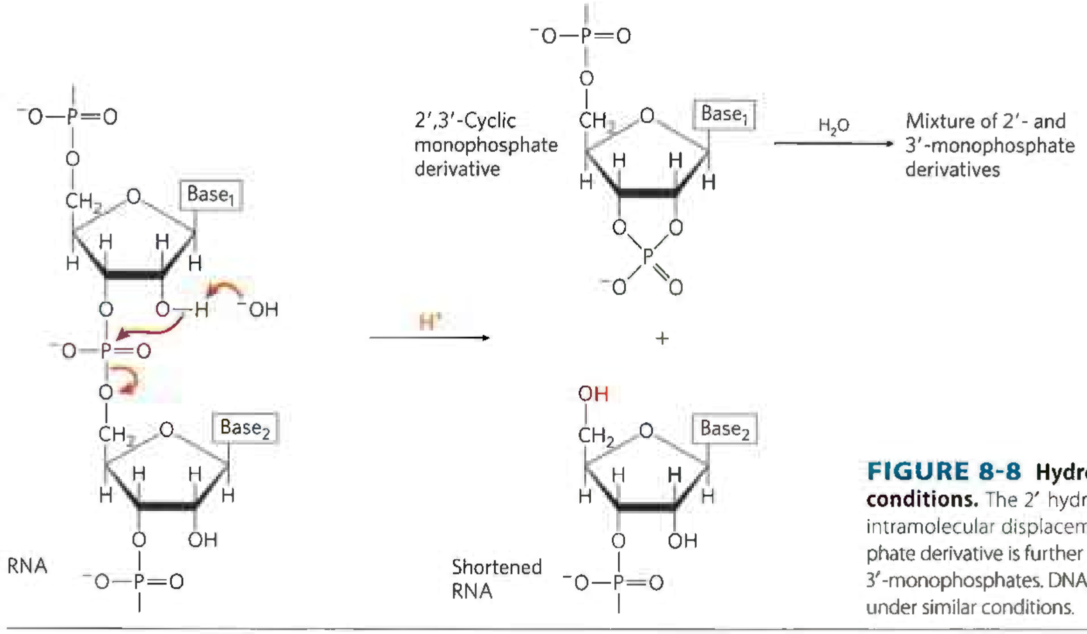

RNA
H^+
2′,3′-Cyclic monophosphate derivative
Base₁
Base₂
+
Shortened RNA
H_2O
Mixture of 2′- and 3′-monophosphate derivatives

FIGURE 8-8 Hydrolysis of RNA under alkaline conditions. The 2′ hydroxyl acts as a nucleophile in an intramolecular displacement. The 2′,3′-cyclic monophosphate derivative is further hydrolyzed to a mixture of 2′- and 3′-monophosphates. DNA, which lacks 2′ hydroxyls, is stable under similar conditions.

Some simpler representations of this pentadeoxyribonucleotide are pA-C-G-T-A_{OH}, pApCpGpTpA, and pACGTA.

KEY CONVENTION The sequence of a single strand of nucleic acid is always written with the 5′ end at the left and the 3′ end at the right—that is, in the 5′⟶3′ direction. ■

A short nucleic acid is referred to as an **oligonucleotide**. The definition of "short" is somewhat arbitrary, but polymers containing 50 or fewer nucleotides are generally called oligonucleotides. A longer nucleic acid is called a **polynucleotide**.

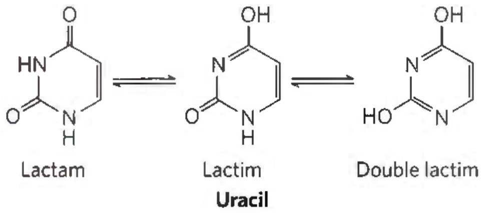


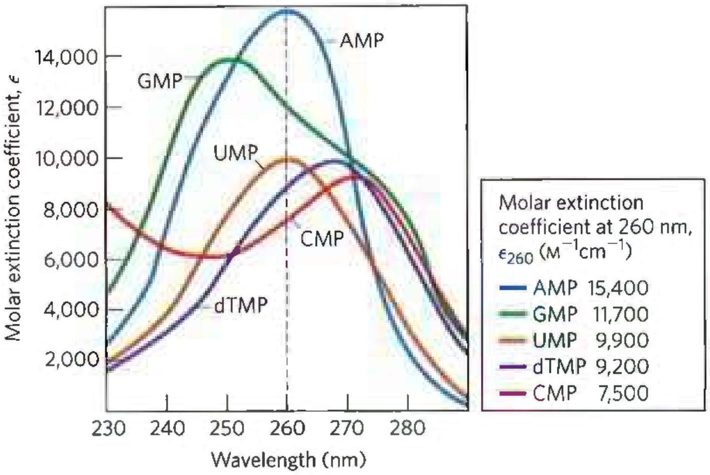
FIGURE 8-9 Tautomeric forms of uracil. The lactam form predominates at pH 7.0; the other forms become more prominent as pH decreases. The other free pyrimidines and the free purines also have tautomeric forms, but they are more rarely encountered.

The Properties of Nucleotide Bases Affect the Three-Dimensional Structure of Nucleic Acids

Free pyrimidines and purines are weakly basic compounds and thus are called bases. The purines and pyrimidines common in DNA and RNA are aromatic molecules (Fig. 8-2), a property with important consequences for the structure, electron distribution, and light absorption of nucleic acids. Electron delocalization among atoms in the ring gives most of the bonds in the ring partial double-bond character. One result is that pyrimidines are planar molecules and purines are very nearly planar, with a slight pucker. Free pyrimidine and purine bases may exist in two or more tautomeric forms depending on the pH. Uracil, for example, occurs in several readily interconverted forms called **tautomers**—lactam, lactim, and double lactim forms (**Fig. 8-9**). The structures shown in Figure 8-2 are the tautomers that predominate at pH 7.0. All nucleotide bases absorb UV light, and nucleic acids are characterized by a strong absorption at wavelengths near 260 nm (**Fig. 8-10**).

The purine and pyrimidine bases are hydrophobic and relatively insoluble in water at the near-neutral pH of the cell. At acidic or alkaline pH, the bases become charged and their solubility in water increases. Hydrophobic stacking interactions in which two or more bases are positioned with the planes of their rings parallel (like a stack of coins) are one of two important modes of interaction between bases in nucleic acids. The stacking also involves a combination of van der Waals and dipole-dipole interactions between the bases. Base stacking helps to minimize contact of the bases with water, and base-stacking interactions are very important in stabilizing the three-dimensional structure of nucleic acids, as described later.

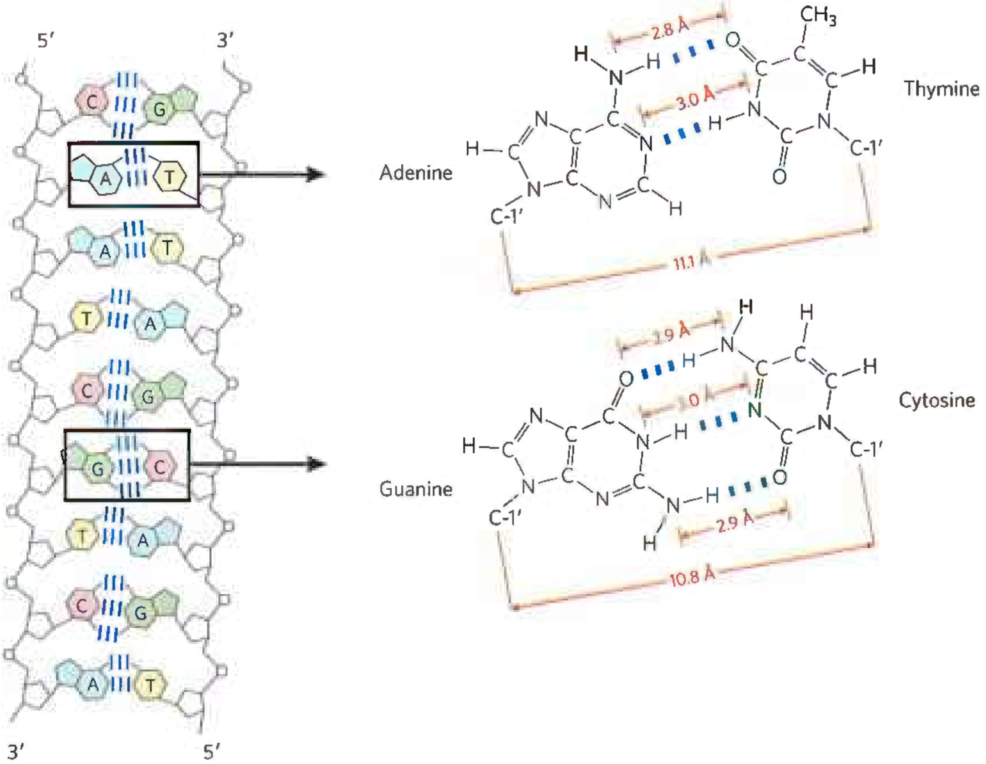
The functional groups of pyrimidines and purines are ring nitrogens, carbonyl groups, and exocyclic amino groups. Hydrogen bonds involving the amino and carbonyl groups are the most important mode of complementary interaction between two (and occasionally three or four) complementary strands of nucleic acid. The most common hydrogen-bonding patterns are those defined by James D. Watson and Francis Crick in 1953, in which A bonds specifically to T (or U) and G bonds to C (**Fig. 8-11**). These two types of **base pairs** predominate in double-stranded DNA and RNA, and the tautomers shown in Figure 8-2 are responsible for these patterns. P1 P2 It is this specific pairing of bases that permits the duplication of genetic information.



Molar extinction coefficient at 260 nm, ε_{260} ($M^{-1}cm^{-1}$)

AMP	15,400
GMP	11,700
UMP	9,900
dTMP	9,200
CMP	7,500

FIGURE 8-10 Absorption spectra of the common nucleotides. The spectra are shown as the variation in molar extinction coefficient with wavelength. The molar extinction coefficients at 260 nm and pH 7.0 (ε_{260}) are listed in the table. The spectra of corresponding ribonucleotides and deoxyribonucleotides, as well as the nucleosides, are essentially identical. For mixtures of nucleotides, a wavelength of 260 nm (dashed vertical line) is used for absorption measurements.



James D. Watson
[Bettmann/Getty Images.]

Francis Crick, 1916–2004
[Bettmann/Getty Images.]

FIGURE 8-11 Hydrogen-bonding patterns in the base pairs defined by Watson and Crick. Here, as elsewhere, hydrogen bonds are represented by three blue lines.

SUMMARY 8.1 *Some Basic Definitions and Conventions*

- A nucleotide consists of a nitrogenous base (purine or pyrimidine), a pentose sugar, and one or more phosphate groups. If no phosphate is present, the combination of the pentose sugar and the nitrogenous base is a nucleoside.
- Nucleic acids are polymers of nucleotides, joined together by phosphodiester linkages between the 5′-hydroxyl group of one pentose and the 3′-hydroxyl group of the next.
- There are two types of nucleic acid: RNA and DNA. The nucleotides in RNA contain ribose, and the common pyrimidine bases are uracil and cytosine. In DNA, the nucleotides contain 2′-deoxyribose, and the common pyrimidine bases are thymine and cytosine. The primary purines are adenine and guanine in both RNA and DNA. The nitrogenous bases have a hydrophobic character and interact via base-stacking interactions.

8.2 Nucleic Acid Structure

The discovery of the structure of DNA by Watson and Crick in 1953 gave rise to entirely new disciplines and influenced the course of many established ones. In this section we focus on DNA structure, some of the events that led to its discovery, and more recent refinements in our understanding of DNA. We also introduce RNA structure.

As in the case of protein structure (Chapter 4), it is sometimes useful to describe nucleic acid structure in terms of hierarchical levels of complexity (primary, secondary, tertiary). The primary structure of a nucleic acid is its covalent structure and nucleotide sequence. Any regular, stable structure taken up by some or all of the nucleotides in a nucleic acid can be referred to as secondary structure. Most structures considered in the remainder of this chapter fall under the heading of secondary structure. The complex folding of large chromosomes within eukaryotic chromatin and bacterial nucleoids, or the elaborate folding of large tRNA or rRNA molecules, is generally considered tertiary structure. DNA tertiary structure is discussed in Chapter 24. RNA tertiary structure is considered briefly in this chapter and more thoroughly in Chapter 26.

DNA Is a Double Helix That Stores Genetic Information

DNA was first isolated and characterized by Friedrich Miescher in 1869. He called the phosphorus-containing substance "nuclein." Not until the 1940s, with the work of Oswald T. Avery, Colin MacLeod, and Maclyn McCarty, was there any compelling evidence that DNA was the genetic material. Avery and his colleagues found that an extract of a virulent strain of the bacterium *Streptococcus pneumoniae* (causing disease in mice) could be used to transform a nonvirulent strain of the same bacterium into a virulent strain. They were able to demonstrate through various chemical tests that it was DNA from the virulent strain (not protein, polysaccharide, or RNA, for example) that carried the genetic information for virulence. Then in 1952, experiments by Alfred D. Hershey and Martha Chase — in which they studied the infection of bacterial cells by a virus (bacteriophage) with radioactively labeled DNA or protein — removed any remaining doubt that DNA, not protein, carried the genetic information.

Another important clue to the structure of DNA came from the work of Erwin Chargaff and his colleagues in the late 1940s. Examining dozens of species, they found that the four nucleotide bases of DNA occur in different ratios in the DNAs of different organisms. However, the base composition remains constant in different tissues of the same species, and does not vary with age, environment, nutritional state, or generation. Furthermore, regardless of the species, the number of adenosine residues is equal to the number of thymidine residues (that is, A = T), and the number of guanosine residues is equal to the number of cytidine residues (G = C). From these relationships it follows that the sum of the purine residues equals the sum of the pyrimidine residues; that is, A + G = T + C. These quantitative relationships, sometimes called "Chargaff's rules," were a key to establishing the three-dimensional structure of DNA.

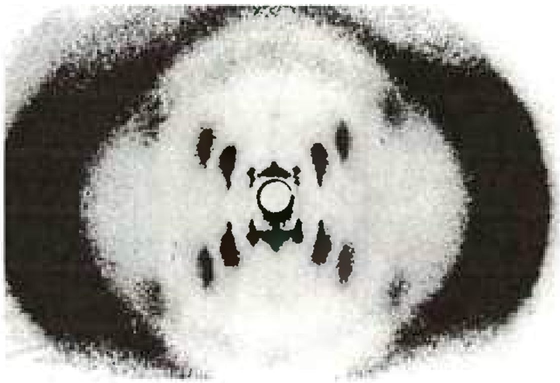


FIGURE 8-12 X-ray diffraction pattern of DNA fibers. The spots forming a cross in the center denote a helical structure. The heavy bands at the left and the right arise from the recurring bases. [Science Source.]

To shed more light on the structure of DNA, in the early 1950s Rosalind Franklin and Maurice Wilkins used the powerful method of x-ray diffraction (see Fig. 4-30) to analyze DNA fibers. Although lacking the molecular definition of diffraction from crystals, the x-ray diffraction pattern generated from the fibers was informative (**Fig. 8-12**). The pattern revealed that DNA molecules are helical, with two periodicities along their long axis: a primary one of 3.4 Å and a secondary one of 34 Å. The problem then was to formulate a three-dimensional model of the DNA molecule that could account not only for the x-ray diffraction data but also for the specific A = T and G = C base equivalences discovered by Chargaff and for the other chemical properties of DNA.

Rosalind Franklin, 1920–1958
[National Library of Medicine/Science Source]

Maurice Wilkins, 1916–2004
[Bettmann/Getty Images.]

James Watson and Francis Crick relied on this accumulated information about DNA to set about deducing its structure. In 1953 they postulated a three-dimensional model of DNA structure that accounted for all the available data. It consists of two helical DNA chains wound around the same axis to form a right-handed double helix. (See Box 4-1 for an explanation of the right- or left-handed sense of a helical structure.) The hydrophilic backbones of alternating deoxyribose and phosphate groups are on the outside of the double helix, facing the surrounding water. The furanose ring of each deoxyribose is in the C-2′ endo conformation. The purine and pyrimidine bases of both strands are stacked inside the double helix, with their hydrophobic and nearly planar ring structures very close together and perpendicular to the long axis. The offset pairing of the two strands creates a **major groove** and a **minor groove** on the surface of the duplex (**Fig. 8-13**). Each nucleotide base of one strand is paired in the same plane with a base of the other strand. Watson and Crick found that the hydrogen-bonded base pairs illustrated in Figure 8-11, G with C and A with T, are those that fit best within the structure, providing a rationale for Chargaff's rule that in any DNA, G = C and A = T. It is important to note that three hydrogen bonds can form between G and C, symbolized G≡C, but only two can form between A and T, symbolized A=T. Pairings of bases other than G with C and A with T tend (to varying degrees) to destabilize the double-helical structure.

When Watson and Crick constructed their model, they had to decide at the outset whether the strands of DNA should be **parallel** or **antiparallel**—whether their 3′,5′-phosphodiester bonds should run in the same or opposite directions. An antiparallel orientation produced the most convincing model, and later work with DNA polymerases (Chapter 25) provided experimental evidence that the strands are indeed antiparallel, a finding ultimately confirmed by x-ray analysis.

To account for the periodicities observed in the x-ray diffraction patterns of DNA fibers, Watson and Crick manipulated molecular models to arrive at a structure in which the vertically stacked bases inside the double helix would be 3.4 Å apart; the secondary repeat distance of about 34 Å was accounted for by the presence of 10 base pairs (bp) in each complete turn of the double helix. The structure in aqueous solution differs slightly from that in fibers, having 10.5 bp per helical turn (Fig. 8-13).

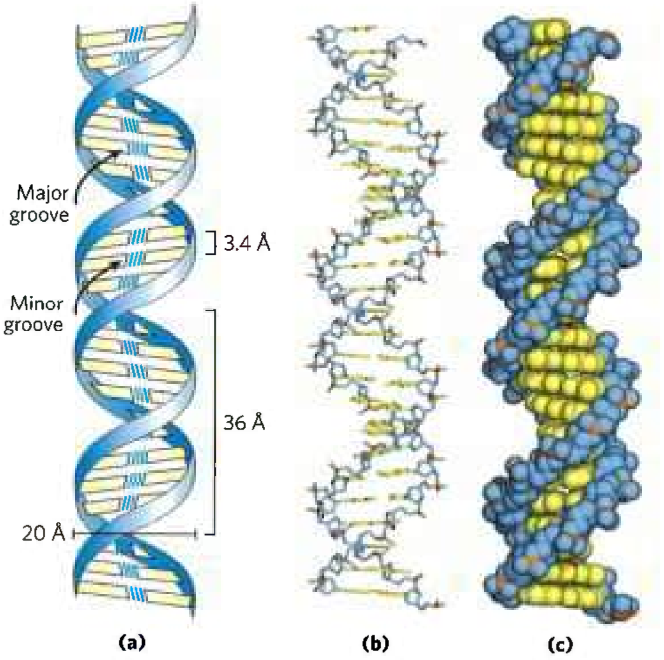


FIGURE 8-13 Watson-Crick model for the structure of DNA. The original model proposed by Watson and Crick had 10 bp, or 34 Å (3.4 nm), per turn of the helix; subsequent measurements revealed 10.5 bp, or 36 Å (3.6 nm), per turn. (a) Schematic representation, showing dimensions of the helix. (b) Stick representation showing the backbone and stacking of the bases. (c) Space-filling model.

As **Figure 8-14** shows, the two antiparallel polynucleotide chains of double-helical DNA are not identical in either base sequence or composition. Instead they are **complementary** to each other. Wherever adenine occurs in one chain, thymine is found in the other; similarly, wherever guanine occurs in one chain, cytosine is found in the other.

The DNA double helix, or duplex, is held together by hydrogen bonding between complementary base pairs (Fig. 8-11) and by base-stacking interactions. The complementarity between the DNA strands is attributable to the hydrogen bonding between base pairs; however, the hydrogen bonds do not contribute significantly to the stability of the structure. The double helix is primarily stabilized by metal cations, which shield the negative charges of backbone phosphates, and by base-stacking interactions between successive base pairs. Base-stacking interactions between successive G≡C or C≡G pairs are stronger than those between successive A=T and T=A pairs or adjacent pairs including all four bases. Because of this, DNA duplexes with higher G≡C content are more stable.

The important features of the double-helical model of DNA structure are now supported by much chemical and biological evidence. Moreover, the model immediately suggested a mechanism for the transmission of

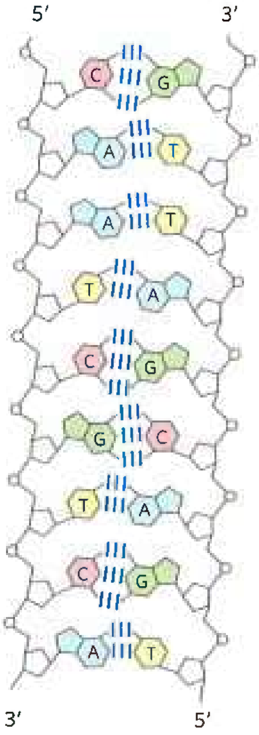


FIGURE 8-14 Complementarity of strands in the DNA double helix. The complementary antiparallel strands of DNA follow the pairing rules proposed by Watson and Crick. The base-paired antiparallel strands differ in base composition: the left strand has the composition $A_3T_2G_1C_3$; the right strand has $A_2T_3G_3C_1$. They also differ in sequence when each chain is read in the 5′→3′ direction. Note the base equivalences: A = T and G = C in the duplex.

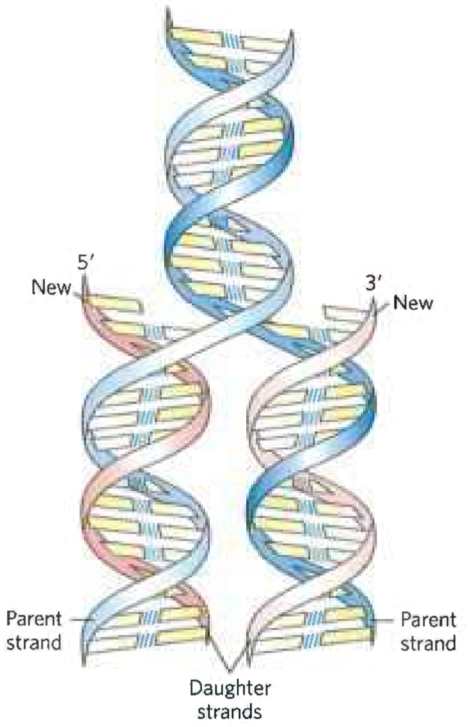


FIGURE 8-15 **Replication of DNA as suggested by Watson and Crick.** The preexisting or "parent" strands become separated, and each is the template for biosynthesis of a complementary "daughter" strand (in pink).

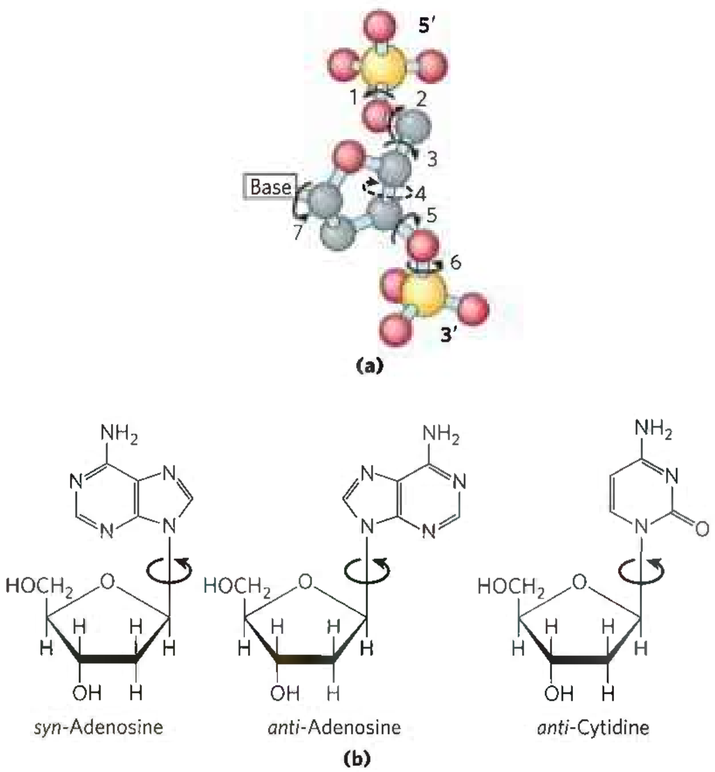


FIGURE 8-16 **Structural variation in DNA.** (a) The conformation of a nucleotide in DNA is affected by rotation about seven different bonds. Six of the bonds rotate freely. The limited rotation about bond 4 gives rise to ring pucker. This conformation is endo or exo, depending on whether the atom is displaced to the same side of the plane as C-5′ or to the opposite side (see Fig. 8-3b). (b) For purine bases in nucleotides, only two conformations with respect to the attached ribose units are sterically permitted: anti or syn. Pyrimidines occur in the anti conformation.

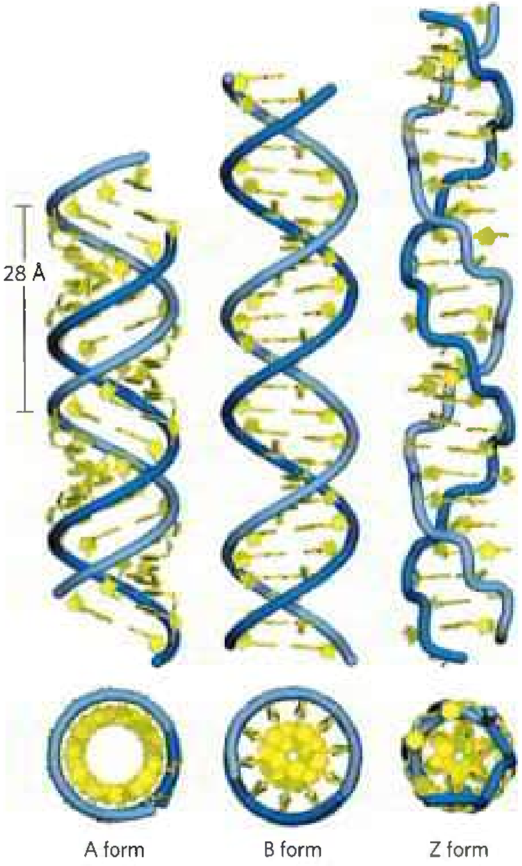
genetic information. The essential feature of the model was the complementarity of the two DNA strands. As Watson and Crick were able to see, well before confirmatory data became available, this structure could logically be replicated by (1) separating the two strands and (2) synthesizing a complementary strand for each. P1 P2 Because nucleotides in each new strand are joined in a sequence specified by the base-pairing rules stated above, each preexisting strand functions as a template to guide the synthesis of one complementary strand (**Fig. 8-15**). These expectations were experimentally confirmed, inaugurating a revolution in our understanding of biological inheritance.

DNA Can Occur in Different Three-Dimensional Forms

DNA is a remarkably flexible molecule. Considerable rotation is possible around several types of bonds in the sugar–phosphate (phosphodeoxyribose) backbone, and thermal fluctuation can produce bending, stretching, and unpairing (melting) of the strands. Many significant deviations from the Watson-Crick DNA structure are found in cellular DNA, some or all of which may be important in DNA metabolism. These structural variations generally do not affect the key properties of DNA defined by Watson and Crick: strand complementarity, antiparallel strands, and the requirement for A=T and G≡C base pairs.

Structural variation in DNA reflects three things: the different possible conformations of the deoxyribose, rotation about the contiguous bonds that make up the phosphodeoxyribose backbone (**Fig. 8-16a**), and free rotation about the C-1′–*N*-glycosyl bond (Fig. 8-16b). Because of steric constraints, purines in purine nucleotides are restricted to two stable conformations with respect to deoxyribose, called syn and anti (Fig. 8-16b). Pyrimidines are generally restricted to the anti conformation because of steric interference between the sugar and the carbonyl oxygen at C-2 of the pyrimidine.

The Watson-Crick structure is also referred to as **B-form DNA**, or B-DNA. The B form is the most stable structure for a random-sequence DNA molecule under physiological conditions and is therefore the standard point of reference in any study of the properties of DNA. Two structural variants that have been well characterized in crystal structures are the **A** and **Z forms**. These three DNA conformations are shown in **Figure 8-17**, with a summary of their properties. The A form is favored in many solutions that are relatively devoid of water. The DNA is still arranged in a right-handed double helix, but the helix is wider and the number of base pairs per helical turn is 11, rather than 10.5 as in B-DNA. The plane of the base pairs in A-DNA is tilted about 20° relative to B-DNA base pairs, thus the base pairs in A-DNA are not perfectly perpendicular to the helix axis. These structural changes deepen the major groove while making the minor groove



	A form	B form	Z form
Helical sense	Right-handed	Right-handed	Left-handed
Diameter	~26 Å	~20 Å	~18 Å
Base pairs per helical turn	11	10.5	12
Helix rise per base pair	2.6 Å	3.4 Å	3.7 Å
Base tilt normal to the helix axis	20°	6°	7°
Sugar pucker conformation	C-3′ endo	C-2′ endo	C-2′ endo for pyrimidines; C-3′ endo for purines
Glycosyl bond conformation	Anti	Anti	Anti for pyrimidines; syn for purines

FIGURE 8-17 Comparison of A, B, and Z forms of DNA. Each structure shown here has 36 bp. The riboses and bases are shown in yellow. The phosphodiester backbone is represented as a blue rope. Blue is the color used to represent DNA strands in later chapters. The table summarizes some properties of the three forms of DNA.

shallower. The reagents used to promote crystallization of DNA tend to dehydrate it, and thus most short DNA molecules tend to crystallize in the A form.

Z-form DNA is a more radical departure from the B structure; the most obvious distinction is the left-handed helical rotation. There are 12 bp per helical turn, and the structure appears more slender and elongated. The DNA backbone takes on a zigzag appearance. Certain nucleotide sequences fold into left-handed Z helices much more readily than others. Prominent examples are sequences in which pyrimidines alternate with purines, especially alternating C and G (that is, in the helix, alternating C≡G and G≡C pairs) or 5-methyl-C and G residues. To form the left-handed helix in Z-DNA, the purine residues flip to the syn conformation, alternating with pyrimidines in the anti conformation. The major groove is barely apparent in Z-DNA, and the minor groove is narrow and deep.

Whether A-DNA occurs in cells is uncertain, but there is evidence for some short stretches (tracts) of Z-DNA in both bacteria and eukaryotes. These Z-DNA tracts may play a role (as yet undefined) in regulating the expression of some genes or in genetic recombination.

Certain DNA Sequences Adopt Unusual Structures

Other sequence-dependent structural variations found in larger chromosomes may affect the function and metabolism of the DNA segments in their immediate vicinity. For example, bends occur in the DNA helix wherever four or more adenosine residues appear sequentially in one strand. Six adenosines in a row produce a bend of about 18°. The bending observed with this and other sequences may be important in the binding of some proteins to DNA.

A common type of DNA sequence is a **palindrome**. A palindrome is a word, phrase, or sentence that is spelled identically when read either forward or backward; two examples are ROTATOR and NURSES RUN. In DNA, the term is applied to regions of DNA with **inverted repeats**, such that an inverted, self-complementary sequence in one strand is repeated in the opposite orientation in the paired strand, as in **Figure 8-18**. The self-complementarity within each strand confers the potential to form **hairpin** or **cruciform** (cross-shaped) structures (**Fig. 8-19**). When the inverted repeat occurs within each individual strand of the DNA, the sequence is called a **mirror repeat**. Mirror repeats do not have complementary sequences within the same strand and thus cannot form hairpin or cruciform structures. Sequences of these types are found in almost every large DNA molecule and can encompass a few base pairs or thousands. The extent to which palindromes occur as cruciforms in cells is not known, although some cruciform structures have been demonstrated in vivo in *Escherichia coli*. Self-complementary sequences cause isolated single strands of DNA (or RNA) in solution to fold into complex structures containing multiple hairpins.

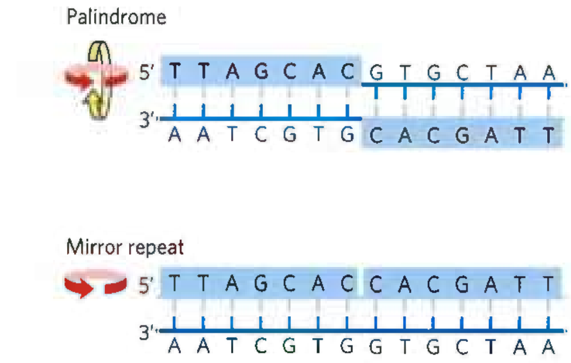

Palindrome
5′ T T A G C A C G T G C T A A
3′ A A T C G T G C A C G A T T
Mirror repeat
5′ T T A G C A C C A C G A T T
3′ A A T C G T G G T G C T A A

FIGURE 8-18 **Palindromes and mirror repeats.** Palindromes are sequences of double-stranded nucleic acids with twofold symmetry. To superimpose one repeat (shaded sequence) on the other, it must be rotated 180° about the horizontal axis and then 180° about the vertical axis, as shown by the colored arrows. A mirror repeat, on the other hand, has a symmetric sequence within each strand. Superimposing one repeat on the other requires only a single 180° rotation about the vertical axis.

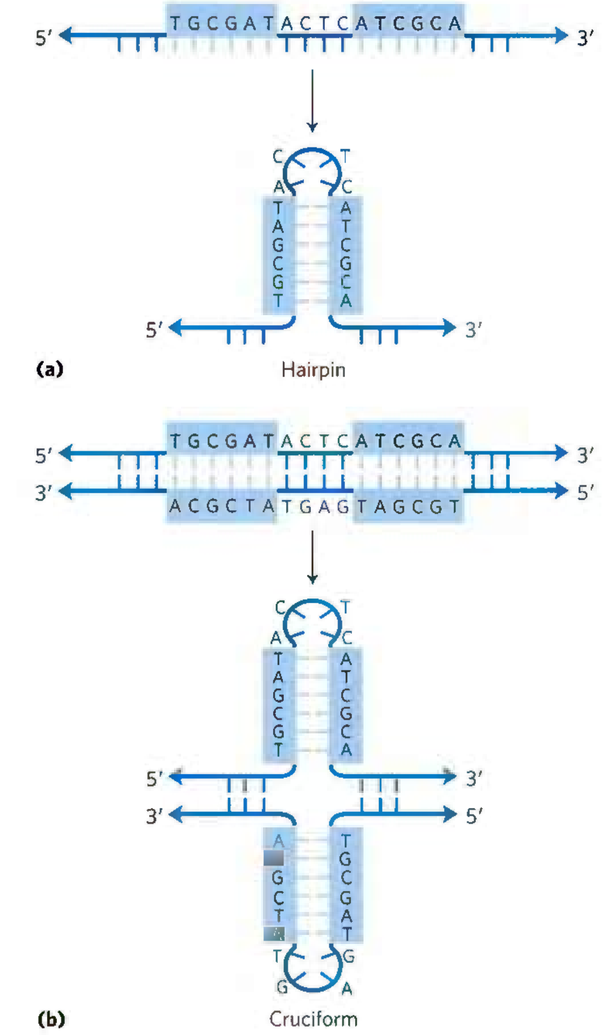


FIGURE 8-19 **Hairpins and cruciforms.** Palindromic DNA (or RNA) sequences can form alternative structures with intrastrand base pairing. (a) Hairpin structures involve a single DNA or RNA strand. (b) Cruciform structures involve both strands of a duplex DNA. Blue shading highlights asymmetric sequences that can pair with the complementary sequence either in the same strand or in the complementary strand.

Several unusual DNA structures are formed from three or even four DNA strands. Nucleotides participating in a Watson-Crick base pair (Fig. 8-11) can form additional hydrogen bonds with a third strand, particularly with functional groups arrayed in the major groove. For example, the guanosine residue of a $G\equiv C$ nucleotide pair can pair with a cytidine residue (if protonated) on a third strand (**Fig. 8-20a**); the adenosine of an $A{=}T$ pair can pair with a thymidine residue. The N-7, O^6, and N^6 of purines, the atoms that participate in the hydrogen bonding with a third DNA strand, are often referred to as **Hoogsteen positions**, and the non-Watson-Crick pairing is called **Hoogsteen pairing**, after Karst Hoogsteen, who in 1963 first recognized the potential for these unusual pairings. Hoogsteen pairing allows the formation of **triplex DNAs**. The triplexes shown in Figure 8-20 (a, b) are most stable at low pH because the $C\equiv G \cdot C^+$ triplet requires a protonated cytosine. In the triplex, the pK_a of this cytosine is >7.5, altered from its normal value of 4.2. The triplexes also form most readily within long sequences containing only pyrimidines or only purines in a given strand. Some triplex DNAs contain two pyrimidine strands and one purine strand; others contain two purine strands and one pyrimidine strand.

Four DNA strands can also pair to form a tetraplex (quadruplex), but this occurs readily only for DNA sequences with a very high proportion of guanosine residues (Fig. 8-20c, d). The guanosine tetraplex, or **G tetraplex**, is quite stable over a broad range of conditions. The orientation of strands in the tetraplex can vary as shown in Figure 8-20e.

In the DNA of living cells, sites recognized by many sequence-specific DNA-binding proteins (Chapter 28) are arranged as palindromes, and polypyrimidine or polypurine sequences that can form triple helices are found within regions involved in the regulation of expression of some eukaryotic genes.

Messenger RNAs Code for Polypeptide Chains

We now turn our attention to the expression of the genetic information that DNA contains. Given that the DNA of eukaryotes is largely confined to the nucleus, whereas protein synthesis occurs on ribosomes in the cytoplasm, some molecule other than DNA must carry the genetic message from the nucleus to the cytoplasm. As early as the 1950s, RNA was considered the logical candidate: RNA is found in both the nucleus and the cytoplasm, and an increase in protein synthesis is accompanied by an increase in the amount of cytoplasmic RNA and an increase in its rate of turnover. These and other observations led several researchers to suggest that RNA carries genetic information from DNA to the protein-synthesizing machinery of the ribosome. In 1961, François Jacob and Jacques Monod presented a unified (and essentially correct) picture of many aspects of this process. They proposed the name "messenger RNA" (mRNA) for that portion of the total

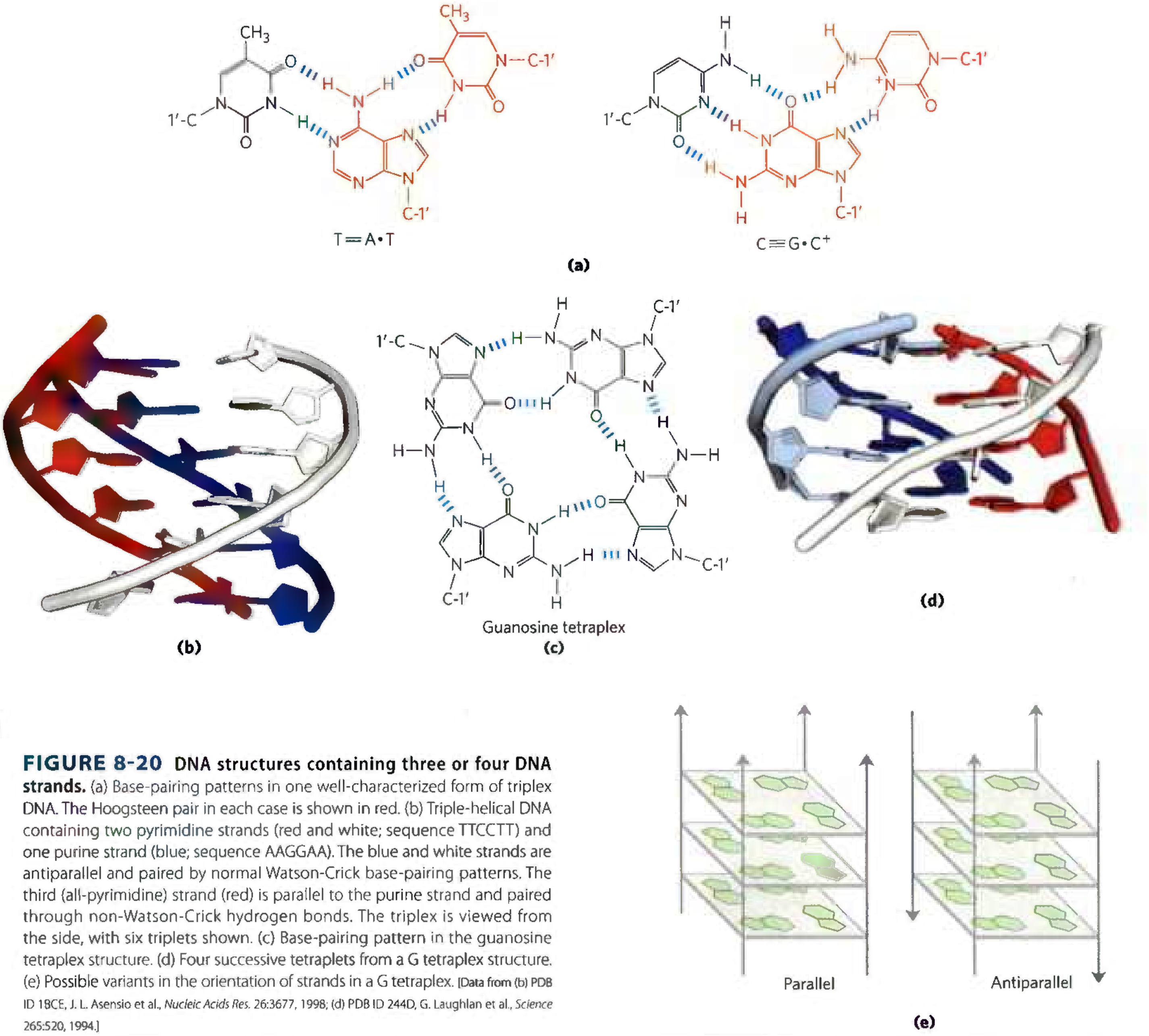
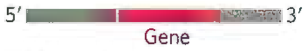


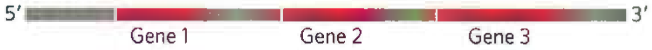
FIGURE 8-20 DNA structures containing three or four DNA strands. (a) Base-pairing patterns in one well-characterized form of triplex DNA. The Hoogsteen pair in each case is shown in red. (b) Triple-helical DNA containing two pyrimidine strands (red and white; sequence TTCCTT) and one purine strand (blue; sequence AAGGAA). The blue and white strands are antiparallel and paired by normal Watson-Crick base-pairing patterns. The third (all-pyrimidine) strand (red) is parallel to the purine strand and paired through non-Watson-Crick hydrogen bonds. The triplex is viewed from the side, with six triplets shown. (c) Base-pairing pattern in the guanosine tetraplex structure. (d) Four successive tetraplets from a G tetraplex structure. (e) Possible variants in the orientation of strands in a G tetraplex. [Data from (b) PDB ID 1BCE, J. L. Asensio et al., *Nucleic Acids Res.* 26:3677, 1998; (d) PDB ID 244D, G. Laughlan et al., *Science* 265:520, 1994.]

cellular RNA carrying the genetic information from DNA to the ribosomes. P1 P2 The mRNAs are formed on a DNA template by the process of **transcription**. Once they reach the ribosomes, the messengers provide the templates that specify amino acid sequences in polypeptide chains. Although mRNAs from different genes can vary greatly in length, the mRNAs from a particular gene generally have a defined size.

In bacteria and archaea, a single mRNA molecule may code for one or several polypeptide chains. If it carries the code for only one polypeptide, the mRNA is **monocistronic**; if it codes for two or more different polypeptides, the mRNA is **polycistronic**. In eukaryotes, most mRNAs are monocistronic. (For the purposes of this discussion, "cistron" refers to a gene. The term itself has historical roots in the science of genetics, and its formal genetic definition is beyond the scope of this text.) The minimum length of an mRNA is set in part by the length of the polypeptide chain for which it codes. For example, a polypeptide chain of 100 amino acid residues requires an RNA coding sequence of at least 300 nucleotides, because each amino acid is coded by a nucleotide triplet (this and other details of protein synthesis are discussed in Chapter 27). However, mRNAs transcribed from DNA are always somewhat longer than the length needed simply to code for a polypeptide sequence (or sequences). The additional, noncoding RNA includes sequences required to begin and end translation by the ribosome, as well as regulatory sequences. **Figure 8-21** summarizes the general structure of bacterial mRNAs.



(a) Monocistronic



(b) Polycistronic

FIGURE 8-21 Bacterial mRNA. Schematic diagrams show (a) monocistronic and (b) polycistronic mRNAs of bacteria. Red segments represent RNA coding for a gene product; gray segments represent noncoding RNA. In the polycistronic transcript, noncoding RNA separates the three genes.

Many RNAs Have More Complex Three-Dimensional Structures

Messenger RNA is only one of several classes of cellular RNA. Transfer RNAs are adapter molecules that act in protein synthesis; covalently linked to an amino acid at one end, each tRNA pairs with the mRNA in such a way that amino acids are joined to a growing polypeptide in the correct sequence. Ribosomal RNAs are components of ribosomes. There is also a wide variety of noncoding RNAs, including some (called ribozymes) that have enzymatic activity. All the RNAs are considered in detail in Chapter 26. The diverse and often complex functions of these RNAs reflect a diversity of structure much richer than that observed in DNA molecules.

The product of transcription of DNA is always single-stranded RNA. The single strand tends to assume a right-handed helical conformation dominated by base-stacking interactions (**Fig. 8-22**), which are stronger between two purines than between a purine and a pyrimidine or between two pyrimidines. The purine-purine interaction is so strong that a pyrimidine separating two purines is often displaced from the stacking pattern so that the purines can interact. Any self-complementary sequences in the molecule trigger folding into structures with more complexity. RNA can base-pair with complementary regions of either RNA or DNA. Base pairing matches the pattern for DNA: G pairs with C and A pairs with U (or with the occasional T residue in some RNAs). One difference is that base pairing between G and U residues is allowed in RNA (see Fig. 8-24) when complementary sequences in two single strands of RNA (or within a single strand of RNA that folds back on itself to align the residues) pair with each other. The paired strands in RNA or RNA-DNA duplexes are antiparallel, as in DNA.

When two strands of RNA with perfectly complementary sequences are paired, the predominant double-stranded structure is an A-form right-handed double helix. However, strands of RNA that are perfectly paired over long regions of sequence are uncommon. The three-dimensional structures of many RNAs, like those of proteins, are complex and unique. Weak interactions, especially base-stacking interactions, help stabilize RNA structures, just as they do in DNA. Z-form helices have been made in the laboratory (under very high-salt or high-temperature conditions). The B form of RNA has not been observed. Breaks in the regular A-form helix caused by mismatched or unmatched bases in one or both strands are common and result in bulges or internal loops (**Fig. 8-23**). Hairpin loops form between

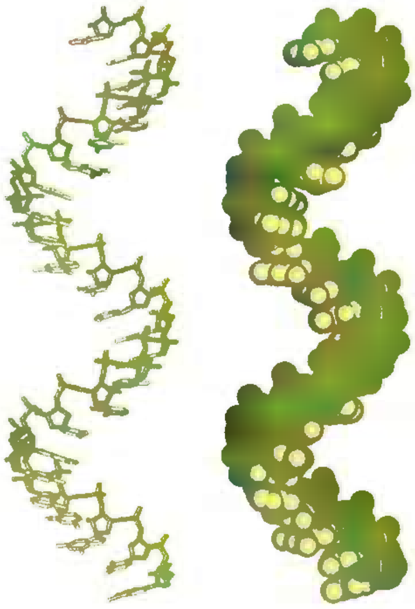


FIGURE 8-22 Typical right-handed stacking pattern of single-stranded RNA. The bases are shown in yellow, the phosphorus atoms in orange, and the riboses and phosphate oxygens in green. Green is used to represent RNA strands in succeeding chapters, just as blue is used for DNA.

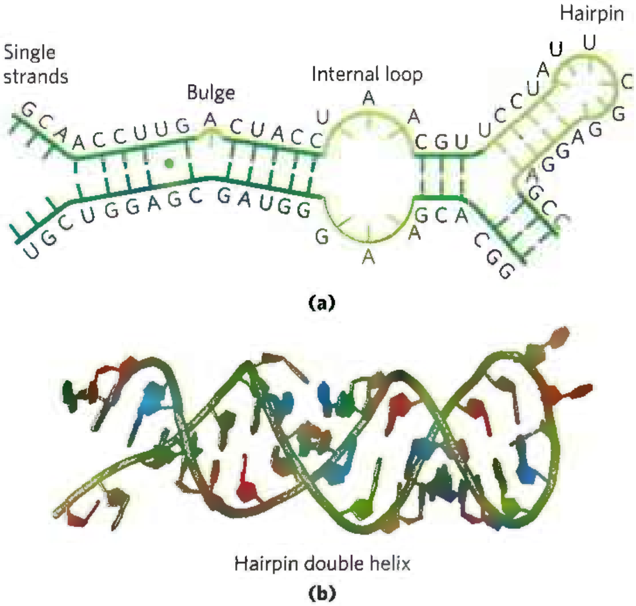


FIGURE 8-23 Secondary structure of RNAs. (a) Bulge, internal loop, and hairpin loop. (b) The paired regions generally have an A-form right-handed helix, as shown for a hairpin. The single UG base pair is identified with a green dot. [(b) Data from PDB ID 1GID, J. H. Cate et al., *Science* 273:1678, 1996.]

nearby self-complementary (palindromic) sequences. Extensive base-paired helical segments are formed in many RNAs (**Fig. 8-24**), and the resulting hairpins are the most common type of secondary structure in RNA. Specific short base sequences (such as UUCG) are often found at the ends of RNA hairpins and are known to form particularly tight and stable loops. Such sequences may act as starting points for the folding of an RNA molecule into its precise three-dimensional structure. Other contributions are made by hydrogen bonds that are not part of standard Watson-Crick base pairs. For example, the 2′-hydroxyl group of ribose can hydrogen-bond with other groups. Some of these properties are evident in the tertiary structure of the phenylalanine transfer RNA of yeast—the tRNA responsible for inserting Phe residues into polypeptides—and in two RNA enzymes, or ribozymes, whose functions, like those of protein enzymes, depend on their three-dimensional structures (**Fig. 8-25**).

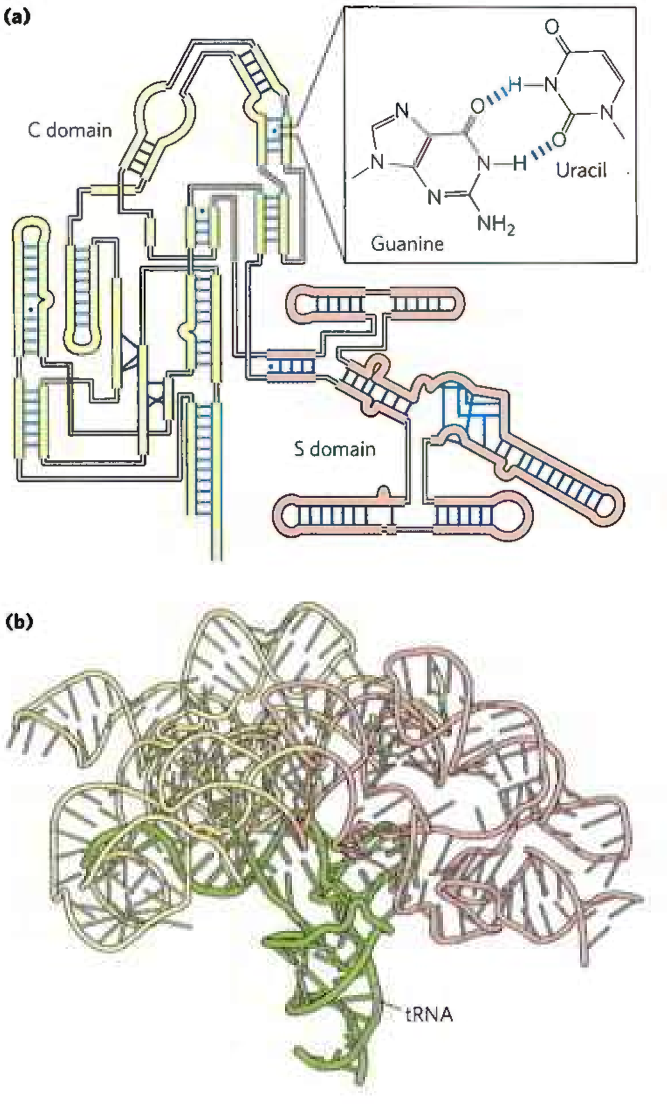


FIGURE 8-24 Base-paired helical structures in an RNA. Shown here are (a) the secondary structure and (b) the three-dimensional structure of the P RNA component of the RNase P of *Thermotoga maritima*. RNase P, which also contains a protein component (not shown), functions in the processing of transfer RNAs. A complexed tRNA is also shown in (b). Separate C (catalytic) and S (specificity) domains are denoted with yellow and light red backbones in both images. The blue dots in (a) indicate non-Watson-Crick G–U base pairs (boxed inset). Note that G–U base pairs are allowed only when presynthesized strands of RNA fold up or anneal with each other. [(a) Information from N. J. Reiter et al., *Nature* 468:784, 2010, Fig. 2a. (b) Data from PDB ID 3Q1R, N. J. Reiter et al., *Nature* 468:784, 2010.]

The analysis of RNA structure and the relationship between its structure and its function remains a robust field of inquiry that has many of the same complexities as the analysis of protein structure. The importance of understanding RNA structure grows as we become increasingly aware of the large number of functional roles for RNA molecules.

SUMMARY 8.2 *Nucleic Acid Structure*

- Many lines of evidence show that DNA bears genetic information. Some of the earliest evidence came from the Avery-MacLeod-McCarty experiment, which showed that DNA isolated from one bacterial strain can enter and transform the cells of another strain, endowing the second strain with some of the inheritable characteristics of the donor. The Hershey-Chase experiment showed that the DNA of a bacterial virus, but not its protein coat, carries the genetic message for replication of the virus in a host cell.
- Putting together the available data, Watson and Crick postulated that native DNA consists of two antiparallel chains in a right-handed double-helical arrangement. Complementary base pairs, $A{=}T$ and $G{\equiv}C$, are formed by hydrogen bonding between chains in the helix. The base pairs are stacked perpendicular to the long axis of the double helix, 3.4 Å apart, with 10.5 bp per turn.
- DNA can exist in several structural forms. Two variations of the Watson-Crick form, or B-DNA, are A- and Z-DNA.
- Some sequence-dependent structural variations cause bends in the DNA molecule. DNA strands with appropriate sequences can form hairpin or cruciform structures or triplex or tetraplex DNA.
- Messenger RNA transfers genetic information from DNA to ribosomes for protein synthesis.
- Transfer RNA and ribosomal RNA are also involved in protein synthesis. RNA can be structurally complex; single RNA strands can fold into hairpins, double-stranded regions, or complex loops. Additional noncoding RNAs have a variety of special functions.

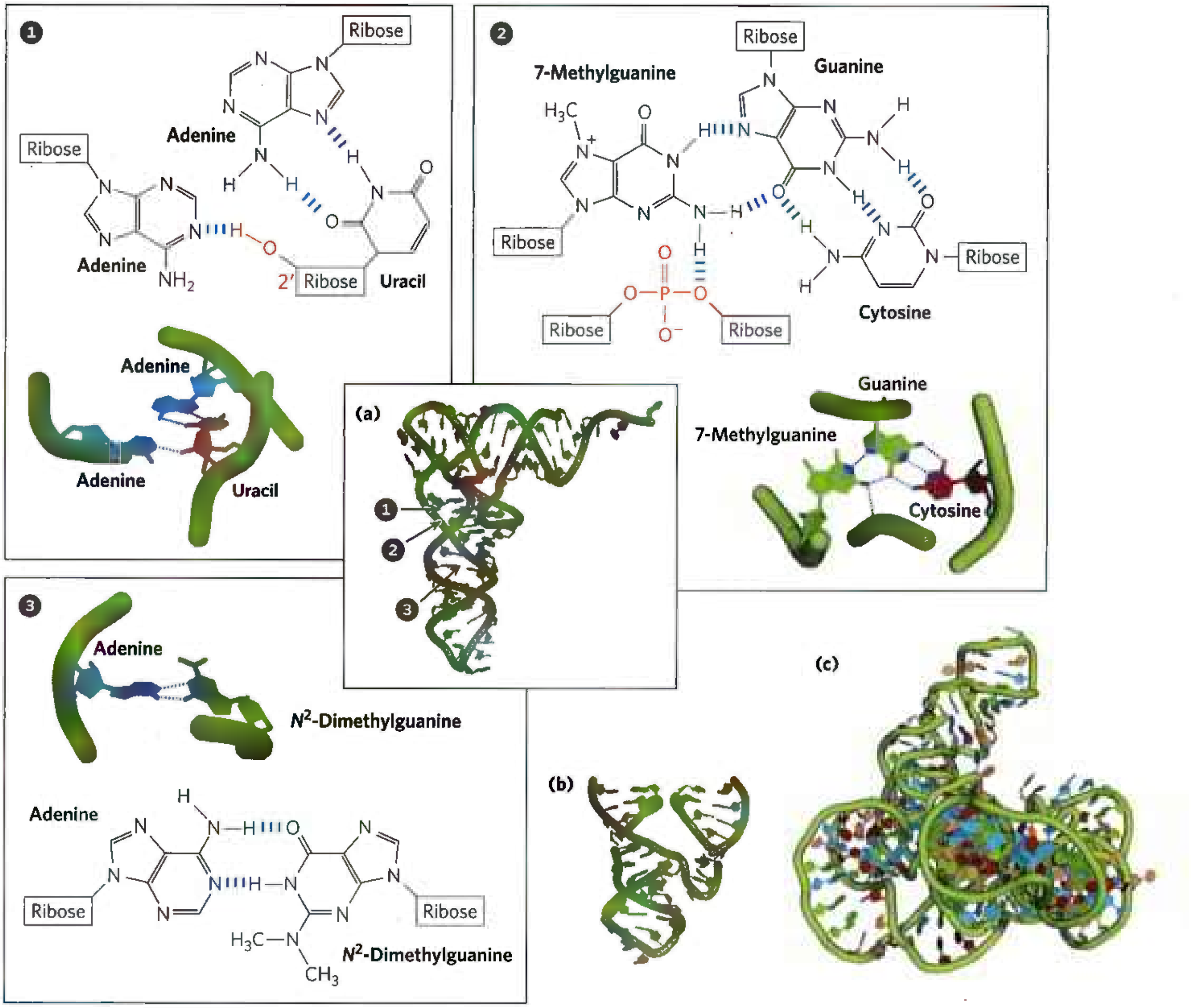


FIGURE 8-25 Three-dimensional structure in RNA. (a) Three-dimensional structure of phenylalanine tRNA of yeast. Some unusual base-pairing patterns found in this tRNA are shown in the numbered insets. Note in ① a hydrogen bond with a ribose 2′-hydroxyl group and in ② a hydrogen bond with the oxygen of a ribose phosphodiester (both shown in red). (b) A hammerhead ribozyme (so named because the secondary structure at the active site looks like the head of a hammer), derived from certain plant viruses. Ribozymes, or RNA enzymes, catalyze a variety of reactions, primarily in RNA metabolism and protein synthesis. The complex three-dimensional structures of these RNAs reflect the complexity inherent in catalysis, as described for protein enzymes in Chapter 6. (c) A segment of mRNA known as an intron, from the ciliated protozoan *Tetrahymena thermophila*. This intron (a ribozyme) catalyzes its own excision from between exons in an mRNA strand (discussed in Chapter 26). [Data from (a) PDB ID 1TRA, E. Westhof and M. Sundaralingam, *Biochemistry* 25:4868, 1986; (b) PDB ID 1MME, W. G. Scott et al., *Cell* 81:991, 1995; (c) PDB ID 1GRZ, B. L. Golden et al., *Science* 282:259, 1998.]

8.3 Nucleic Acid Chemistry

P1 P3 The role of DNA as a repository of genetic information depends in part on its inherent stability. The chemical transformations that do occur are generally very slow in the absence of an enzyme catalyst. The long-term storage of information without alteration is so important to a cell, however, that even very slow reactions that alter DNA structure can be physiologically significant. Processes such as carcinogenesis and aging may be intimately linked to slowly accumulating, irreversible alterations of DNA. Other, nondestructive alterations also occur and are essential to function, such as the strand separation that must precede DNA replication or transcription. In addition to providing insights into physiological processes, our understanding of nucleic acid chemistry has given us a powerful array of technologies that have applications in molecular biology, medicine, agriculture, and forensic science. We now examine the chemical properties of DNA and a few of these technologies.

Double-Helical DNA and RNA Can Be Denatured

Solutions of carefully isolated, native DNA are highly viscous at pH 7.0 and room temperature (25 °C). When such a solution is subjected to extremes of pH or to temperatures above 80 °C, its viscosity decreases sharply, indicating that the DNA has undergone a physical change. Just as heat and extremes of pH denature globular proteins, they also cause denaturation, or melting, of double-helical DNA. Disruption of the hydrogen bonds between paired bases and of base-stacking interactions causes unwinding of the double helix to form two single strands, completely separate from each other along the entire length or part of the length (partial denaturation) of the molecule. No covalent bonds in the DNA are broken (**Fig. 8-26**).

When the temperature or pH is returned to the range in which most organisms live, the unwound segments of the two strands spontaneously rewind, or **anneal**, to yield the intact duplex (Fig. 8-26). However, if the two strands are completely separated, renaturation occurs in two steps. In the first, relatively slow step, the two strands "find" each other by random collisions and form a short segment of complementary double helix. The second step is much faster: the remaining unpaired bases successively come into register as base pairs, and the two strands "zipper" themselves together to form the double helix.

The close interaction between stacked bases in a nucleic acid has the effect of decreasing its absorption of UV light relative to that of a solution with the same concentration of free nucleotides, and the absorption is decreased further when two complementary nucleic acid strands are paired. This is called the **hypochromic effect**. Denaturation of a double-stranded nucleic acid produces the opposite result: an increase in absorption called the **hyperchromic effect**. The transition from double-stranded DNA to the denatured, single-stranded form can thus be detected by monitoring UV absorption at 260 nm.

Viral or bacterial DNA molecules in solution denature when they are heated slowly (**Fig. 8-27**). Each species of DNA has a characteristic denaturation temperature, or melting point (t_m; formally, the temperature at which half the DNA is present as separated single strands): the higher its content of $G\equiv C$ base pairs, the higher the melting point of the DNA. This is primarily because, as we saw earlier, $G\equiv C$ base pairs make greater contributions to base stacking than do $A{=}T$ base pairs. Thus, the melting point of a DNA molecule, determined under fixed conditions of pH and ionic strength, can yield an estimate of its base composition. If denaturation conditions are carefully controlled, regions that are rich in $A{=}T$ base pairs will denature while most of the DNA remains double-stranded. Such denatured regions (called bubbles) can be visualized with electron microscopy (**Fig. 8-28**). In the strand separation of DNA that occurs in vivo during processes

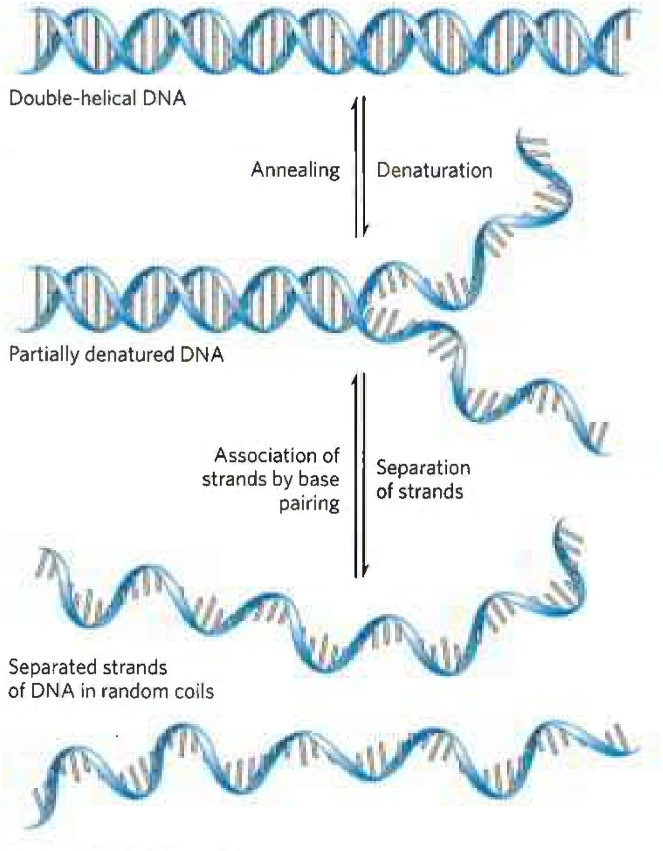


FIGURE 8-26 **Reversible denaturation and annealing (renaturation) of DNA.**

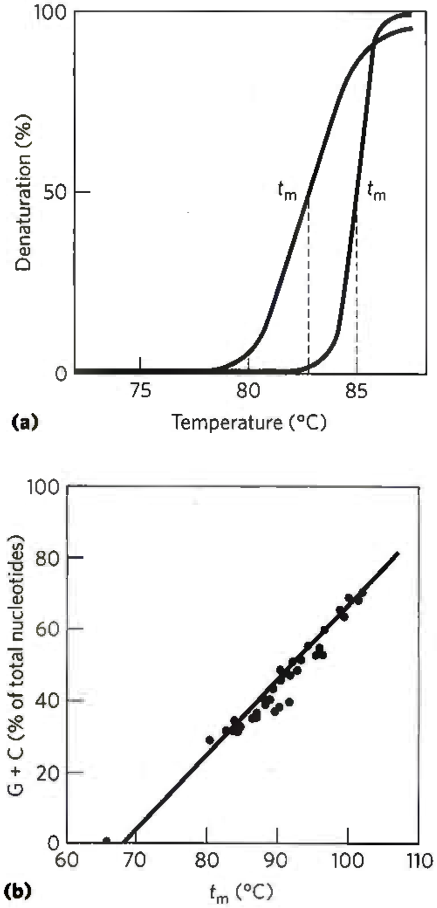


FIGURE 8-27 **Heat denaturation of DNA.** (a) The denaturation, or melting, curves of two DNA specimens. The temperature at the midpoint of the transition (t_m) is the melting point; it depends on pH and ionic strength and on the size and base composition of the DNA. (b) Relationship between t_m and the G+C content of a DNA. [(b) Data from J. Marmur and P. Doty, *J. Mol. Biol.* 5:109, 1962.]

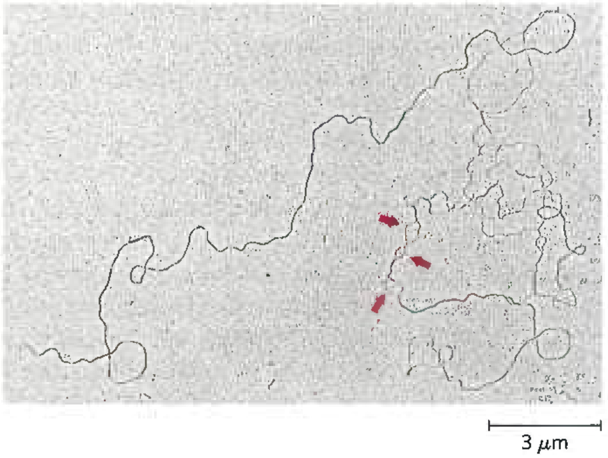


FIGURE 8-28 Partially denatured DNA. This DNA was partially denatured, then fixed to prevent renaturation during sample preparation. Although the shadowing method used to visualize the DNA in this electron micrograph obliterates many details, single-stranded and double-stranded regions are readily distinguishable. The arrows point to some single-stranded bubbles where denaturation has occurred. The regions that denature are highly reproducible and are rich in A═T base pairs. [Ross B. Inman.]

such as DNA replication and transcription, the site where strand separation is initiated is often rich in A═T base pairs, as we shall see.

Duplexes of two RNA strands or of one RNA strand and one DNA strand (RNA-DNA hybrids) can also be denatured. Notably, RNA duplexes are more stable to heat denaturation than DNA duplexes. At neutral pH, denaturation of a double-helical RNA often requires temperatures at least 20 °C higher than those required for denaturation of a DNA molecule with a comparable sequence, assuming that the strands in each molecule are perfectly complementary. The stability of an RNA-DNA hybrid is generally intermediate between that of RNA and DNA duplexes. The physical basis for these differences in thermal stability is not known.

WORKED EXAMPLE 8-1 *DNA Base Pairs and DNA Stability*

In samples of DNA isolated from two unidentified species of bacteria, X and Y, adenine makes up 32% and 17%, respectively, of the total bases. What relative proportions of adenine, guanine, thymine, and cytosine would you expect to find in the two DNA samples? What assumptions have you made? One of these species was isolated from a hot spring (64 °C). Which species is most likely the thermophilic bacterium, and why?

SOLUTION: For any double-helical DNA, A = T and G = C. The DNA from species X has 32% A and therefore must contain 32% T. This accounts for 64% of the bases and leaves 36% as G≡C pairs: 18% G and 18% C. The sample from species Y, with 17% A, must contain 17% T, accounting for 34% of the base pairs. The remaining 66% of the bases are thus equally distributed as 33% G and 33% C. This calculation is based on the assumption that both DNA molecules are double-stranded.

The higher the G+C content of a DNA molecule, the higher the melting temperature. Species Y, having the DNA with the higher G+C content (66%), most likely is the thermophilic bacterium; its DNA has a higher melting temperature and thus is more stable at the temperature of the hot spring.

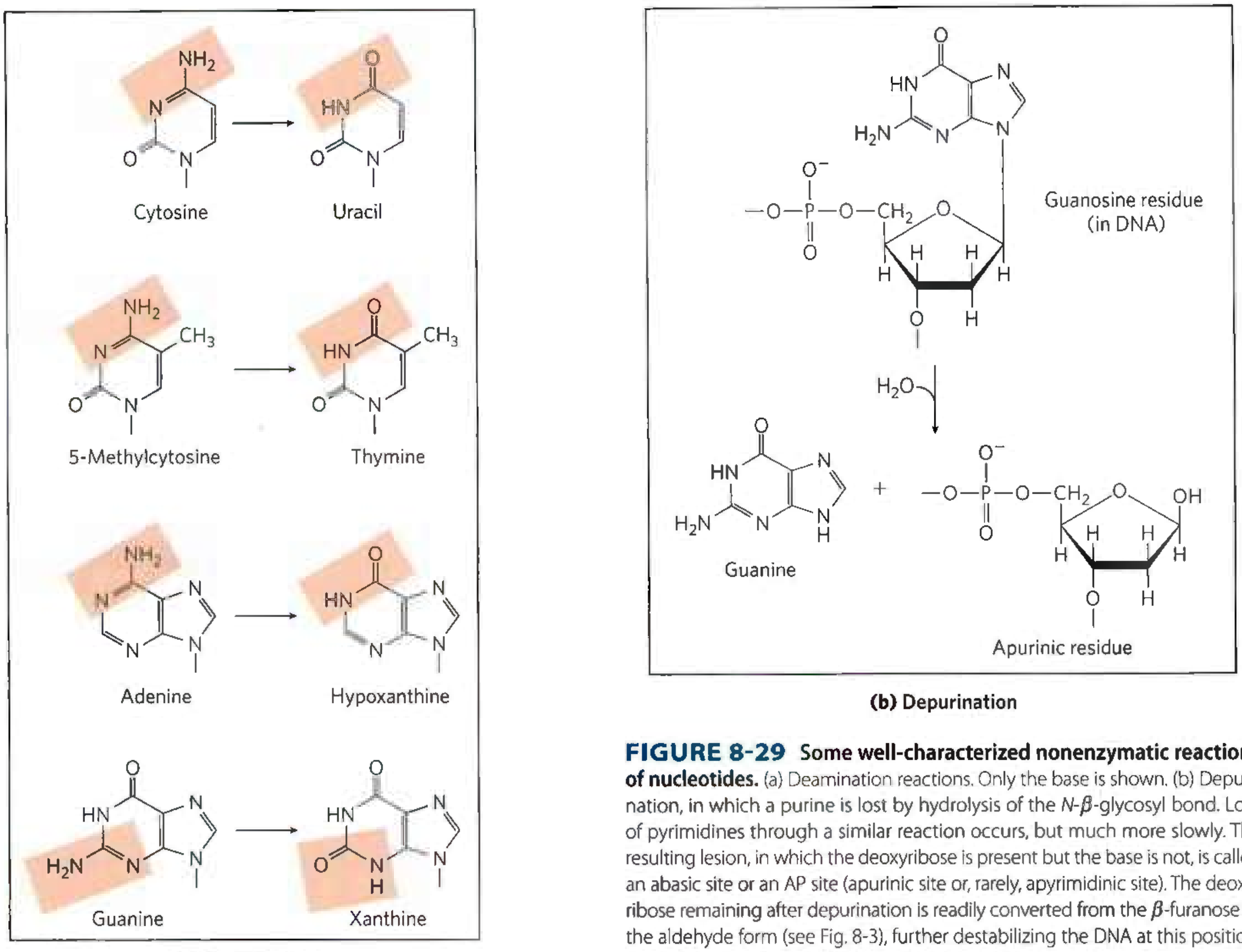
Nucleotides and Nucleic Acids Undergo Nonenzymatic Transformations

P3 Purines and pyrimidines, along with the nucleotides of which they are a part, undergo spontaneous alterations in their covalent structure. The rate of these reactions is generally *very slow*, but they are physiologically significant because of the cell's very low tolerance for alterations in its genetic information. Alterations in DNA structure that produce permanent changes in the genetic information encoded therein are called **mutations**. In higher organisms, much evidence suggests an intimate link between the accumulation of mutations in an individual and the processes of aging and carcinogenesis.

Several nucleotide bases undergo spontaneous loss of their exocyclic amino groups (deamination) (**Fig. 8-29a**). For example, under typical cellular conditions, deamination of cytosine (in DNA) to uracil occurs in about one of every 10^7 cytidine residues in 24 hours. This rate of deamination corresponds to about 100 spontaneous events per day, on average, in a mammalian cell. Deamination of adenine and guanine occurs at about 1/100th this rate.

The slow cytosine deamination reaction seems innocuous enough, but it is almost certainly the reason why DNA contains thymine rather than uracil. The product of cytosine deamination (uracil) is readily recognized as foreign in DNA and is removed by a repair system (Chapter 25). If DNA normally contained uracil, recognition of uracils resulting from cytosine deamination would be more difficult, and unrepaired uracils would lead to permanent sequence changes as they were paired with adenines during replication. Cytosine deamination would gradually lead to a decrease in G≡C base pairs and an increase in A═U base pairs in the DNA of all cells. Over the millennia, cytosine deamination could eliminate G≡C base pairs and the genetic code that depends on them. P3 Establishing thymine as one of the four bases in DNA may well have been one of the crucial turning points in evolution, making the long-term storage of genetic information possible.

Another important reaction in deoxyribonucleotides is the hydrolysis of the *N*-β-glycosyl bond between the base and the pentose. The base is lost, creating a DNA lesion called an AP (apurinic, apyrimidinic) site or abasic site (Fig. 8-29b). Purines are lost at a higher rate than pyrimidines. As many as one in 10^5 purines



(a) Deamination

(b) Depurination

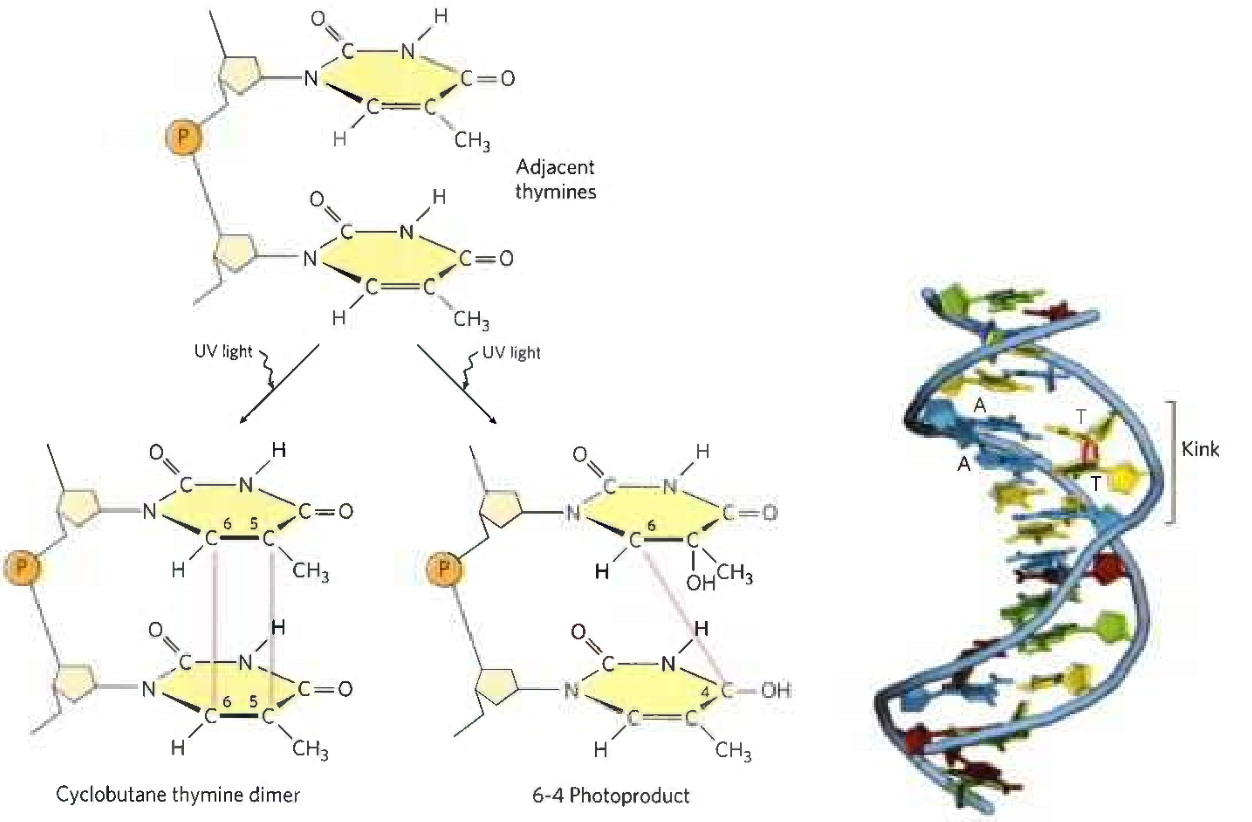
FIGURE 8-29 Some well-characterized nonenzymatic reactions of nucleotides. (a) Deamination reactions. Only the base is shown. (b) Depurination, in which a purine is lost by hydrolysis of the *N*-*β*-glycosyl bond. Loss of pyrimidines through a similar reaction occurs, but much more slowly. The resulting lesion, in which the deoxyribose is present but the base is not, is called an abasic site or an AP site (apurinic site or, rarely, apyrimidinic site). The deoxyribose remaining after depurination is readily converted from the *β*-furanose to the aldehyde form (see Fig. 8-3), further destabilizing the DNA at this position. More nonenzymatic reactions are illustrated in Figures 8-30 and 8-31.

(10,000 per mammalian cell) are lost from DNA every 24 hours under typical cellular conditions. Depurination of ribonucleotides and RNA is much slower and less physiologically significant. In the test tube, loss of purines can be accelerated by dilute acid. Incubation of DNA at pH 3 causes selective removal of the purine bases, resulting in a derivative called apurinic acid.

Other reactions are promoted by radiation. UV light induces the condensation of two ethylene groups to form a cyclobutane ring. In the cell, the same reaction between adjacent pyrimidine bases in nucleic acids forms cyclobutane pyrimidine dimers. This happens most frequently between adjacent thymidine residues on the same DNA strand (**Fig. 8-30**). A second type of pyrimidine dimer, called a 6-4 photoproduct, is also formed during UV irradiation. Ionizing radiation (x-rays and gamma rays) can cause ring opening and fragmentation of bases as well as breaks in the covalent backbone of nucleic acids.

Virtually all forms of life are exposed to energy-rich radiation capable of causing chemical changes in DNA. Near-UV radiation (with wavelengths of 200 to 400 nm), which makes up a significant portion of the solar spectrum, is known to cause pyrimidine dimer formation and other chemical changes in the DNA of bacteria and of human skin cells. We are subjected to a constant field of ionizing radiation in the form of cosmic rays, which can penetrate deep into the earth, as well as radiation emitted from radioactive elements, such as radium, plutonium, uranium, radon, ^{14}C, and ^{3}H. X-rays used in medical and dental examinations and in radiation therapy of cancer and other diseases are another form of ionizing radiation. It is estimated that UV and ionizing radiations are responsible for about 10% of all DNA damage caused by environmental agents.

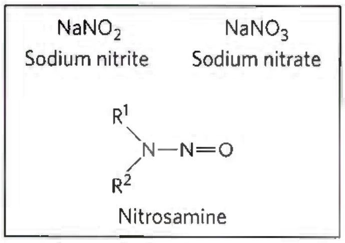
DNA also may be damaged by reactive chemicals introduced into the environment as products of industrial activity. Such products may not be injurious per se but may be metabolized by cells into forms that are. There are two prominent classes of such agents (**Fig. 8-31**): (1) deaminating agents, particularly nitrous acid (HNO_2) or compounds that can be metabolized to nitrous acid or nitrites, and (2) alkylating agents.


Adjacent thymines
UV light
UV light
P
Cyclobutane thymine dimer
6-4 Photoproduct
A
A
T
T
Kink

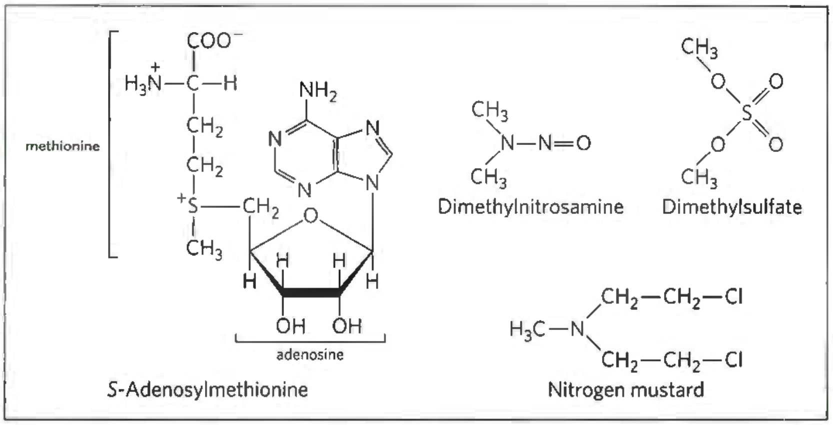
(a)

(b)

FIGURE 8-30 Formation of pyrimidine dimers induced by UV light. (a) One type of reaction (on the left) results in the formation of a cyclobutyl ring involving C-5 and C-6 of adjacent pyrimidine residues. An alternative reaction (on the right) results in a 6-4 photoproduct, with a linkage between C-6 of one pyrimidine and C-4 of its neighbor. (b) Formation of a cyclobutane pyrimidine dimer introduces a bend or kink into the DNA. [(b) Data from PDB ID 1TTD, K. McAteer et al., *J. Mol. Biol.* 282:1013, 1998.]


$NaNO_2$
Sodium nitrite
$NaNO_3$
Sodium nitrate
Nitrosamine

(a) Nitrous acid precursors

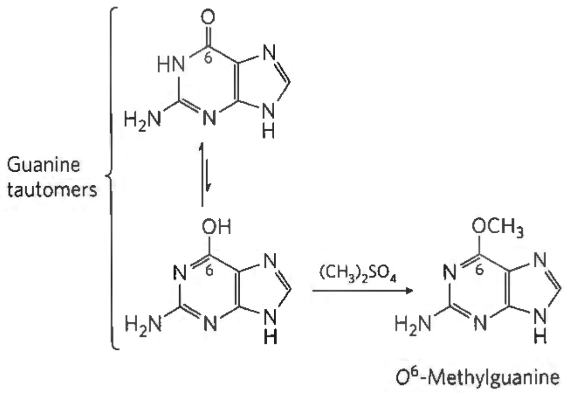

methionine
adenosine
S-Adenosylmethionine
Dimethylnitrosamine
Dimethylsulfate
Nitrogen mustard

(b) Alkylating agents

FIGURE 8-31 Chemical agents that cause DNA damage. (a) Precursors of nitrous acid, which promotes deamination reactions. (b) Alkylating agents. Most generate modified nucleotides nonenzymatically.

Nitrous acid, formed from organic precursors such as nitrosamines and from nitrite and nitrate salts, is a potent accelerator of the deamination of bases. Bisulfite has similar effects. Both agents are used as preservatives in processed foods to prevent the growth of toxic bacteria. They do not seem to increase cancer risks significantly when used in this way, perhaps because they are used in only small amounts and make only a minor contribution to the overall levels of DNA damage. (The potential health risk from food spoilage if these preservatives were not used is much greater.)

Alkylating agents can alter certain bases of DNA. For example, the highly reactive chemical dimethylsulfate (Fig. 8-31b) can methylate a guanine to yield O^6-methylguanine, which cannot base-pair with cytosine.



Some alkylation of bases is a normal part of the regulation of gene expression. The enzymatic methylation of certain bases using *S*-adenosyl methionine is one example discussed below.

The most important source of mutagenic alterations in DNA is oxidative damage. Reactive oxygen species such as hydrogen peroxide, hydroxyl radicals, and superoxide radicals arise during irradiation or (more commonly) as a byproduct of aerobic metabolism. These species damage DNA through any of a large, complex group of reactions, ranging from oxidation of deoxyribose and base moieties to strand breaks. Of these species, the hydroxyl radicals are responsible for most oxidative DNA damage. Cells have an elaborate defense system to destroy reactive oxygen species, including enzymes such as catalase and superoxide dismutase that convert reactive oxygen species to harmless products. A fraction of these oxidants inevitably escape cellular defenses, however, and are able to damage DNA. Accurate estimates for the extent of this damage are not yet available, but every day the DNA of each human cell is subjected to thousands of damaging oxidative reactions.

This is merely a sampling of the best-understood reactions that damage DNA. Many carcinogenic compounds in food, water, and air exert their cancer-causing effects by modifying bases in DNA. Nevertheless, the integrity of DNA as a polymer is better maintained than that of either RNA or protein, because DNA is the only macromolecule that has the benefit of extensive biochemical repair systems. These repair processes (described in Chapter 25) greatly lessen the impact of damage to DNA. ■

Some Bases of DNA Are Methylated

Certain nucleotide bases in DNA molecules are enzymatically methylated. Adenine and cytosine are methylated more often than guanine and thymine. Methylation is generally confined to certain sequences or regions of a DNA molecule. In some cases, the function of methylation is well understood; in others, the function remains unclear. All known DNA methylases use *S*-adenosylmethionine as a methyl group donor (Fig. 8-31b). *E. coli* has two prominent DNA methylation systems. One serves in a defense role, allowing the cell to distinguish its DNA from foreign DNA by marking its own DNA with methyl groups. The cell can then identify as foreign and destroy DNA without the methyl groups (this is known as a restriction-modification system; see p. 303). The other enzyme system methylates adenosine residues within the sequence (5′)GATC(3′) to N^6-methyladenosine (Fig. 8-5a). Methyl groups are added by the Dam (*D*NA *a*denine *m*ethylation) methylase shortly after DNA replication, allowing the cell to distinguish newly replicated DNA from older cellular DNA (see Fig. 25-20).

In eukaryotic cells, about 5% of cytidine residues in DNA are methylated to 5-methylcytidine (Fig. 8-5a). Methylation is most common at CpG sequences, producing methyl-CpG symmetrically on both strands of the DNA. The extent of methylation of CpG sequences varies by region in large eukaryotic DNA molecules, affecting DNA metabolism and gene expression.

The Chemical Synthesis of DNA Has Been Automated

An important practical advance in nucleic acid chemistry was the rapid and accurate synthesis of short oligonucleotides of known sequence. The methods were pioneered by H. Gobind Khorana and his colleagues in the 1970s. Refinements by Robert Letsinger and Marvin Caruthers led to the chemistry now in widest use, called the phosphoramidite method (**Fig. 8-32**). The synthesis is carried out with the growing strand attached to a solid support, using principles similar to those used by Merrifield for peptide synthesis (see Fig. 3-30), and is readily automated. The efficiency of each addition step is very high, allowing the routine synthesis of polymers containing 70 or 80 nucleotides and, in some laboratories, much longer strands. The availability of relatively inexpensive DNA polymers with predesigned sequences revolutionized all areas of biochemistry.

Gene Sequences Can Be Amplified with the Polymerase Chain Reaction

Genome projects, as described in Chapter 9, have given rise to online databases containing the complete genome sequences of thousands of organisms. This archive of sequence information allows researchers to greatly amplify any DNA segment they might be interested in with the **polymerase chain reaction (PCR)**, a process conceived by Kary Mullis in 1983. Even DNA segments with unknown sequences can be amplified if the sequences flanking them are known. The amplified DNA can then be used for a multitude of purposes, as we shall see.

The PCR procedure, shown in **Figure 8-33**, relies on **DNA polymerases**, enzymes that synthesize DNA strands from deoxyribonucleotides (dNTPs), using a DNA template. DNA polymerases do not synthesize DNA de novo, but instead must add nucleotides to the 3′ ends of preexisting strands, referred to as **primers** (see Chapter 25). In PCR, two synthetic oligonucleotides are prepared for use as

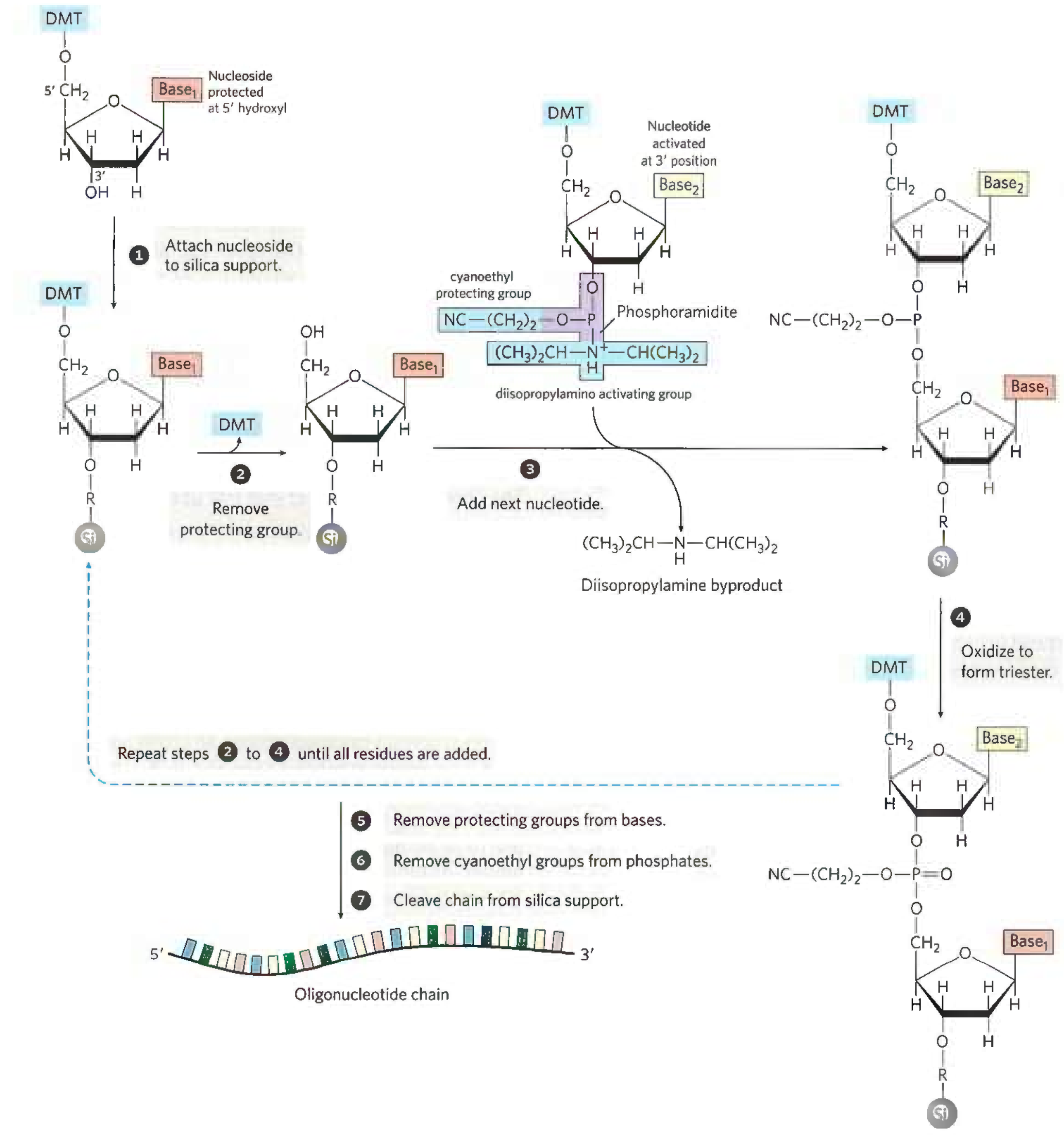

DMT
5′ CH2
Base1
Nucleoside protected at 5′ hydroxyl
3′
OH
① Attach nucleoside to silica support.
DMT
R
② Remove protecting group.
OH
Nucleotide activated at 3′ position
Base2
cyanoethyl protecting group
NC—(CH2)2—O—P
Phosphoramidite
(CH3)2CH—N+—CH(CH3)2
diisopropylamino activating group
③ Add next nucleotide.
(CH3)2CH—N—CH(CH3)2
Diisopropylamine byproduct
④ Oxidize to form triester.
NC—(CH2)2—O—P=O
Repeat steps ② to ④ until all residues are added.
⑤ Remove protecting groups from bases.
⑥ Remove cyanoethyl groups from phosphates.
⑦ Cleave chain from silica support.
5′
3′
Oligonucleotide chain

FIGURE 8-32 Chemical synthesis of DNA by the phosphoramidite method. Automated DNA synthesis is conceptually similar to the synthesis of polypeptides on a solid support. The oligonucleotide is built up on the solid support (silica), one nucleotide at a time, in a repeated series of chemical reactions with suitably protected nucleotide precursors. ① The first nucleoside (which will be the 3′ end) is attached to the silica support at the 3′ hydroxyl (through a linking group, R) and is protected at the 5′ hydroxyl with an acid-labile dimethoxytrityl group (DMT). The reactive groups on all bases are also chemically protected. ② The protecting DMT group is removed by washing the column with acid (the DMT group is colored, so this reaction can be followed spectrophotometrically). ③ The next nucleotide has a reactive phosphoramidite at its 3′ position: a trivalent phosphite (as opposed to the more oxidized pentavalent phosphate normally present in nucleic acids) with one linked oxygen replaced by an amino group or a substituted amine. In the common variant shown, one of the phosphoramidite oxygens is bonded to the deoxyribose, the other is protected by a cyanoethyl group, and the third position is occupied by a readily displaced diisopropylamino group. Reaction with the immobilized nucleotide forms a 5′,3′ linkage, and the diisopropylamino group is eliminated. In step ④, the phosphite linkage is oxidized with iodine to produce a phosphotriester linkage. Reactions 2 through 4 are repeated until all nucleotides are added. At each step, excess nucleotide is removed before addition of the next nucleotide. In steps ⑤ and ⑥ the remaining protecting groups on the bases and the phosphates are removed, and in ⑦ the oligonucleotide is separated from the solid support and purified. The chemical synthesis of RNA is somewhat more complicated because of the need to protect the 2′ hydroxyl of ribose without adversely affecting the reactivity of the 3′ hydroxyl.

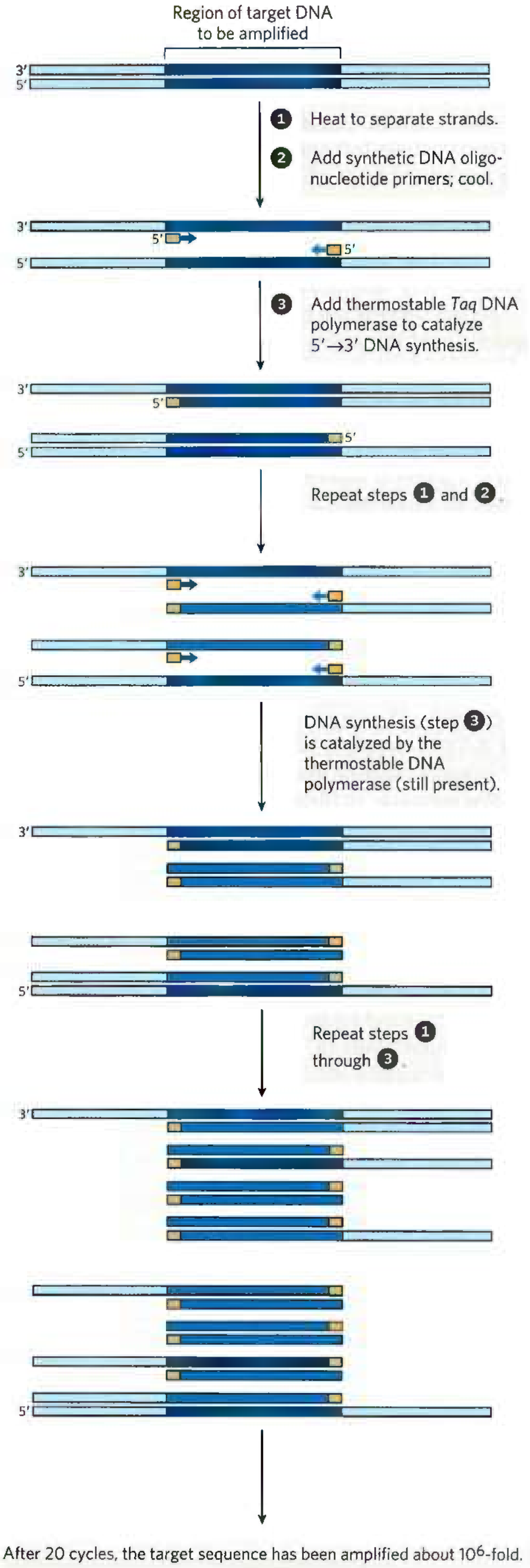

Region of target DNA to be amplified
3′
5′
① Heat to separate strands.
② Add synthetic DNA oligonucleotide primers; cool.
3′
5′
5′
5′
③ Add thermostable *Taq* DNA polymerase to catalyze 5′→3′ DNA synthesis.
3′
5′
5′
5′
Repeat steps ① and ②.
3′
5′
DNA synthesis (step ③) is catalyzed by the thermostable DNA polymerase (still present).
3′
5′
Repeat steps ① through ③.
3′
5′
After 20 cycles, the target sequence has been amplified about 10^6-fold.

FIGURE 8-33 Amplification of a DNA segment by the polymerase chain reaction (PCR). The PCR procedure has three steps: DNA strands are ① separated by heating, then ② annealed to an excess of short synthetic DNA primers (orange) that flank the region to be amplified (dark blue); ③ new DNA is synthesized by polymerization catalyzed by DNA polymerase. The thermostable *Taq* DNA polymerase is not denatured by the heating steps. The three steps are repeated for 25 or 30 cycles in an automated process carried out in a small benchtop instrument called a thermocycler.

replication primers that can be extended by a DNA polymerase. These oligonucleotide primers are complementary to sequences on opposite strands of the target DNA, positioned so that their 5′ ends define the ends of the segment to be amplified, and they become part of the amplified sequence. The 3′ ends of the annealed primers are oriented toward each other and positioned to prime DNA synthesis across the targeted DNA segment.

The PCR procedure has an elegant simplicity. Basic PCR requires four components: a DNA sample containing the segment to be amplified, the pair of synthetic oligonucleotide primers, a pool of deoxynucleoside triphosphates, and a DNA polymerase. There are three steps (Fig. 8-33). In step ①, the reaction mixture is heated briefly to denature the DNA, separating the two strands. In step ②, the mixture is cooled so that the primers can anneal to the DNA. The high concentration of primers increases the likelihood that they will anneal to each strand of the denatured DNA before the two DNA strands (present at a much lower concentration) can reanneal to each other. Then, in step ③, the primed segment is replicated selectively by the DNA polymerase, using the pool of dNTPs. The cycle of heating, cooling, and replication is repeated 25 to 30 times over a few hours in an automated process, amplifying the DNA segment between the primers until the sample is large enough to be readily analyzed or cloned (described in Chapter 9).

Each replication cycle doubles the number of target DNA segment copies, so the concentration grows exponentially. The flanking DNA sequences increase in number linearly, but this effect is quickly rendered insignificant. After 20 cycles, the targeted DNA segment has been amplified more than a millionfold (2^{20}); after 30 cycles, more than a billionfold. Step ③ of PCR uses a heat-stable DNA polymerase such as the *Taq* polymerase, isolated from a thermophilic bacterium (*Thermus aquaticus*) that thrives in hot springs where temperatures approach the boiling point of water. The *Taq* polymerase remains active after every heating step (step ①) and does not have to be replenished.

P4 This technology is highly sensitive: PCR can detect and amplify just one DNA molecule in almost any type of sample—including some ancient ones. The double-helical structure of DNA is highly stable, but as we have seen, DNA does degrade slowly over time through various nonenzymatic reactions. PCR has allowed the successful cloning of rare, undegraded DNA segments isolated from samples more than 40,000 years old. Investigators have used the technique to clone DNA fragments from the mummified remains of humans and extinct animals, such as the woolly mammoth, creating the research fields of molecular archaeology and molecular paleontology. DNA from burial sites has been amplified by PCR and used to trace ancient human migrations (see Fig. 9-31). Epidemiologists use PCR-enhanced DNA samples from human remains to trace the evolution of human pathogenic viruses. Due to its capacity to amplify just a few strands of DNA that might be present in a sample, PCR is a potent tool in forensic medicine (**Box 8-1**). It is also being used to detect viral infections and certain types of cancers before they cause symptoms, as well as in the prenatal diagnosis of genetic diseases.

WORKED EXAMPLE 8-2 *Designing Primers for the Polymerase Chain Reaction*

You set out to amplify the chromosomal sequence between the bases underlined below, using PCR. Only one strand is shown, but keep in mind that it is paired to a complementary strand.

TGGTAGGCCGAT – – – [1,000 bp] – – – TAGCTAAGAATCTTTCTCAGAA

Design single-stranded oligonucleotide primers to amplify only those sequences between (and including) the underlined bases. The optimal length for PCR primers is usually 18 to 22 nucleotides. For this example, simply write the first 6 nucleotides of each primer.

SOLUTION:

Left primer GTAGGC

Right primer AAGATT

Remember, (1) the two strands of DNA are antiparallel, (2) DNA synthesis proceeds uniquely in the 5′→3′ direction, (3) DNA synthesis must be directed across the region to be amplified, and (4) DNA sequences are always written in the 5′→3′ direction. The sequence given is thus oriented 5′→3′, left to right, even though no orientation guides are provided. The left primer must be complementary to the strand not shown, which is in the opposite orientation. Thus, the left primer begins at the **G** and is identical to the sequence shown. The right primer must direct DNA synthesis right to left, synthesizing a strand complementary to the strand provided. It will begin with an A complementary to the **T**, and then continue with additional nucleotides complementary to the strand shown. Although that sequence is written right to left, it is in the 3′→5′ direction and must be flipped to be in the conventional orientation, written 5′→3′, left to right. Companies that provide PCR primers expect orders to be written in the conventional 5′→3′ direction. Doing otherwise is a common (and expensive) mistake.

The Sequences of Long DNA Strands Can Be Determined

P4 In its capacity as a repository of information, a DNA molecule's most important property is its nucleotide sequence. Until the late 1970s, determining the sequence of a nucleic acid containing as few as 5 or 10 nucleotides was very laborious. The development of two techniques in 1977 (one by Allan Maxam and Walter Gilbert, the other by Frederick Sanger) made possible the sequencing of larger DNA molecules. Although the two methods are similar in strategy, **Sanger sequencing**, also known as dideoxy chain-termination sequencing, is both technically easier and more accurate (**Fig. 8-34**).

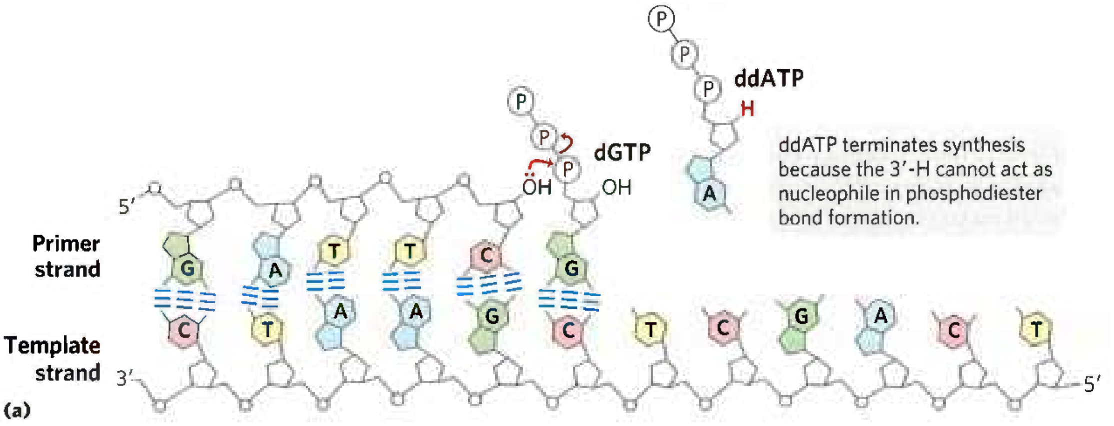
Any protocol for DNA sequencing has two parts. One must first chemically distinguish between G, C, T, and A residues. A second strategy is then needed to determine where the four residues appear in the overall sequence.

Sanger sequencing exploited new (at the time) information about the mechanism of DNA synthesis by DNA polymerase to distinguish between the four nucleotides, making use of nucleotide analogs called dideoxynucleoside triphosphates (ddNTPs) to interrupt synthesis specifically at one or another type of nucleotide. Like PCR, Sanger's method makes use of DNA polymerases and a primer to synthesize a DNA strand complementary to the strand under analysis. Each added deoxynucleotide is complementary, through base pairing, to a base in the template strand. In the reaction catalyzed by DNA polymerase, the 3′-hydroxyl group of the primer reacts with an incoming dNTP to form a new phosphodiester bond (Fig. 8-34a). The ddNTPs interrupt DNA synthesis because they bind to the template strand but lack the 3′-hydroxyl group needed to add the next nucleotide (Fig. 8-34b). Once a ddNTP is added to a growing strand, that strand cannot be extended further.

For instance, to identify C residues, a small amount of ddCTP is added to a reaction system containing a much larger amount of dCTP (along with the other three dNTPs). A competition then occurs every time the DNA polymerase encounters a G in the template strand. Usually, dC is added, and synthesis of the strand continues. Sometimes, ddC will be added instead, and the strand will be terminated at that position. Thus, a small fraction of the synthesized strands are prematurely terminated at every position where dC would normally be added, opposite each template dG. Given the excess of dCTP over ddCTP, the chance that the analog will be incorporated instead of dC is small. But enough ddCTP is present to ensure that some of the strands will be terminated at each G residue in the template.

The result is a solution containing a mixture of fragments, each ending with a ddC residue. The fragments differ in length, and locating the C residues then relies on precise electrophoretic methods that allow separation of DNA strands differing in size by only one nucleotide residue. (See Fig. 3-18 for a description of gel electrophoresis.) Note that in most sequencing protocols, the sequence obtained is that of the newly synthesized strand complementary to the template strand being analyzed.

When this procedure was first developed, the process was repeated separately for each of the four ddNTPs. Radioactively labeled primers allowed researchers to


P
P
P
ddATP
H
ddATP terminates synthesis because the 3′-H cannot act as nucleophile in phosphodiester bond formation.
P
P
P
dGTP
OH
OH
5′
Primer strand
G
A
T
T
C
G
A
Template strand
C
T
A
A
G
C
T
C
G
A
C
T
3′
5′
(a)

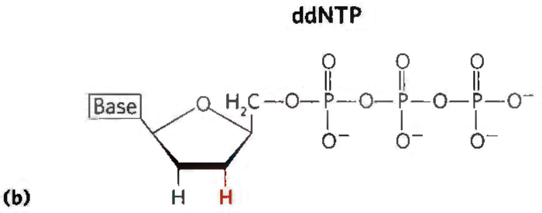
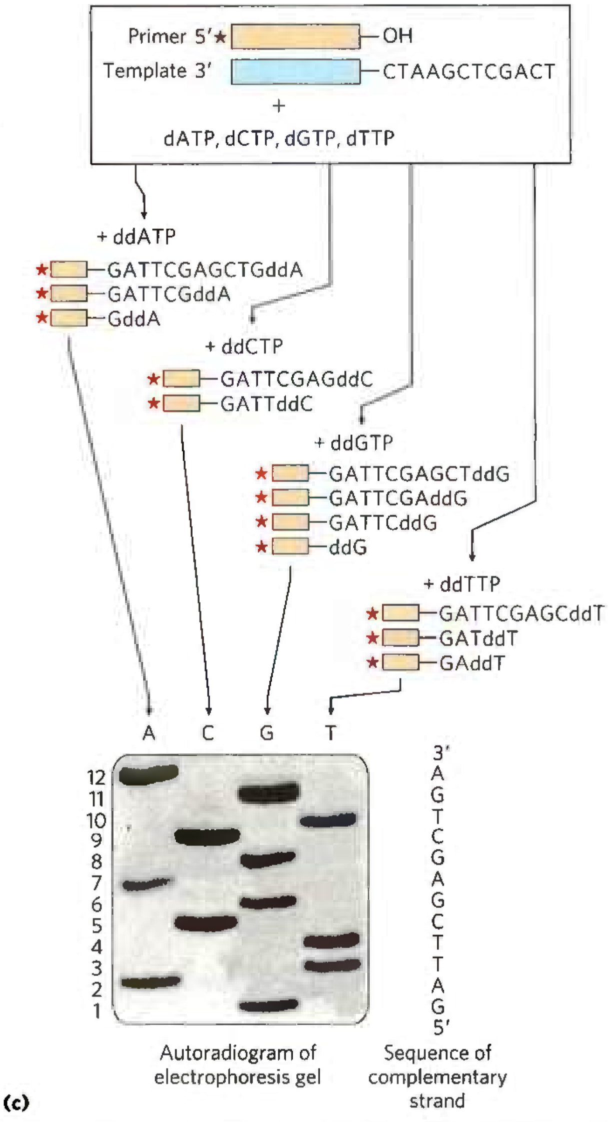

ddNTP
Base
O
H_2C
O
P
O
P
O
P
O
O^-
O^-
O^-
O^-
H
H
(b)

FIGURE 8-34 DNA sequencing by the Sanger method. This method makes use of the mechanism of DNA synthesis by DNA polymerases (Chapter 25). (a) DNA polymerases require both a primer (a short oligonucleotide strand), to which nucleotides are added, and a template strand to guide the selection of each new nucleotide. In cells, the 3′-hydroxyl group of the primer reacts with an incoming deoxynucleoside triphosphate—dGTP in this example—to form a new phosphodiester bond. The Sanger sequencing procedure uses dideoxynucleoside triphosphate (ddNTP) analogs to interrupt DNA synthesis. When a ddNTP—ddATP in this example—is inserted in place of a dNTP, strand elongation is halted after the analog is added, because the analog lacks the 3′-hydroxyl group needed for the next step. (b) Dideoxynucleoside triphosphate analogs have —H (red) rather than —OH at the 3′ position of the ribose ring. (c) The DNA to be sequenced is used as the template strand, and a short primer, radioactively (in the example here) or fluorescently labeled, is annealed to it. The result is a solution containing a mixture of labeled fragments of particular length, each ending with a C residue. The different-sized fragments, separated by electrophoresis, reveal the location of C residues. This procedure is repeated separately for each of the four ddNTPs, and the sequence can be read directly from an autoradiogram of the gel. Because shorter DNA fragments migrate faster, the fragments near the bottom of the gel represent the nucleotide positions closest to the primer (the 5′ end), and the sequence is read (in the 5′→3′ direction) from bottom to top. Note that the sequence obtained is that of the strand *complementary* to the strand being analyzed. [(c) Dr. Lloyd Smith, University of Wisconsin–Madison, Department of Chemistry.]


Primer 5′★
OH
Template 3′
CTAAGCTCGACT
+
dATP, dCTP, dGTP, dTTP
+ ddATP
★GATTCGAGCTGddA
★GATTCGddA
★GddA
+ ddCTP
★GATTCGAGddC
★GATTddC
+ ddGTP
★GATTCGAGCTddG
★GATTCGAddG
★GATTCddG
★ddG
+ ddTTP
★GATTCGAGCddT
★GATddT
★GAddT
A
C
G
T
12
11
10
9
8
7
6
5
4
3
2
1
3′
A
G
T
C
G
A
G
C
T
T
A
G
5′
Autoradiogram of electrophoresis gel
Sequence of complementary strand
(c)

detect the DNA fragments generated during the DNA synthesis reactions. The sequence of the synthesized DNA strand was read directly from an autoradiogram of the resulting gel (Fig. 8-34c). Because shorter DNA fragments migrate faster, the fragments near the bottom of the gel represented the nucleotide positions closest to the primer (the 5′ end), and the sequence was read (in the 5′→3′ direction) from bottom to top.

DNA sequencing was first automated by a variation of the Sanger method, in which each of the four ddNTPs

BOX 8-1

A Potent Weapon in Forensic Medicine

One of the most accurate methods for placing an individual at the scene of a crime is a fingerprint. But with the advent of recombinant DNA technology (see Chapter 9), a much more powerful tool became available: **DNA genotyping** (also called DNA fingerprinting or DNA profiling). As first described by English geneticist Alec Jeffreys in 1985, the method is based on **sequence polymorphisms**, slight sequence differences among individuals—1 in every 1,000 bp, on average. Each difference from the prototype human genome sequence (the first human genome that was sequenced) occurs in some fraction of the human population; every person has some differences from this prototype.

Forensic work focuses on differences in the lengths of **short tandem repeat (STR)** sequences. An STR locus is a specific location on a chromosome where a short DNA sequence (usually 4 bp long) is repeated many times in tandem. The loci most often used in STR genotyping are short—4 to 50 repeats (16 to 200 bp for tetranucleotide repeats)—and have multiple length variants in the human population. More than 20,000 tetranucleotide STR loci have been characterized in the human genome. And more than a million STRs of all types may be present in the human genome, accounting for about 3% of all human DNA.

The length of a particular STR in a given individual can be determined with the aid of the polymerase chain reaction (see Fig. 8-33). The use of PCR also makes the procedure sensitive enough to be applied to the very small samples often collected at crime scenes. The DNA sequences flanking STRs are unique to each STR locus and are identical (except for very rare mutations) in all humans. PCR primers are targeted to this flanking DNA and are designed to amplify the DNA across the

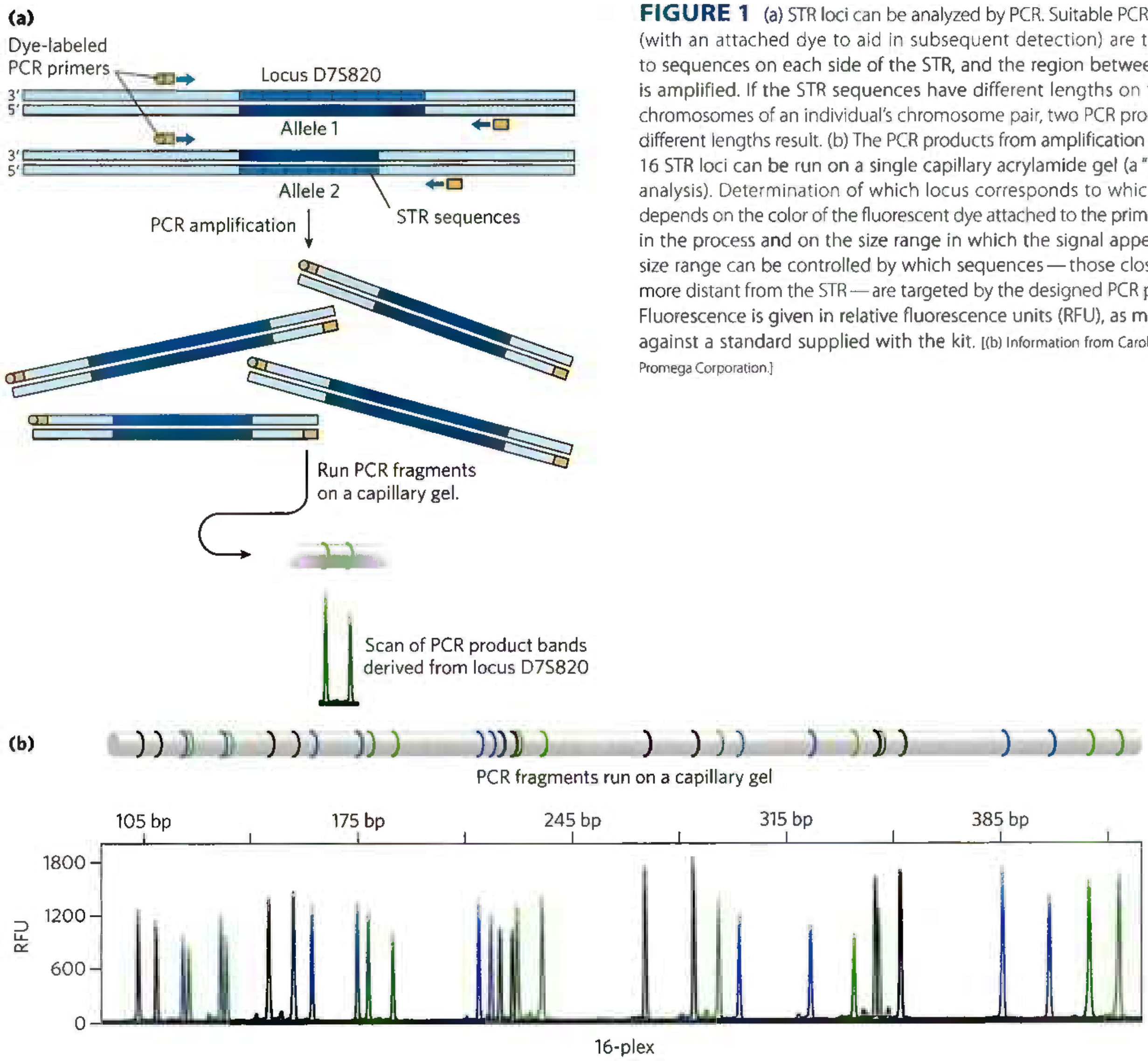


FIGURE 1 (a) STR loci can be analyzed by PCR. Suitable PCR primers (with an attached dye to aid in subsequent detection) are targeted to sequences on each side of the STR, and the region between them is amplified. If the STR sequences have different lengths on the two chromosomes of an individual's chromosome pair, two PCR products of different lengths result. (b) The PCR products from amplification of up to 16 STR loci can be run on a single capillary acrylamide gel (a "16-plex" analysis). Determination of which locus corresponds to which signal depends on the color of the fluorescent dye attached to the primers used in the process and on the size range in which the signal appears (the size range can be controlled by which sequences—those closer to or more distant from the STR—are targeted by the designed PCR primers). Fluorescence is given in relative fluorescence units (RFU), as measured against a standard supplied with the kit. [(b) Information from Carol Bingham, Promega Corporation.]

STR (Fig. 1a). The length of the PCR product then reflects the length of the STR in that sample. Because each human inherits one chromosome of each chromosome pair from each parent, the STR lengths on the two chromosomes are often different, generating two different STR lengths from one individual.

The PCR products are subjected to electrophoresis on a very thin polyacrylamide gel in a capillary tube. The resulting bands are converted into a set of peaks that accurately reveal the size of each PCR fragment and thus the length of the STR in the corresponding allele. Analysis of multiple STR loci can yield a profile that is unique to an individual (Fig. 1b). This is typically done with a commercially available kit that includes PCR primers unique to each locus, linked to colored dyes to help distinguish the different PCR products. PCR amplification enables investigators to obtain STR genotypes from less than 1 ng of partially degraded DNA, an amount that can be obtained from a single hair follicle, a small fraction of a drop of blood, a small semen sample, or samples that might be months or even many years old. When good STR genotypes are obtained, the chance of misidentification is less than 1 in 10^{18} (a quintillion).

The successful forensic use of STR analysis required standardization, first attempted in the United Kingdom in 1995. The U.S. standard, called the Combined DNA Index System (CODIS), established in 1998, was originally based on 13 well-studied STR loci. These continue to be required in any DNA-typing experiment carried out in the United States (Table 1) and are also used internationally. The amelogenin gene is also used as a marker in the analyses. Present on the human sex chromosomes, this gene has a slightly different length on the X and Y chromosomes. PCR amplification across this gene thus generates different-sized products that can reveal the sex of the DNA donor. By mid-2019, the CODIS database contained more than 18 million STR genotypes and had assisted in nearly 500,000 forensic investigations. As the CODIS database has expanded, the chance for adventitious matches has increased. The CODIS standard was expanded in 2017 to include 20 core loci. The new loci were incorporated with international agreement to ensure compatibility. Loci utilized in commercial kits have since expanded from 16 to 24.

DNA genotyping has been used to both convict and acquit suspects, and to establish paternity with an extraordinary degree of certainty. In the United States, there have been many hundreds of postconviction exonerations based on DNA evidence. The impact of these procedures on court cases will continue to grow as standards are refined and as international STR genotyping databases grow. Even very old mysteries can be solved. In 1996, STR genotyping helped confirm identification of the bones of the last Russian czar and his family, who were assassinated in 1918.

TABLE 1 **Properties of the Loci Used for the CODIS Database**

Locus	Chromosome	Repeat motif	Repeat length (range)[a]	Number of alleles seen[b]
CSF1PO	5	TAGA	5–16	20
FGA	4	CTTT	12.2–51.2	80
TH01	11	TCAT	3–14	20
TPOX	2	GAAT	4–16	15
VWA	12	[TCTG][TCTA]	10–25	28
D3S1358	3	[TCTG][TCTA]	8–21	24
D5S818	5	AGAT	7–18	15
D7S820	7	GATA	5–16	30
D8S1179	8	[TCTA][TCTG]	7–20	17
D13S317	13	TATC	5–16	17
D16S539	16	GATA	5–16	19
D18S51	18	AGAA	7–39.2	51
D21S11	21	[TCTA][TCTG]	12–41.2	82
Amelogenin[c]	X, Y	Not applicable		

Data from J. M. Butler, *Forensic DNA Typing: Biology, Technology, and Genetics of STR Markers*, 2nd edn, Elsevier, 2005, p. 96.

[a] Repeat lengths observed in the human population. Partial or imperfect repeats can be included in some alleles.

[b] Number of different alleles observed as of 2005 in the human population. Careful analysis of a locus in many individuals is a prerequisite to its use in forensic DNA typing.

[c] Amelogenin is a gene, of slightly different size on the X and Y chromosomes, that is used to establish gender.

used for a reaction was labeled with a different-colored fluorescent tag (**Fig. 8-35**). With this technology, all four fluorescent ddNTPs could be introduced into a single reaction. The terminated fragments, each of a different size, could be separated by electrophoresis in a single gel lane. The identity of the residue that terminated each fragment was made evident by its fluorescent color. Researchers could sequence DNA molecules containing thousands of nucleotides in a few hours, and the entire genomes of hundreds of organisms were sequenced in this way. For example, in the Human Genome Project, researchers sequenced all 3.2×10^9 bp of the DNA in a human cell (see Chapter 9) in an effort that spanned nearly a decade and included contributions from dozens of laboratories worldwide. This form of Sanger sequencing is still used for routine analysis of short segments of DNA.

DNA Sequencing Technologies Are Advancing Rapidly

P4 The billions of base pairs in a complete human genome can now be sequenced in a day or two, the millions in a bacterial genome in a few hours. With modest expense, a personal genomic sequence can be routinely included in each individual's medical record. These advances have been made possible by methods sometimes referred to as next-generation, or "next-gen," sequencing. The sequencing strategies have some similarities to the Sanger method. Innovations have allowed a miniaturization of the procedure, a massive increase in scale, and a corresponding decrease in cost. Two widely used approaches, **reversible terminator sequencing** and **single molecule real time (SMRT) sequencing**, both developed commercially, are described.

In both approaches, large genomes are sequenced by first collecting the DNA from many cells of the organism or individual. The DNA is sheared at random locations to generate fragments of a particular average size. The individual fragments — many with overlapping sequences — are immobilized on a solid support, and each is sequenced in place. Fluorescent dyes linked to the nucleotides and powerful optical systems that can detect incorporation of each new nucleotide by DNA polymerase allow the sequencing process to be monitored directly. The fluorescent dyes and the design of the methods solve both problems in DNA sequencing at

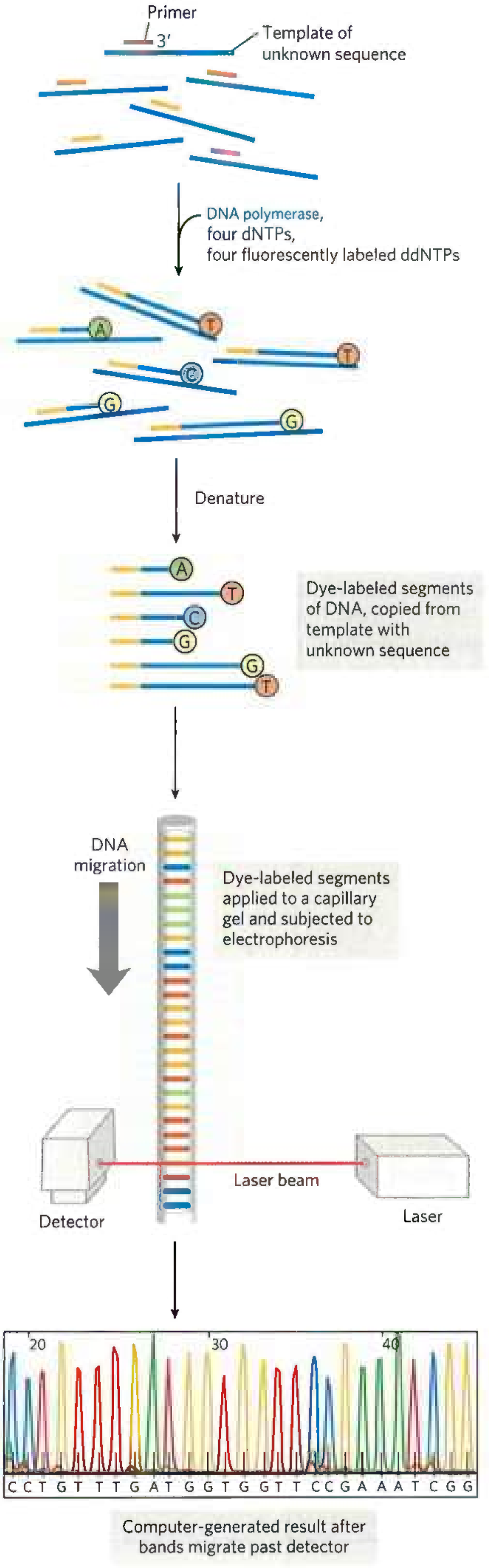

Primer
3′
Template of unknown sequence
DNA polymerase, four dNTPs, four fluorescently labeled ddNTPs
A
T
T
C
G
G
Denature
A
T
C
G
G
T
Dye-labeled segments of DNA, copied from template with unknown sequence
DNA migration
Dye-labeled segments applied to a capillary gel and subjected to electrophoresis
Detector
Laser beam
Laser
20
30
40
C C T G T T T G A T G G T G G T T C C G A A A T C G G
Computer-generated result after bands migrate past detector

FIGURE 8-35 Automation of DNA sequencing reactions. In the Sanger method, each ddNTP can be linked to a fluorescent (dye) molecule that gives the same color to all the fragments terminating in that nucleotide, with a different color for each nucleotide. All four labeled ddNTPs are added to the reaction mix together. The resulting colored DNA fragments are separated by size in an electrophoretic gel in a capillary tube (a refinement of gel electrophoresis that allows faster separations). All fragments of a given length migrate through the capillary gel together in a single band, and the color associated with each band is detected with a laser beam. The DNA sequence is read by identifying the color sequences in the bands as they pass the detector and feeding this information directly to a computer. The amount of fluorescence in each band is represented as a peak in the computer output. [Data from Dr. Lloyd Smith, University of Wisconsin-Madison, Department of Chemistry.]

once, identifying each nucleotide and fixing its location in the large sequence. Individual regions of a genome may be sequenced hundreds, even thousands, of times. The entire genomic sequence is reconstructed by computer programs that align the sequences of overlapping fragments.

In the reversible terminator sequencing method developed by Illumina, the genomic DNA to be sequenced is sheared so as to generate fragments a few hundred base pairs long. Synthetic oligonucleotides of known sequence are ligated to each end of each fragment, providing a point of reference on every DNA molecule. The individual fragments are then immobilized on a solid surface, and each is amplified in place by PCR to form a tight cluster of identical fragments. The solid surface is part of a channel on a flow cell that allows liquid solutions to stream over the samples. The result is a solid surface just a few centimeters wide with millions of attached DNA clusters, each cluster containing multiple copies of a single DNA sequence derived from a random genomic DNA fragment. To provide a starting point for DNA polymerase, an oligonucleotide primer is then added that is complementary to the oligonucleotides of known sequence ligated to the various fragment ends. All of these millions of clusters are sequenced at the same time, with the data from each cluster captured and stored by a computer.

The actual sequencing of each cluster employs reversible terminator sequencing (**Fig. 8-36**). Four different modified deoxynucleotides (A, T, G, and C), each with a particular fluorescent label that identifies the nucleotide by color, are added to the sequencing reaction, along with the DNA polymerase. The labeled nucleotides also incorporate blocking groups attached to their 3′ ends that permit only one nucleotide to be added to each strand. The polymerase adds the appropriate nucleotide to the strands in each cluster, giving each cluster a color that corresponds to the added nucleotide. Next, lasers excite all the fluorescent labels, and an image of the entire surface reveals the color (and thus the identity) of the base added to each cluster. The fluorescent label and the blocking groups are then chemically or photolytically removed. The surface goes dark until the solution with labeled nucleotides and DNA polymerase is again introduced to the surface, allowing the next nucleotide to be added to each cluster. The sequencing proceeds stepwise. Read lengths obtained with this technology (that is, the length of individual DNA sequences that can be accurately determined) are typically 100 to 300 nucleotides. Read length is limited by constraints on cluster density on the flow cell surface and by small inefficiencies in the PCR reactions needed to amplify each cluster accurately. Accuracy is high, with error rates as low as 0.1%.

The relatively short read lengths produced by the Illumina technology are problematic in some situations, such as the sequencing of long stretches of DNA where short sequences are repeated over and over. Pacific Biosciences has pioneered the single-molecule real time (SMRT) sequencing method that allows read lengths averaging up to 30,000 to 40,000 bp (**Fig. 8-37**). The SMRT technology has a lower throughput, a higher cost, and a higher error rate than the Illumina approach. However, the very long read lengths are essential in some applications, particularly to reconstruct the complete genomes of higher organisms that may contain extensive regions of repeated DNA sequences. They also facilitate the detection of genomic alterations—deletions, duplications, or rearrangements of genomic segments—that arise in some cells, such as those in cancerous tumors (see Box 24-2).

SMRT sequencing utilizes SMRT cells with 150,000 pores, each pore smaller in diameter (~70 nm) than the wavelength of visible light. Attenuated light from an excitation beam penetrates only the lower 20 to 30 nm in each pore, producing a light volume small enough to accommodate just one DNA polymerase molecule (Fig. 8-37a). A single DNA polymerase is immobilized at the bottom of each pore. Genomic DNA is sheared to generate random fragments that are tens of thousands of base pairs in length. Hairpin oligonucleotides are ligated to both ends of each fragment, so that the two DNA strands are joined in one continuous circle (Fig. 8-37b). A primer is annealed to the open single-stranded DNA at one end of the fragment, and this is captured by a DNA polymerase in one of the pores to initiate DNA synthesis.

Fluorescent nucleotides are introduced, with A, T, G, and C each having a specific colored dye attached to the triphosphate. When a nucleotide binds to the DNA polymerase in the pore, it is immobilized long enough to produce a brief fluorescent light pulse that can be read by a detector (Fig. 8-37c). The fluorescent dye is released with the pyrophosphate as each new phosphodiester bond is formed. The error rate is high, about 10% to 15%. However, as the DNA template is a continuous circle, one DNA polymerase can replicate the same fragment over and over. The light pulses continue without interruption as new nucleotides are added in real time, and each pore thus generates a movie of light pulses (sometimes several hours long) that corresponds to the repeated sequencing of the DNA fragment bound in that pore. A computer program deletes the known sequences of the hairpin ends. Error is reduced by compiling a consensus sequence of the fragment by automated alignment of the many repeated sequencing passes and acceptance of the most common nucleotide signal detected at each position.

Translating the sequences of millions of short DNA fragments into a complex and contiguous genomic sequence requires the computerized alignment of overlapping fragments (**Fig. 8-38**). The number of times that a particular nucleotide in a genome is sequenced, on average, is referred to as the **sequencing depth** or sequencing coverage. In many cases, a sufficiently large number of random fragments are sequenced so that each nucleotide in the genome is sequenced an average of hundreds to thousands of times (100× to 1,000× coverage). Although

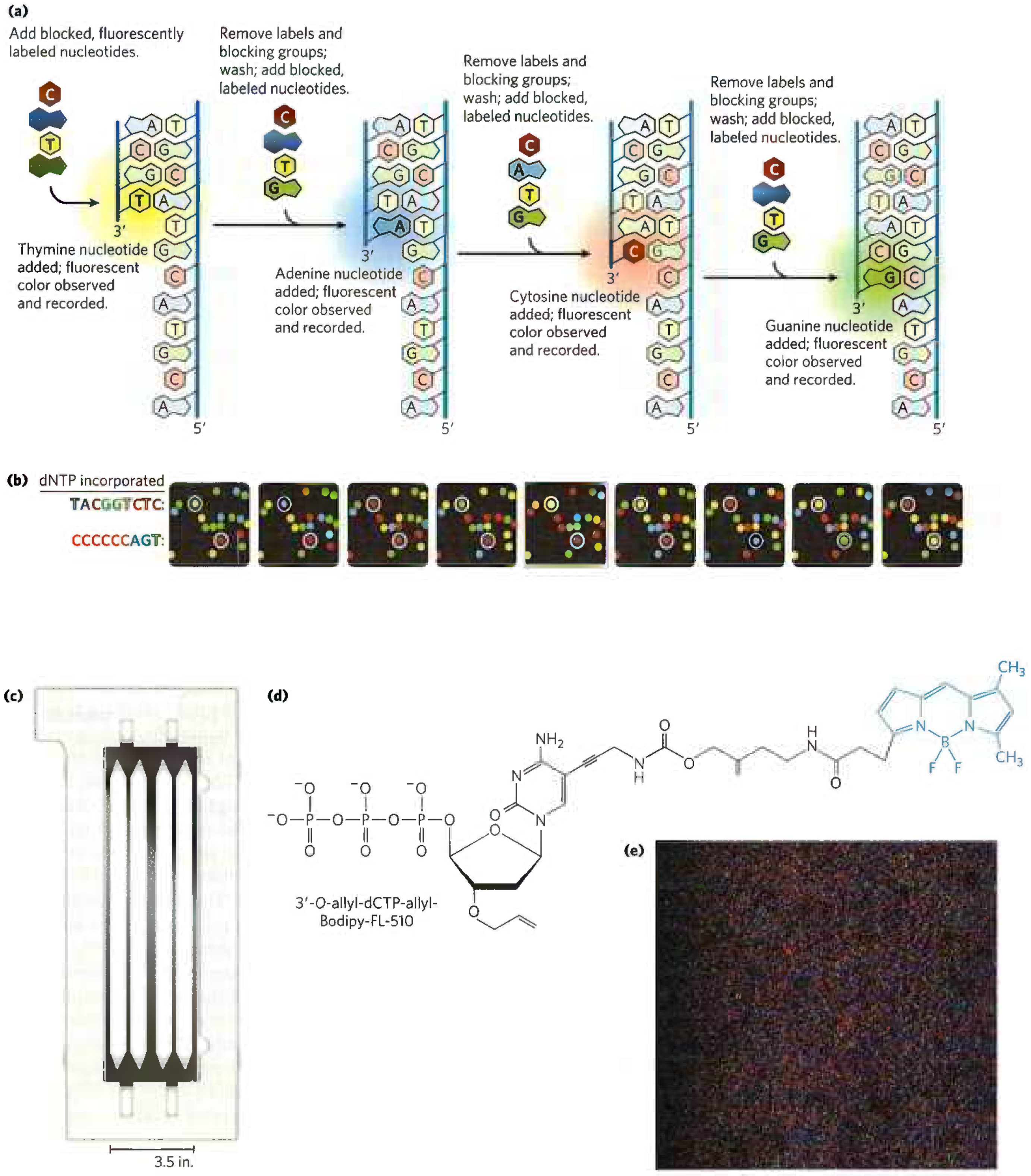

(a)
Add blocked, fluorescently labeled nucleotides.
Remove labels and blocking groups; wash; add blocked, labeled nucleotides.
Remove labels and blocking groups; wash; add blocked, labeled nucleotides.
Remove labels and blocking groups; wash; add blocked, labeled nucleotides.
Thymine nucleotide added; fluorescent color observed and recorded.
Adenine nucleotide added; fluorescent color observed and recorded.
Cytosine nucleotide added; fluorescent color observed and recorded.
Guanine nucleotide added; fluorescent color observed and recorded.
3′
5′
(b) dNTP incorporated
TACGGTCTC:
CCCCCCAGT:
(c)
3.5 in.
(d)
3′-O-allyl-dCTP-allyl-Bodipy-FL-510
(e)

FIGURE 8-36 Next-generation reversible terminator sequencing. (a) Blocking groups on each fluorescently labeled nucleotide prevent multiple nucleotides from being added in a single cycle. (b) Artist's rendition of nine successive cycles from one very small part of an Illumina sequencing run. Each colored spot represents the location of a cluster of immobilized identical oligonucleotides affixed to the surface of the flow cell. The white-circled spots represent the same two clusters on the surface over successive cycles, with the sequences indicated. Data are recorded and analyzed digitally. (c) Typical flow cell used for a next-generation sequencer. Millions of DNA fragments can be sequenced simultaneously in each of the four channels. (d) A dCTP molecule modified with a fluorescent dye and a 3′ blocking group for use in reversible terminator sequencing. Both the dye and the 3′-end-blocking group can be removed, either chemically or photolytically, leaving a free 3′-OH group for addition of the next nucleotide. The modified nucleotides currently used in reversible terminator sequencing are proprietary. (e) Part of the surface of one channel during a sequencing reaction. [(c, e) Used under license from Illumina, Inc. All Rights Reserved.]

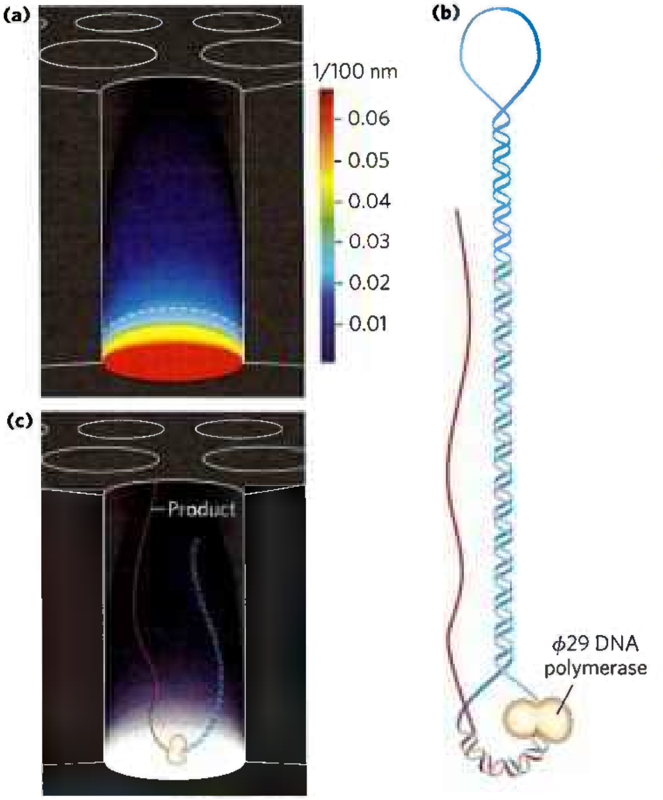


FIGURE 8-37 SMRT sequencing. (a) One pore in an SMRT cell. The pore is smaller in diameter than the wavelength of visible light, so that light projected at the bottom penetrates only a short distance into the pore. (b) DNA fragments sequenced by SMRT technology. Hairpin oligonucleotides are ligated to both ends. A primer for DNA synthesis is annealed to one and sometimes both single-stranded regions in the hairpin ends. (c) DNA synthesis by a DNA polymerase immobilized within a SMRT pore. A fluorescent dye is attached to the triphosphate of the nucleoside triphosphates used. The dye is released from each nucleotide incorporated into the growing DNA strand and its wavelength recorded.

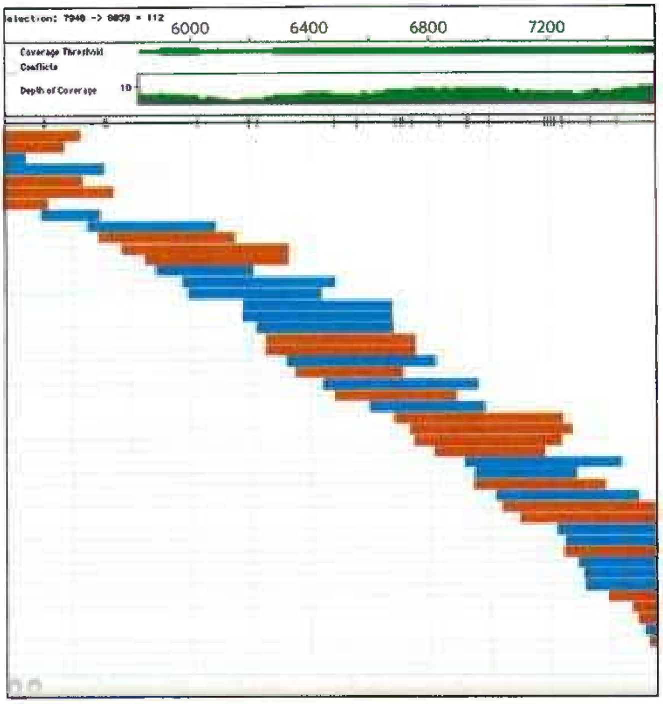


FIGURE 8-38 Sequence assembly. In a genomic sequence, each base pair of the genome is usually represented in multiple sequenced fragments, referred to as reads. This schematic shows how the overlaps between reads are used to assemble a contiguous segment of the genomic sequence, or contig. The numbers at the top represent base-pair positions in the genome, relative to an arbitrarily defined reference point. All sequence fragments come from a particular long contig. The reads are represented by horizontal colored bars. DNA strand segments are sequenced at random, with sequences obtained from one strand (5′ to 3′, left to right) or the other strand (5′ to 3′, right to left) represented by blue lines or red lines, respectively. The coverage bar at the top indicates how many times the sequence at a particular position has appeared in a sequenced read, with higher numbers corresponding to increased quality of the output sequence data.

the coverage of particular nucleotides may vary, a high level of coverage ensures that most sequencing errors will be detected and eliminated. The overlaps allow the computer to trace the sequence through a chromosome, from one overlapping fragment to another, permitting the assembly of long, contiguous sequences called **contigs**. In a successful genomic sequencing exercise, many contigs can extend over millions of base pairs.

P4 The rapid evolution of DNA sequencing technologies shows no signs of slowing. As costs plummet and sensitivity increases, new applications come online to enhance medicine, forensics, archaeology, and many other fields. We present some of those applications in Chapter 9.

SUMMARY 8.3 *Nucleic Acid Chemistry*

- Native DNA undergoes reversible unwinding and separation of strands (melting) upon heating or at extremes of pH. DNAs rich in G≡C pairs have higher melting points than DNAs rich in A═T pairs.
- DNA is a relatively stable polymer. Spontaneous reactions such as deamination of certain bases, hydrolysis of base-sugar *N*-glycosyl bonds, radiation-induced formation of pyrimidine dimers, and oxidative damage occur at very low rates, yet are important because of a cell's very low tolerance for changes in its genetic material.
- DNA is subject to enzymatic modification of nucleotide bases at particular locations. Methylated bases are common.
- Oligonucleotides of known sequence can be synthesized rapidly and accurately.
- The polymerase chain reaction (PCR) provides a convenient and rapid method for amplifying segments of DNA if the sequences of the ends of the targeted DNA segment are known.
- Routine DNA sequencing of genes or short DNA segments is carried out using an automated variation of Sanger dideoxy sequencing.
- DNA sequences, including entire genomes, can be efficiently determined in hours or days using commercial next-gen sequencing technologies.

8.4 Other Functions of Nucleotides

In addition to their roles as the subunits of nucleic acids, nucleotides have cellular functions as energy carriers, components of enzyme cofactors, and chemical messengers.

Nucleotides Carry Chemical Energy in Cells

The phosphate group covalently linked at the 5′ hydroxyl of a ribonucleotide may have one or two additional phosphates attached. The resulting molecules are referred to as nucleoside mono-, di-, and triphosphates (**Fig. 8-39**). Starting from the ribose, the three phosphates are generally labeled α, β, and γ. **P5** Hydrolysis of nucleoside triphosphates provides the chemical energy to drive many cellular reactions. Adenosine 5′-triphosphate, ATP, is by far the most widely used nucleoside triphosphate for this purpose, but UTP, GTP, and CTP are also used in some reactions. Nucleoside triphosphates also serve as the activated precursors of DNA and RNA synthesis, as described in Chapters 25 and 26 (see also Fig. 8-34).

The energy released by hydrolysis of ATP and the other nucleoside triphosphates is accounted for by the structure of the triphosphate group. The bond between the ribose and the α phosphate is an ester linkage. The α, β and β, γ linkages are phosphoanhydrides (**Fig. 8-40**). Hydrolysis of the ester linkage yields about 14 kJ/mol under standard conditions, whereas hydrolysis of each anhydride bond yields about 30 kJ/mol. ATP hydrolysis often plays an important thermodynamic role in biosynthesis. **P5** When coupled to a reaction with a positive free-energy change, ATP hydrolysis shifts the equilibrium of the overall process to favor product formation (recall the relationship between the equilibrium constant and free-energy change described by Eqn 6-3 on p. 182).

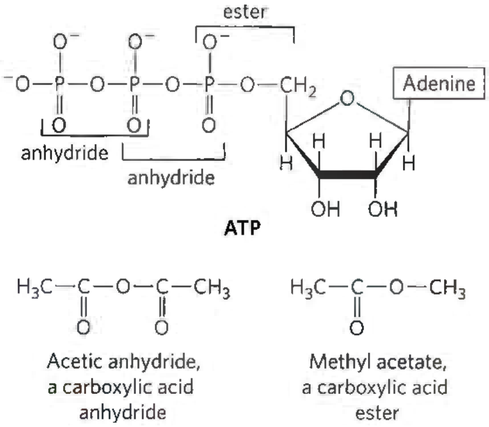
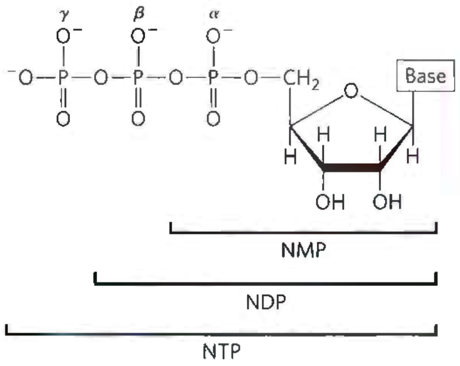

ester
Adenine
anhydride
anhydride
ATP
Acetic anhydride, a carboxylic acid anhydride
Methyl acetate, a carboxylic acid ester

FIGURE 8-40 The phosphate ester and phosphoanhydride bonds of ATP. Hydrolysis of an anhydride bond yields more energy than hydrolysis of the ester. A carboxylic acid anhydride and a carboxylic acid ester are shown for comparison.

Adenine Nucleotides Are Components of Many Enzyme Cofactors

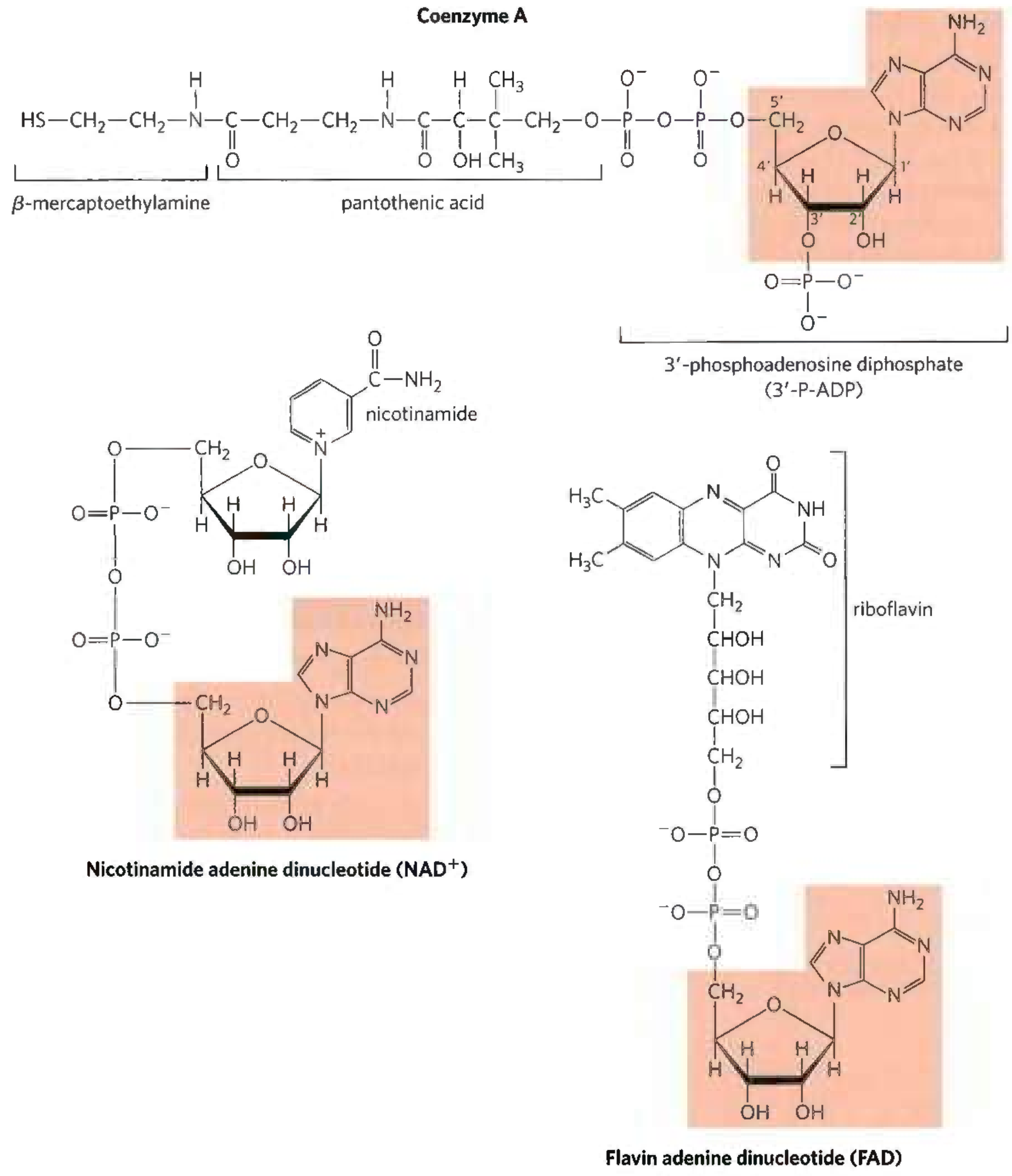
Enzyme cofactors serving a wide range of chemical functions include adenosine as part of their structure (**Fig. 8-41**). They are unrelated structurally except for the presence of adenosine. In none of these cofactors does the adenosine portion participate directly in the primary function, but removal of adenosine generally results in a drastic reduction of cofactor activities. For example, removal of the adenine nucleotide (3′-phosphoadenosine diphosphate) from acetoacetyl-CoA, the coenzyme A derivative of acetoacetate, reduces its reactivity as a substrate for β-ketoacyl-CoA transferase (an enzyme of lipid metabolism) by a factor of 10^6. Although this requirement for adenosine has not been investigated in detail, it must involve the binding energy between enzyme and substrate (or cofactor) that is used both in catalysis and in stabilizing



Abbreviations of ribonucleoside 5′-phosphates			
Base	Mono-	Di-	Tri-
Adenine	AMP	ADP	ATP
Guanine	GMP	GDP	GTP
Cytosine	CMP	CDP	CTP
Uracil	UMP	UDP	UTP

Abbreviations of deoxyribonucleoside 5′-phosphates			
Base	Mono-	Di-	Tri-
Adenine	dAMP	dADP	dATP
Guanine	dGMP	dGDP	dGTP
Cytosine	dCMP	dCDP	dCTP
Thymine	dTMP	dTDP	dTTP

FIGURE 8-39 Nucleoside phosphates. General structure of the nucleoside 5′-mono-, di-, and triphosphates (NMPs, NDPs, and NTPs) and their standard abbreviations. In the deoxyribonucleoside phosphates (dNMPs, dNDPs, and dNTPs), the pentose is 2′-deoxy-D-ribose.


Coenzyme A

Nicotinamide adenine dinucleotide (NAD$^+$)

Flavin adenine dinucleotide (FAD)

FIGURE 8-41 Some coenzymes containing adenosine. The adenosine portion is shaded in light red. Coenzyme A (CoA) functions in acyl group transfer reactions; the acyl group (such as the acetyl or acetoacetyl group) is attached to the CoA through a thioester linkage to the β-mercaptoethylamine moiety. NAD$^+$ functions in hydride transfers, and FAD, the active form of vitamin B_2 (riboflavin), functions in electron transfers. Another coenzyme incorporating adenosine is 5′-deoxyadenosylcobalamin, the active form of vitamin B_{12} (see Box 17-2), which participates in intramolecular group transfers between adjacent carbons.

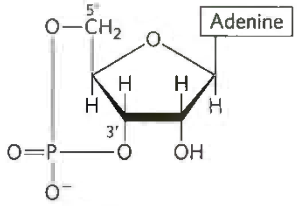
the initial enzyme-substrate complex (Chapter 6). In the case of β-ketoacyl-CoA transferase, the nucleotide moiety of coenzyme A seems to be a binding "handle" that helps to pull the substrate (acetoacetyl-CoA) into the active site. Similar roles may be found for the nucleoside portion of other nucleotide cofactors.

Why is adenosine, rather than some other large molecule, used in these structures? The answer here may involve a form of evolutionary economy. Adenosine is certainly not unique in the amount of potential binding energy it can contribute. The importance of adenosine probably lies not so much in some special chemical characteristic as in the evolutionary advantage of using one compound for multiple roles. Once ATP became the universal source of chemical energy, systems developed to synthesize ATP in greater abundance than the other nucleotides; because it is abundant, it becomes the logical choice for incorporation into a wide variety of structures. The economy extends to protein structure. A single protein domain that binds adenosine can be used in different enzymes. Such a domain, called a **nucleotide-binding fold**, is found in many enzymes that bind ATP and nucleotide cofactors.

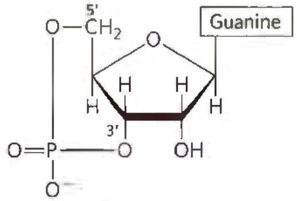
Some Nucleotides Are Regulatory Molecules

Cells respond to their environment by taking cues from hormones or other external chemical signals. The interaction of these extracellular chemical signals ("first messengers") with receptors on the cell surface often leads to the production of **second messengers** inside the cell, which in turn leads to adaptive changes in the cell interior (Chapter 12). Often, the second messenger is a nucleotide (**Fig. 8-42**). One of the most common is **adenosine 3′,5′-cyclic monophosphate (cyclic AMP**, or **cAMP)**, formed from ATP in a reaction catalyzed by adenylyl cyclase, an enzyme associated with the inner face of the plasma membrane. Cyclic AMP serves regulatory functions in virtually every cell outside the plant kingdom. Guanosine 3′,5′-cyclic monophosphate (cGMP) also has regulatory functions in many cells.

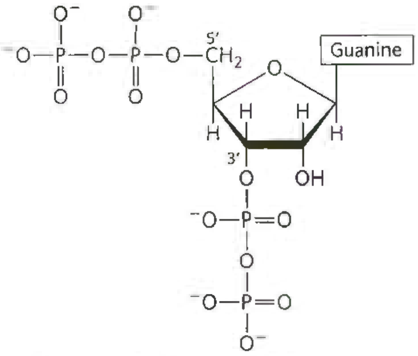
Another regulatory nucleotide, ppGpp (Fig. 8-42), is produced in bacteria in response to a slowdown in protein synthesis during amino acid starvation. This nucleotide inhibits the synthesis of the rRNA and tRNA molecules (see Fig. 28-22) needed for protein synthesis, preventing the unnecessary production of nucleic acids.



Adenosine 3′,5′-cyclic monophosphate
(cyclic AMP; cAMP)



Guanosine 3′,5′-cyclic monophosphate
(cyclic GMP; cGMP)



Guanosine 5′-diphosphate,3′-diphosphate
(guanosine tetraphosphate; ppGpp)

FIGURE 8-42 Three regulatory nucleotides.

Adenine Nucleotides Also Serve as Signals

ATP and ADP also serve as signaling molecules in many unicellular and multicellular organisms, including humans. In mammals, certain neurons release ATP at synapses. The ATP binds P_{2X} receptors on the postsynaptic cell, triggering changes in membrane potential or the release of an intracellular second messenger that initiates diverse physiological processes, including taste, inflammation, and smooth muscle contraction. One important class of ATP receptors that mediate the sensation of pain is an obvious target for drug development. Extracellular ADP is a signaling molecule that acts through P_{2Y} receptors in sensitive cell types. By preventing ADP from binding the P_{2Y} receptors of platelets, the drug clopidogrel (Plavix) inhibits undesirable blood clotting in patients with cardiac disease. Signaling pathways are discussed in more detail in Chapter 12. ■

SUMMARY 8.4 *Other Functions of Nucleotides*

- ATP is the central carrier of chemical energy in cells.
- The presence of an adenosine moiety in a variety of enzyme cofactors may be related to binding-energy requirements.
- Cyclic AMP, formed from ATP in a reaction catalyzed by adenylyl cyclase, is a common second messenger produced in response to hormones and other chemical signals.
- ATP and ADP serve as neurotransmitters in a variety of signaling pathways.

KEY TERMS

Terms in bold are defined in the glossary.

deoxyribonucleic acid (DNA)	263
ribonucleic acid (RNA)	263
gene	263
ribosomal RNA (rRNA)	264
messenger RNA (mRNA)	264
transfer RNA (tRNA)	264
nucleotide	264
nucleoside	264
pyrimidine	264
purine	264
deoxyribonucleotides	265
ribonucleotide	265
phosphodiester linkage	267
5′ end	267
3′ end	267
oligonucleotide	268
polynucleotide	268
tautomers	268
base pair	268
major groove	271
minor groove	271
B-form DNA	272
A-form DNA	272
Z-form DNA	272
palindrome	273
hairpin	273
cruciform	273
triplex DNA	274
G tetraplex	274
transcription	275
monocistronic mRNA	275
polycistronic mRNA	275
mutation	280
polymerase chain reaction (PCR)	283
DNA polymerases	283
primer	283
Sanger sequencing	286

sequence polymorphisms 288
short tandem repeat (STR) 288
reversible terminator sequencing 290
single-molecule real-time (SMRT) sequencing 290
sequencing depth 291
contig 293
nucleotide-binding fold 295
second messenger 296
adenosine 3′,5′-cyclic monophosphate (cyclic AMP, cAMP) 296

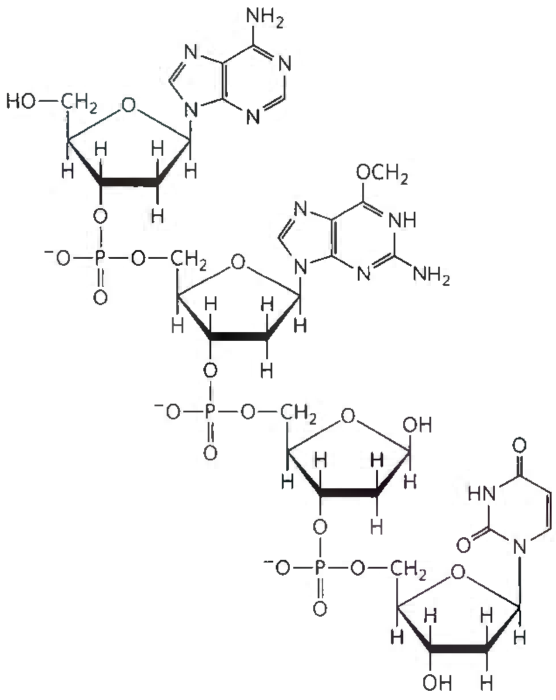
PROBLEMS

1. Nucleotide Structure Which positions in the purine ring of a purine nucleotide in DNA have the potential to form hydrogen bonds but are not involved in Watson-Crick base pairing?

2. Base Sequence of Complementary DNA Strands One strand of a double-helical DNA has the sequence (5′)GCG CAATATTTCTCAAAATATTGCGC(3′). Write the base sequence of the complementary strand. What special type of sequence is contained in this DNA segment? Does the double-stranded DNA have the potential to form any alternative structures?

3. DNA of the Human Body If completely unraveled, all of a human's DNA would be able to reach a distance of nearly 3.2×10^5 km, the distance from Earth to the moon. Given that each base pair in a DNA helix extends a distance of 3.4 Å, calculate the number of base pairs found within the entirety of a human's DNA.

4. Nucleic Acids A damaged tetranucleotide structure is shown below. **(a)** Name each of the nucleotides (or the type of damaged site as appropriate), proceeding from top left to lower right. **(b)** Indicate which end (upper left or lower right) is the 3′ end and which is the 5′ end. **(c)** Is this tetranucleotide DNA or RNA?



5. Distinction between DNA Structure and RNA Structure Secondary structures called hairpins may form at palindromic sequences in single strands of either RNA or DNA. The fully base-paired portions of hairpins form helices. How do RNA hairpins differ from DNA hairpins?

6. Nucleotide Chemistry The cells of many eukaryotic organisms have highly specialized systems that specifically repair G–T mismatches in DNA. The mismatch is repaired to form a G≡C, not A=T, base pair. This G–T mismatch repair mechanism occurs in addition to a more general system that repairs virtually all mismatches. Suggest why cells might require a specialized system to repair G–T mismatches.

7. Denaturation of Nucleic Acids A duplex DNA oligonucleotide in which one of the strands has the sequence TAATACGACT CACTATAGGG has a melting temperature (t_m) of 59 °C. If an RNA duplex oligonucleotide of identical sequence (substituting U for T) is constructed, will its melting temperature be higher or lower?

8. Spontaneous DNA Damage Hydrolysis of the *N*-glycosyl bond between deoxyribose and a purine in DNA creates an apurinic (AP) site. An AP site is more thermodynamically destabilizing to a DNA molecule than is a mismatched base pair. Examine the structure of an AP site (see Fig. 8-29b) and describe some chemical consequences of base loss.

9. Prediction of Nucleic Acid Structure from Its Sequence A part of a sequenced chromosome has the sequence (on one strand) ATTGCATCCGCGCGTGCGCGCGCGATCCCGT TACTTTCCG. What is the longest part of this sequence that is likely to take up the Z conformation?

10. Nucleic Acid Identity Explain how RNA nucleotides differ from DNA nucleotides.

11. Nucleic Acid Structure Explain why the absorption of UV light by double-stranded DNA increases (the hyperchromic effect) when the DNA is denatured.

12. Solubility of the Components of DNA Draw the structures of deoxyribose, guanine, and phosphate and rate their relative solubilities in water (most soluble to least soluble). How are these solubilities consistent with the three-dimensional structure of double-stranded DNA?

13. Polymerase Chain Reaction An investigator has one strand of a chromosomal DNA whose sequence is shown. She wants to use polymerase chain reaction (PCR) to amplify and isolate the DNA fragment defined by the segment shown in red. Her first step is to design two PCR primers, each 20 nucleotides long, that can be used to amplify this DNA segment. The final PCR product generated from the primers should include no sequences outside the segment in red.

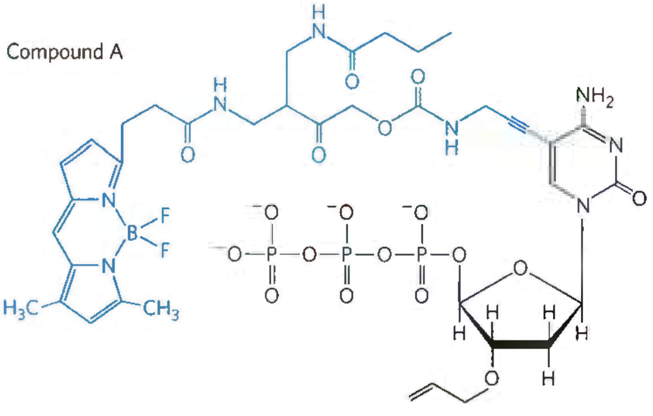
5′ – – – AATGCCGTCAGCCGATCTGCCTCGAGTCAATCGAT
GCTGGTAACTTGGGGTATAAAGCTTACCCATGGTATCGTAGT
TAGATTGATTGTTAGGTTCTTAGGTTTAGGTTTCTGGTATTG
GTTTAGGGTCTTTGATGCTATTAATTGTTTGGTTTTGATTTG
GTCTTTATATGGTTTATGTTTTAAGCCGGGTTTTGTCTGG
GATGGTTCGTCTGATGTGCGCGTAGCGTGCGGCG – – – 3′

What are the sequences of the investigator's forward primer and reverse primer? Recall that the forward primer binds to the strand of DNA running in the 3′ to 5′ direction, whereas the reverse primer binds to the opposite strand.

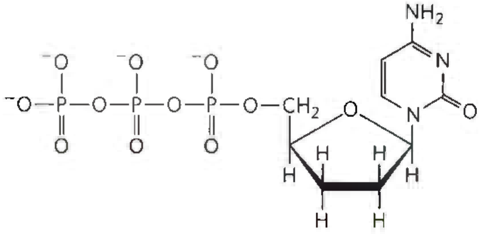
14. DNA Sequencing Reagents Indicate which of the modified cytidine nucleotide triphosphates shown might be used for each procedure: **(a)** Classical Sanger sequencing

(b) Automated Sanger sequencing **(c)** Next-generation DNA sequencing (Illumina). Linked fluorescent dyes, where present, are highlighted.

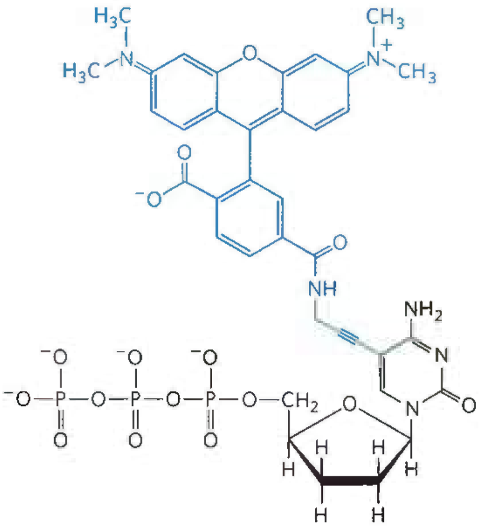
Compound A



Compound B



Compound C



15. Genomic Sequencing In large-genome sequencing projects, the initial data usually reveal gaps between contigs where no sequence information has been obtained. To close the gaps, DNA primers complementary to the 5′-ending strand at the end of each contig are especially useful. Explain how researchers could use these primers to close the gaps between contigs.

16. Next-Generation Sequencing In reversible terminator sequencing, how would the sequencing process be affected if the 3′-end-blocking group of each nucleotide were replaced with the 3′-H present in the dideoxynucleotides used in Sanger sequencing?

17. Sanger Sequencing Logic In the Sanger (dideoxy) method for DNA sequencing, researchers add a small amount of a dideoxynucleoside triphosphate, such as ddCTP, to the sequencing reaction along with a larger amount of the corresponding deoxynucleoside, such as dCTP. What result would researchers observe if they omitted CTP from the sequencing reaction?

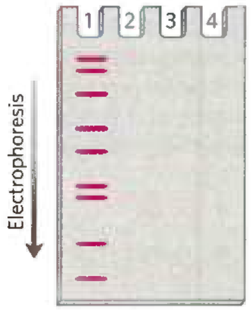
18. DNA Sequencing A researcher used the Sanger method to sequence the DNA fragment shown. The red asterisk indicates a fluorescent label.

*5′ ——— 3′-OH
3′ ——— ATTACGCAAGGACATTAGAC---5′

She reacted a sample of the DNA with DNA polymerase and each of the four nucleotide mixtures (in an appropriate buffer) listed. Some of the mixtures included dideoxynucleotides (ddNTPs) in relatively small amounts.

1. dATP, dTTP, dCTP, dGTP, ddTTP
2. dATP, dTTP, dCTP, dGTP, ddGTP
3. dATP, dCTP, dGTP, ddTTP
4. dATP, dTTP, dCTP, dGTP

The researcher then separated the resulting DNA by electrophoresis on a polyacrylamide gel and located the fluorescent bands on the gel. The image of the gel shows the band pattern resulting from nucleotide mixture 1. Assuming that all mixtures were run on the same gel, what did the remaining lanes of the gel look like?



19. Snake Venom Phosphodiesterase An exonuclease is an enzyme that sequentially cleaves nucleotides from the end of a polynucleotide strand. Snake venom phosphodiesterase, which hydrolyzes nucleotides from the 3′ end of any oligonucleotide with a free 3′-hydroxyl group, cleaves between the 3′ hydroxyl of the ribose or deoxyribose and the phosphoryl group of the next nucleotide. It acts on single-stranded DNA or RNA and has no base specificity. This enzyme was used in sequence determination experiments before the development of modern nucleic acid sequencing techniques. What are the products of partial digestion by snake venom phosphodiesterase of an oligonucleotide with the sequence (5′)GCGCCAUUGC(3′)—OH?

20. Preserving DNA in Bacterial Endospores Bacterial endospores form when the environment is no longer conducive to active cell metabolism. The soil bacterium *Bacillus subtilis*, for example, begins the process of sporulation when one or more nutrients are depleted. The end product is a small, metabolically dormant structure that can survive almost indefinitely with no detectable metabolism. Spores have mechanisms to prevent accumulation of potentially lethal mutations in their DNA over periods of dormancy that can exceed 1,000 years. *B. subtilis* spores are much more resistant than are the organism's growing cells to heat, UV radiation, and oxidizing agents, all of which promote mutations.

(a) One factor that prevents potential DNA damage in spores is their greatly decreased water content. How would this affect some types of mutations?
(b) Endospores have a category of proteins called small acid-soluble proteins (SASPs) that bind to their DNA, preventing formation of cyclobutane-type dimers. What causes cyclobutane dimers, and why do bacterial endospores need mechanisms to prevent their formation?

21. Oligonucleotide Synthesis As shown in the scheme of Figure 8-34, oligonucleotide synthesis involves adding modified bases, one at a time, to a growing chain. The modified bases contain an activated 3′ hydroxyl and have a dimethoxytrityl (DMT) group attached to the 5′ hydroxyl. What is the function of the DMT group on the incoming base?

BIOCHEMISTRY ONLINE

22. The Structure of DNA Elucidation of the three-dimensional structure of DNA helped researchers understand how this molecule conveys information that can be faithfully replicated from one generation to the next. To see the secondary structure of double-stranded DNA, go to the Protein Data Bank website (www.rcsb.org). Use the PDB identifiers provided in parts (a) and (b) below to retrieve the structure summary for a double-stranded DNA segment. View the 3D structure using JSmol. The viewer select menu is below the right corner of the image box. Once in JSmol, you will need to use both the display menus on the screen and the scripting controls in the JSmol menu. Access the JSmol menu by clicking on the JSmol logo in the lower right corner of the image screen. Refer to the JSmol help links as needed.
(a) Access PDB ID 141D, a highly conserved, repeated DNA sequence from the end of the genome of HIV-1 (the virus that causes AIDS). Set the Style to Ball and Stick. Then use the scripting controls to color by element (Color > Atoms > By Scheme > Element (CPK)). Identify the sugar–phosphate backbone for each strand of the DNA duplex. Locate and identify individual bases. Identify the 5′ end of each strand. Locate the major and minor grooves. Is this a right- or left-handed helix?
(b) Access PDB ID 145D, a DNA with the Z conformation. Set the Style to Ball and Stick. Then use the scripting controls to color by element (Main Menu > Color > Atoms > By Scheme > Element (CPK)). Identify the sugar–phosphate backbone for each strand of the DNA duplex. Is this a right- or left-handed helix?
(c) To fully appreciate the secondary structure of DNA, view the molecules in stereo. From the scripting control Main Menu select Style > Stereographic > Cross-eyed viewing or Wall-eyed viewing. (If you have stereographic glasses available, select the appropriate option.) You will see two images of the DNA molecule. Sit with your nose approximately 10 inches from the screen and focus on the tip of your nose (cross-eyed) or on the opposite edges of the screen (wall-eyed). In the background you should see three images of the DNA helix. Shift your focus to the middle image, which should appear three-dimensional. (Note that only one of the authors can make this work.)

DATA ANALYSIS PROBLEM

23. Chargaff's Studies of DNA Structure The main findings of Erwin Chargaff and his coworkers ("Chargaff's rules") are summarized on page 270. In this problem, you will examine the data Chargaff collected in support of his conclusions.

In one paper, Chargaff (1950) described his analytical methods and some early results. Briefly, he treated DNA samples with acid to remove the bases, separated the bases by paper chromatography, and measured the amount of each base with UV spectroscopy. His results are shown in the three tables below. The *molar ratio* is the ratio of the number of moles of each base in the sample to the number of moles of phosphate in the sample—this gives the fraction of the total number of bases represented by each particular base. The *recovery* is the sum of all four bases (the sum of the molar ratios); full recovery of all bases in the DNA would give a recovery of 1.0.

Molar ratios in ox DNA

	Thymus			Spleen		Liver
Base	**Prep. 1**	**Prep. 2**	**Prep. 3**	**Prep. 1**	**Prep. 2**	**Prep. 1**
Adenine	0.26	0.28	0.30	0.25	0.26	0.26
Guanine	0.21	0.24	0.22	0.20	0.21	0.20
Cytosine	0.16	0.18	0.17	0.15	0.17	
Thymine	0.25	0.24	0.25	0.24	0.24	
Recovery	*0.88*	*0.94*	*0.94*	*0.84*	*0.88*	

Molar ratios in human DNA

	Sperm		Thymus	Liver	
Base	**Prep. 1**	**Prep. 2**	**Prep. 1**	**Normal**	**Carcinoma**
Adenine	0.29	0.27	0.28	0.27	0.27
Guanine	0.18	0.17	0.19	0.19	0.18
Cytosine	0.18	0.18	0.16		0.15
Thymine	0.31	0.30	0.28		0.27
Recovery	*0.96*	*0.92*	*0.91*		*0.87*

Molar ratios in DNA of microorganisms

	Yeast		Avian tubercle bacilli
Base	**Prep. 1**	**Prep. 2**	**Prep. 1**
Adenine	0.24	0.30	0.12
Guanine	0.14	0.18	0.28
Cytosine	0.13	0.15	0.26
Thymine	0.25	0.29	0.11
Recovery	*0.76*	*0.92*	*0.77*

(a) Based on these data, Chargaff concluded that "no differences in composition have so far been found in DNA from different tissues of the same species." However, a skeptic looking at the data might say, "They certainly look different to me!" If you were Chargaff, how would you use the data to change the skeptic's mind?
(b) The base composition of DNA from normal and cancerous liver cells (hepatocarcinoma) was not distinguishably different. Would you expect Chargaff's technique to be capable of detecting a difference between the DNA of normal and cancerous cells? Explain your reasoning.

As you might expect, Chargaff's data were not completely convincing. He went on to improve his techniques, as described in his 1951 paper, in which he reported molar ratios of bases in DNA from a variety of organisms.

Source	A:G	T:C	A:T	G:C	Purine:pyrimidine
Ox	1.29	1.43	1.04	1.00	1.1
Human	1.56	1.75	1.00	1.00	1.0
Hen	1.45	1.29	1.06	0.91	0.99
Salmon	1.43	1.43	1.02	1.02	1.02
Wheat	1.22	1.18	1.00	0.97	0.99
Yeast	1.67	1.92	1.03	1.20	1.0
Haemophilus influenzae type c	1.74	1.54	1.07	0.91	1.0
E. coli K-12	1.05	0.95	1.09	0.99	1.0
Avian tubercle bacillus	0.4	0.4	1.09	1.08	1.1
Serratia marcescens	0.7	0.7	0.95	0.86	0.9
Bacillus schatz	0.7	0.6	1.12	0.89	1.0

(c) According to Chargaff, "The base composition of DNA generally varies from one species to another." Provide an argument, based on the data presented so far, that supports this conclusion.

(d) According to Chargaff's rules, "In *all* cellular DNAs, regardless of the species, . . . A + G = T + C." Provide an argument, based on the data presented, that supports this conclusion.

References

Chargaff, E. 1950. Chemical specificity of nucleic acids and mechanism of their enzymatic degradation. *Experientia* 6:201–209.

Chargaff, E. 1951. Structure and function of nucleic acids as cell constituents. *Fed. Proc.* 10:654–659.

Chapter 9

DNA-BASED INFORMATION TECHNOLOGIES

9.1 Studying Genes and Their Products *302*

9.2 Exploring Protein Function on the Scale of Cells or Whole Organisms *317*

9.3 Genomics and the Human Story *326*

The complexity of the molecules and systems revealed in this book can sometimes conceal a scientific reality: what we have learned is just a beginning. Novel proteins and lipids and carbohydrates and nucleic acids are discovered every day, and we often have no clue as to their functions. How many have yet to be encountered, and what might they do? Even well-characterized biomolecules continue to challenge researchers with countless unresolved mechanistic and functional questions. A new era, defined by technologies that provide broad access to the entirety of a cell's DNA, the genome, has accelerated progress.

This is a methods chapter, a necessary prelude to much of what comes later in this book. It is organized around just a few straightforward principles:

P1 **An organism's DNA—its genome—is the ultimate source of biological information.** Genomic information is a resource of unparalleled importance for investigators studying any aspect of biology. Genomes vary in size but all are large enough to direct all aspects of an organism's structure and function. To approach them often requires tools to break them into small parts that are experimentally digestible.

P2 **Genomic information is accessible.** Advances in DNA sequencing (Chapter 8) are being matched by new approaches to understanding how chromosomal information is expressed and regulated on a genomic and cellular scale. Important clues to protein function are embedded in the sequences of the genes that encode them.

P3 **Genomic information is malleable.** We can not only elucidate cellular genomic information; we can also change it. That capacity provides a path to altering any aspect of cellular metabolism, structure, or function.

The word "genome," coined by German botanist Hans Winkler in 1920, was derived from the Greek words *genesis* and *soma* to describe a body of genes. A **genome** today is defined as the complete haploid genetic complement of an organism. In essence, a genome is one copy of the hereditary information required to specify the organism. For sexually reproducing organisms, the genome includes one set of autosomes and one of each type of sex chromosome. When cells have organelles that also contain DNA, the genetic content of the organelles is not considered part of the nuclear genome. Mitochondria, found in most eukaryotic cells, and chloroplasts, in the light-harvesting cells of photosynthetic organisms, each have their own distinct genome. For viruses, which can have genetic material composed of DNA or RNA, the genome is a complete copy of the nucleic acid required to specify the virus.

As objects of study, DNA molecules present a special problem: their size. Chromosomes are far and away the largest biomolecules in any cell. How do researchers find the information they seek when it is just a small part of a chromosome that can include millions or even billions of contiguous base pairs? Decades of advances by thousands of scientists working in genetics, biochemistry, cell biology, and physical chemistry came together in the laboratories of Paul Berg, Herbert Boyer, and Stanley Cohen to yield the first techniques for locating, isolating, preparing, and studying small segments of DNA derived from much larger chromosomes. The science of **genomics** is dedicated to the study of DNA on a cellular scale. In turn, genomics contributes to **systems biology**, the study of biochemistry on the scale of whole cells and organisms.

The methods described in this chapter were built on advances in our understanding of DNA and RNA metabolism that are not presented in this text until Part III. Fundamental concepts of DNA replication, RNA transcription, protein synthesis, and gene regulation are intrinsic to an appreciation for how these methods work. Yet all facets of modern biochemistry rely on these same methods to such an extent that a current treatment of any aspect of the discipline becomes very difficult without a proper introduction to them. By presenting these technologies early in the book, we acknowledge that they are inextricably interwoven with both the advances that gave rise to them and the newer discoveries they now make possible. The background we necessarily provide makes the discussion here not just an introduction to technology but also a preview of many of the fundamentals of DNA and RNA biochemistry encountered in later chapters.

We begin by outlining the principles of DNA cloning, then illustrate the range of applications and the potential of many newer technologies that support and accelerate the advance of biochemistry.

9.1 Studying Genes and Their Products

A researcher has isolated a new enzyme that she knows is the key to a human disease. She hopes to isolate large amounts of the protein to crystallize it for structural analysis and to study it. She wants to alter amino acid residues at its active site so that she can understand the reaction it catalyzes. She plans an elaborate research program to elucidate how this enzyme interacts with, and is regulated by, other proteins in the cell. All of this, and much more, becomes possible if she can isolate the gene encoding her enzyme. Unfortunately, that gene consists of just a few thousand base pairs within a human chromosome with a size measured in hundreds of millions of base pairs. How does she isolate the small segment that she needs and then study it? The answer lies in DNA cloning and methods developed to manipulate cloned genes.

Genes Can Be Isolated by DNA Cloning

A *clone* is an identical copy. This term originally applied to cells of a single type, isolated and allowed to reproduce to create a population of identical cells. When applied to DNA, a clone represents many identical copies of a particular gene segment. P1 In brief, our researcher must separate the gene from the larger chromosome, attach it to a much smaller piece of carrier DNA, and allow microorganisms to make many copies of it. This is the process of **DNA cloning**. The result is selective amplification of a particular gene or DNA segment so that its genetic information may be studied and utilized. Classically, the cloning of DNA from any organism entails five general procedures:

1. *Obtaining the DNA segment to be cloned.* Enzymes called restriction endonucleases act as precise molecular scissors, recognizing specific sequences in DNA and cleaving genomic DNA into smaller fragments suitable for cloning. Alternatively, genomic DNA can be sheared randomly into fragments of a desired size. Since the sequence of targeted genomic regions is often known (available in databases), DNA segments to be cloned are most often amplified by the polymerase chain reaction (PCR) or are simply synthesized (both methods are described in Chapter 8).
2. *Selecting a small molecule of DNA capable of autonomous replication.* These small DNAs are called **cloning vectors** (a vector is a carrier or delivery agent). Most cloning vectors used in the laboratory are modified versions of naturally occurring small DNA molecules found in bacteria or eukaryotes. Viral DNAs may also play this role.
3. *Joining two DNA fragments covalently.* The enzyme DNA ligase links the cloning vector to the DNA fragment to be cloned. Composite DNA molecules of this type, comprising covalently linked segments from two or more sources, are called **recombinant DNAs**.
4. *Moving recombinant DNA from the test tube to a host organism.* The host organism provides the enzymatic machinery for DNA replication.
5. *Selecting or identifying host cells that contain recombinant DNA.* The cloning vector generally has features that allow the host cells to survive in an environment in which cells lacking the vector would die. Cells containing the vector are thus "selectable" in that environment.

The methods used to accomplish these and related tasks are collectively referred to as **recombinant DNA technology** or, more informally, **genetic engineering**.

Much of our initial discussion focuses on DNA cloning in the bacterium *Escherichia coli*, the first organism used for recombinant DNA work and still the most common host cell. *E. coli* has many advantages: its DNA metabolism (like many other of its biochemical processes) is well understood; many naturally occurring cloning vectors associated with *E. coli*, such as plasmids and bacteriophages (bacterial viruses; also called phages), are readily available; and techniques are available for moving DNA expeditiously from one bacterial cell to another. The principles discussed here are broadly applicable to DNA cloning in other organisms, a topic discussed more fully later in the section.

Restriction Endonucleases and DNA Ligases Yield Recombinant DNA

A set of enzymes (**Table 9-1**) made available through decades of research on nucleic acid metabolism is indispensable for generating and propagating a recombinant DNA molecule (**Fig. 9-1**). First, P1 **restriction endonucleases** (also called restriction enzymes)

TABLE 9-1 Some Enzymes Used in Recombinant DNA Technology

Enzyme(s)	Function
Type II restriction endonucleases	Cleave DNA molecules at specific base sequences
DNA ligase	Joins two DNA molecules or fragments
DNA polymerase I (*E. coli*)	Fills gaps in duplexes by stepwise addition of nucleotides to 3′ ends
Reverse transcriptase	Makes a DNA copy of an RNA molecule
Polynucleotide kinase	Adds a phosphate to the 5′-OH end of a polynucleotide to label it or to permit ligation
Terminal transferase	Adds homopolymer tails to the 3′-OH ends of a linear duplex
Exonuclease III	Removes nucleotide residues from the 3′ ends of a DNA strand
Bacteriophage λ exonuclease	Removes nucleotides from the 5′ ends of a duplex to expose single-stranded 3′ ends
Alkaline phosphatase	Removes terminal phosphates from the 5′ end or 3′ end (or both)

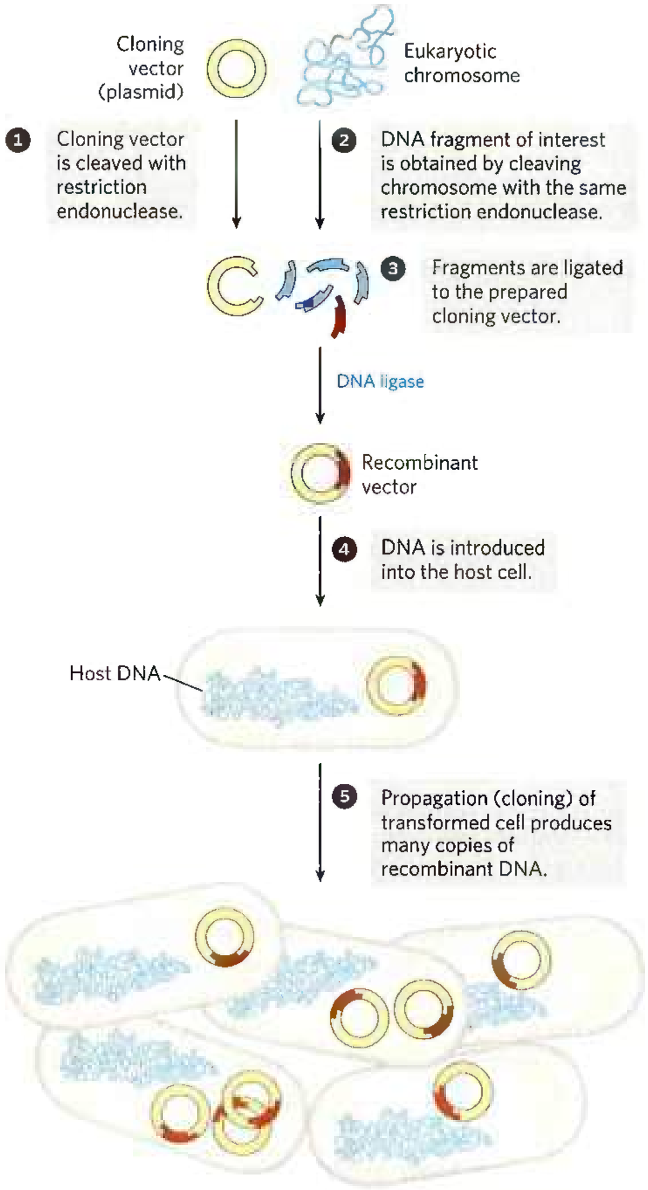

Cloning vector (plasmid)
Eukaryotic chromosome
1 Cloning vector is cleaved with restriction endonuclease.
2 DNA fragment of interest is obtained by cleaving chromosome with the same restriction endonuclease.
3 Fragments are ligated to the prepared cloning vector.
DNA ligase
Recombinant vector
4 DNA is introduced into the host cell.
Host DNA
5 Propagation (cloning) of transformed cell produces many copies of recombinant DNA.

FIGURE 9-1 Schematic illustration of DNA cloning. A cloning vector and eukaryotic chromosomes are separately cleaved with the same restriction endonuclease. (A single chromosome is shown here for simplicity.) The fragments to be cloned are then ligated to the cloning vector. The resulting recombinant DNA (only one recombinant vector is shown here) is introduced into a host cell, where it can be propagated (cloned). Note that this drawing is not to scale: the size of the *E. coli* chromosome relative to that of a typical cloning vector (such as a plasmid) is much greater than depicted here.

recognize and cleave DNA at specific sequences (recognition sequences or restriction sites) to generate a set of smaller fragments. Second, the DNA fragment to be cloned is joined to a suitable cloning vector by using **DNA ligases** to link the DNA molecules together. The recombinant vector is then introduced into a host cell, which amplifies the fragment in the course of many generations of cell division.

Restriction endonucleases are found in a wide range of bacterial species. As Werner Arber discovered in the early 1960s, the biological function of restriction endonucleases is to recognize and cleave foreign DNA (the DNA of an infecting virus, for example); such DNA is said to be *restricted*. In the host cell's DNA, the sequence that would be recognized by one of its own restriction endonucleases is protected from digestion by methylation of the DNA, catalyzed by a specific DNA methylase. The restriction endonuclease and the corresponding methylase are sometimes referred to as a **restriction-modification system**.

There are three types of restriction endonucleases, designated I, II, and III. Types I and III are generally large, multisubunit complexes containing both the endonuclease and methylase activities. **Type II restriction endonucleases**, first isolated by Hamilton Smith in 1970, are simpler, require no ATP, and catalyze the hydrolytic cleavage of particular phosphodiester bonds in the DNA within the recognition sequence itself. The extraordinary utility of this group of restriction endonucleases was demonstrated by Daniel Nathans, who first used them to develop novel methods for mapping and analyzing genes and genomes.

Thousands of type II restriction endonucleases have been discovered in different bacterial species, and more than 100 different DNA sequences are recognized by one or more of these enzymes. The recognition sequences are usually 4 to 6 bp long and are palindromic (see Fig. 8-18). **Table 9-2** lists sequences recognized by a few type II restriction endonucleases.

Some restriction endonucleases make staggered cuts on the two DNA strands, leaving two to four

TABLE 9-2 Recognition Sequences for Some Type II Restriction Endonucleases

BamHI	↓ * (5′) G G A T C C (3′) C C T A G G * ↑	HindIII	↓ (5′) A A G C T T (3′) T T C G A A ↑
ClaI	↓ * (5′) A T C G A T (3′) T A G C T A * ↑	NotI	↓ (5′) G C G G C C G C (3′) C G C C G G C G ↑
EcoRI	↓ * (5′) G A A T T C (3′) C T T A A G * ↑	PstI	* ↓ (5′) C T G C A G (3′) G A C G T C ↑ *
EcoRV	↓ (5′) G A T A T C (3′) C T A T A G ↑	PvuII	↓ (5′) C A G C T G (3′) G T C G A C ↑
HaeIII	↓* (5′) G G C C (3′) G G C C *↑	Tth111I	↓ (5′) G A C N N N G T C (3′) C T G N N N C A G ↑

Note: Arrows indicate the phosphodiester bonds cleaved by each restriction endonuclease. Asterisks indicate bases that are methylated by the corresponding methylase (where known). N denotes any base. Note that the name of each enzyme consists of a three-letter abbreviation of the bacterial species from which it is derived, sometimes followed by a strain designation and roman numerals to distinguish different restriction endonucleases isolated from the same bacterial species. Thus BamHI is the first (I) restriction endonuclease characterized from *Bacillus amyloliquefaciens*, strain H.

nucleotides of one strand unpaired at each resulting end. These unpaired strands are referred to as **sticky ends** (**Fig. 9-2a**) because they can base-pair with each other or with complementary sticky ends of other DNA fragments. Other restriction endonucleases cleave both strands of DNA straight across, at opposing phosphodiester bonds, leaving no unpaired bases on the ends, often called **blunt ends** (Fig. 9-2b).

P2 The gene or DNA segment to be cloned is most often generated by the polymerase chain reaction. Careful design of the primers used for PCR (see Fig. 8-33) can alter the amplified segment by the inclusion, at each end, of additional DNA not present in the chromosome that is being targeted. For example, including restriction endonuclease cleavage sites can facilitate the subsequent cloning of the amplified DNA (Fig. 9-2c).

P3 After the target DNA fragment is prepared and digested with the appropriate restriction enzyme, DNA ligase can be used to join it to a vector digested by the *same* restriction endonuclease; a fragment generated by EcoRI, for example, generally will not link to a fragment generated by BamHI. As described in more detail in Chapter 25 (see Fig. 25-15), DNA ligase catalyzes the formation of new phosphodiester bonds in a reaction that uses ATP or a similar cofactor. The base pairing of complementary sticky ends greatly facilitates the ligation reaction (Fig. 9-2a). Blunt ends can also be ligated, albeit less efficiently. Researchers can create new DNA sequences for a wide range of purposes by inserting synthetic DNA fragments, called **linkers**, to bridge the ends that are being ligated. An inserted DNA fragment with multiple recognition sequences for restriction endonucleases (often useful later as points for inserting additional DNA by cleavage and ligation) is called the **multiple cloning site (MCS)** (Fig. 9-2d).

The effectiveness of sticky ends in selectively joining two DNA fragments was apparent in the earliest recombinant DNA experiments. Before restriction endonucleases were widely available, some investigators found they could generate sticky ends by the combined action of the bacteriophage λ exonuclease and terminal transferase (Table 9-1). The fragments to be joined were given complementary homopolymeric tails. Peter Lobban and Dale Kaiser used this method in 1971 in the first experiments to join naturally occurring DNA fragments. Similar methods were used soon after in Paul Berg's laboratory to join DNA segments from simian virus 40 (SV40) to DNA derived from bacteriophage λ, thereby creating the first recombinant DNA molecule with DNA segments from different species.

Cloning Vectors Allow Amplification of Inserted DNA Segments

The factors that govern the delivery of recombinant DNA in clonable form to a host cell, and its subsequent amplification in the host, are well illustrated in three popular cloning vectors: plasmids and bacterial

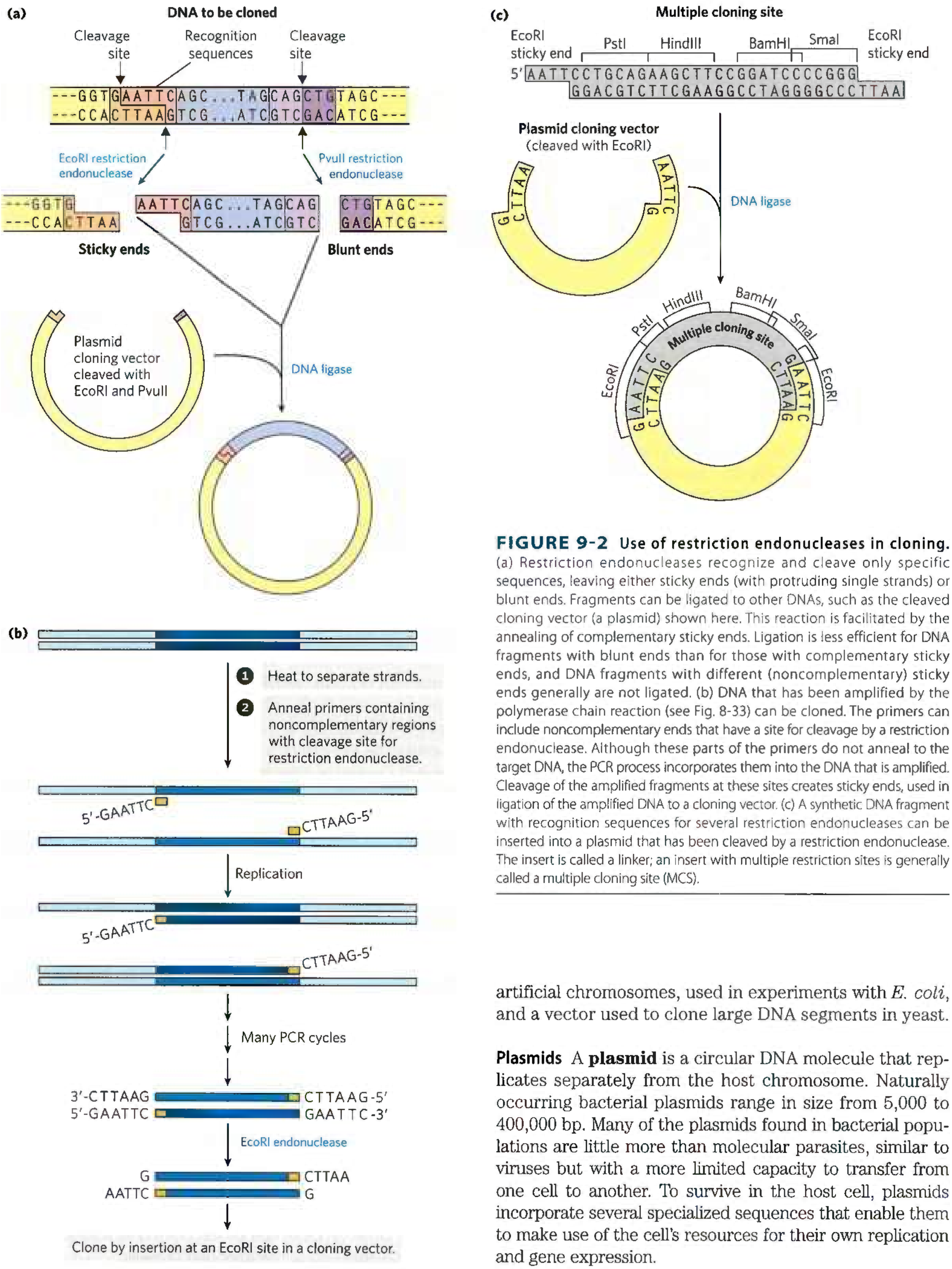

(a)
DNA to be cloned
Cleavage site
Recognition sequences
Cleavage site
---GGTGAATTCAGC...TAGCAGCTGTAGC---
---CCACTTAAGTCG...ATCGTCGACATCG---
EcoRI restriction endonuclease
PvuII restriction endonuclease
---GGTG
---CCACTTAA
AATTCAGC...TAGCAG
GTCG...ATCGTC
CTGTAGC---
GACATCG---
Sticky ends
Blunt ends
Plasmid cloning vector cleaved with EcoRI and PvuII
DNA ligase
(b)
1 Heat to separate strands.
2 Anneal primers containing noncomplementary regions with cleavage site for restriction endonuclease.
5′-GAATTC
CTTAAG-5′
Replication
5′-GAATTC
CTTAAG-5′
Many PCR cycles
3′-CTTAAG
5′-GAATTC
CTTAAG-5′
GAATTC-3′
EcoRI endonuclease
G
AATTC
CTTAA
G
Clone by insertion at an EcoRI site in a cloning vector.
(c)
Multiple cloning site
EcoRI sticky end
PstI
HindIII
BamHI
SmaI
EcoRI sticky end
5′ AATTCCTGCAGAAGCTTCCGGATCCCCGGG
GGACGTCTTCGAAGGCCTAGGGGCCCTTAA
Plasmid cloning vector (cleaved with EcoRI)
CTTAA G
AATTC G
DNA ligase
PstI
HindIII
BamHI
SmaI
EcoRI
EcoRI
Multiple cloning site
GAATTC
CTTAAG
GAATTC
CTTAAG

FIGURE 9-2 Use of restriction endonucleases in cloning. (a) Restriction endonucleases recognize and cleave only specific sequences, leaving either sticky ends (with protruding single strands) or blunt ends. Fragments can be ligated to other DNAs, such as the cleaved cloning vector (a plasmid) shown here. This reaction is facilitated by the annealing of complementary sticky ends. Ligation is less efficient for DNA fragments with blunt ends than for those with complementary sticky ends, and DNA fragments with different (noncomplementary) sticky ends generally are not ligated. (b) DNA that has been amplified by the polymerase chain reaction (see Fig. 8-33) can be cloned. The primers can include noncomplementary ends that have a site for cleavage by a restriction endonuclease. Although these parts of the primers do not anneal to the target DNA, the PCR process incorporates them into the DNA that is amplified. Cleavage of the amplified fragments at these sites creates sticky ends, used in ligation of the amplified DNA to a cloning vector. (c) A synthetic DNA fragment with recognition sequences for several restriction endonucleases can be inserted into a plasmid that has been cleaved by a restriction endonuclease. The insert is called a linker; an insert with multiple restriction sites is generally called a multiple cloning site (MCS).

artificial chromosomes, used in experiments with *E. coli*, and a vector used to clone large DNA segments in yeast.

Plasmids A **plasmid** is a circular DNA molecule that replicates separately from the host chromosome. Naturally occurring bacterial plasmids range in size from 5,000 to 400,000 bp. Many of the plasmids found in bacterial populations are little more than molecular parasites, similar to viruses but with a more limited capacity to transfer from one cell to another. To survive in the host cell, plasmids incorporate several specialized sequences that enable them to make use of the cell's resources for their own replication and gene expression.

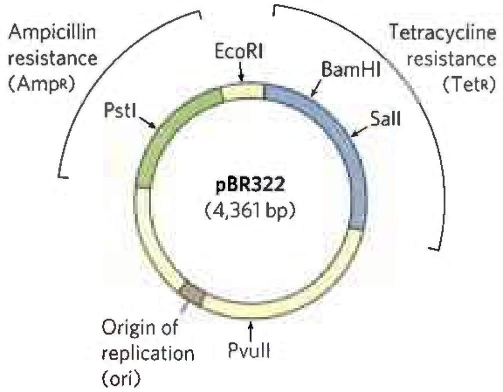

Ampicillin resistance (AmpR)
EcoRI
BamHI
Tetracycline resistance (TetR)
PstI
SalI
pBR322
(4,361 bp)
Origin of replication (ori)
PvuII

FIGURE 9-3 The constructed *E. coli* plasmid pBR322. Notice the location of some important restriction sites, for PstI, EcoRI, BamHI, SalI, and PvuII; genes for ampicillin and tetracycline resistance (Amp^R and Tet^R); and the replication origin (ori). Constructed in 1977, this was one of the early plasmids designed expressly for cloning in *E. coli*.

Naturally occurring plasmids usually have a symbiotic role in the cell. They may provide genes that confer resistance to antibiotics or that perform new functions for the cell. For example, the Ti plasmid of *Agrobacterium tumefaciens* allows the host bacterium to colonize the cells of a plant and make use of the plant's resources. The same properties that enable plasmids to grow and survive in a bacterial or eukaryotic host are useful to molecular biologists who want to engineer a vector for cloning a specific DNA segment. Constructed in 1977, one of the first recombinant vectors—*E. coli* plasmid pBR322—illustrates some key features that define a useful cloning vector (**Fig. 9-3**):

1. The plasmid pBR322 has an **origin of replication**, or **ori**, a sequence where replication is initiated by cellular enzymes (see Chapter 25). This sequence is required to propagate the plasmid. An associated regulatory system is present that limits replication to maintain pBR322 at a level of 10 to 20 copies per cell.
2. The plasmid contains genes that confer resistance to the antibiotics ampicillin (Amp^R) and tetracycline (Tet^R), allowing the selection of cells that contain the intact plasmid or a recombinant version of the plasmid (discussed below).
3. Several unique recognition sequences in pBR322 are targets for restriction endonucleases (PstI, EcoRI, BamHI, SalI, and PvuII), providing sites where the plasmid can be cut to insert foreign DNA.
4. The small size of the plasmid (4,361 bp) facilitates its entry into cells and the biochemical manipulation of the DNA. This small size was the result of trimming away many DNA segments from a larger, parent plasmid—sequences that the biochemist does not need.

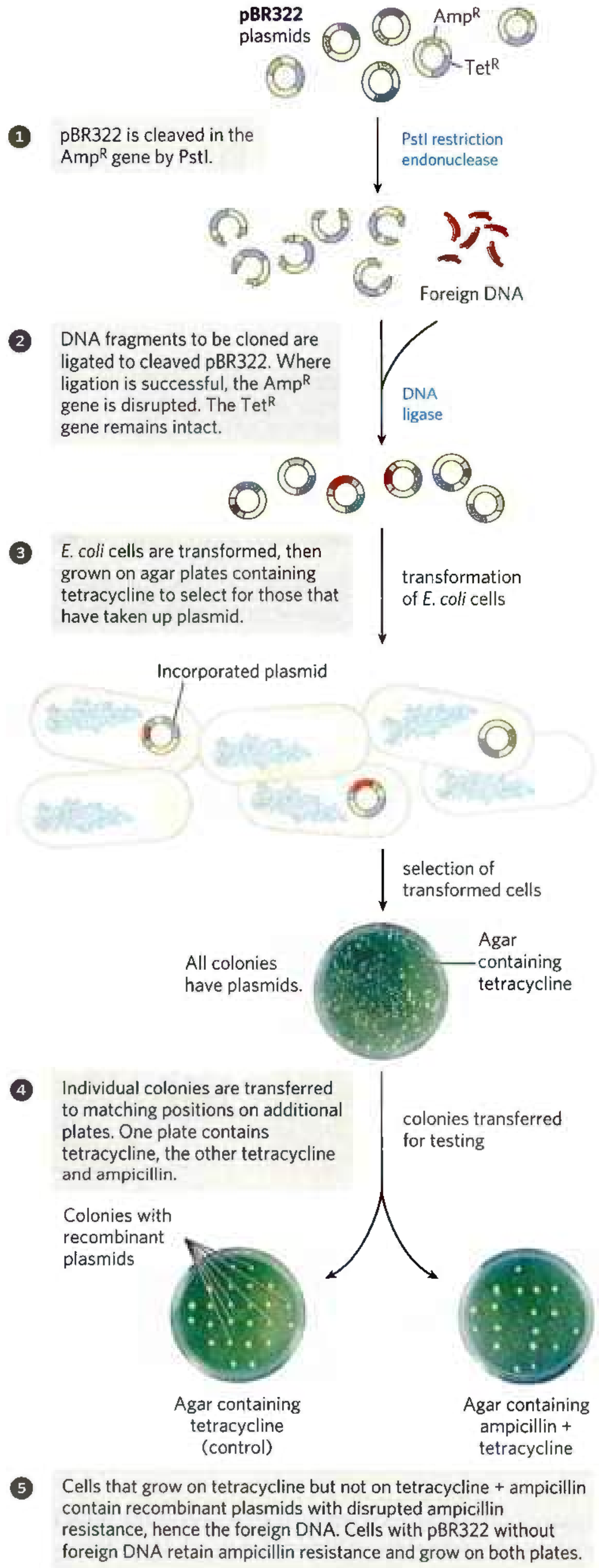
The replication origins inserted in common plasmid vectors were originally derived from naturally occurring plasmids. As in pBR322, each of these origins is regulated to maintain a particular plasmid copy number. Depending on the origin used, the plasmid copy number can vary from one to hundreds or thousands per cell, providing many options for investigators. Two different plasmids cannot function in the same cell if they use the same origin of replication, because the regulation of one will interfere with the replication of the other. Such plasmids are said to be incompatible. When a researcher wants to introduce two or more different plasmids into a bacterial cell, each plasmid must have a different replication origin.

In the laboratory, small plasmids can be introduced into bacterial cells by a process called **transformation**. The cells (often *E. coli*, but other bacterial species are also used) and plasmid DNA are incubated together at 0 °C in a calcium chloride solution, then are subjected to heat shock by rapidly shifting the temperature to between 37 °C and 43 °C. For reasons not well understood, some of the cells treated in this way take up the plasmid DNA. Some species of bacteria, such as *Acinetobacter baylyi*, are naturally competent for DNA uptake and do not require the calcium chloride–heat shock treatment. In an alternative method, called **electroporation**, cells incubated with the plasmid DNA are subjected to a high-voltage pulse, which transiently renders the bacterial membrane permeable to large molecules.

Regardless of the approach, relatively few cells take up the plasmid DNA, so a method is needed to identify those that do. The usual strategy is to utilize one of two types of genes in the plasmid, referred to as selectable and screenable markers. A **selectable marker** either permits the growth of a cell (positive selection) or kills the cell (negative selection) under a defined set of conditions. The plasmid pBR322 provides markers for both positive and negative selection (**Fig. 9-4**). A **screenable marker** is a gene encoding a protein that causes the cell to produce a colored or fluorescent molecule. Cells are not harmed when the gene is present, and the cells that carry the plasmid are easily identified by the colored or fluorescent colonies they produce.

Transformation of typical bacterial cells with purified DNA (never a very efficient process) becomes less successful as plasmid size increases, and it is difficult to clone DNA segments longer than about 15,000 bp when plasmids are used as the vector.

To illustrate the use of a plasmid as a cloning vector, consider the bacterial gene encoding a recombinase called the RecA protein (see Chapter 25). In most bacteria, the gene encoding RecA is one of the thousands of genes on a chromosome millions of base pairs long. The *recA* gene is just over 1,000 bp long. A plasmid would be a good choice for cloning a gene of this size. As described later, the cloned gene can be altered in a variety of ways, and the gene variants can be expressed at high levels to enable purification of the encoded protein.


pBR322
plasmids
Amp^R
Tet^R
PstI restriction endonuclease
Foreign DNA
DNA ligase
transformation of *E. coli* cells
Incorporated plasmid
selection of transformed cells
All colonies have plasmids.
Agar containing tetracycline
colonies transferred for testing
Colonies with recombinant plasmids
Agar containing tetracycline (control)
Agar containing ampicillin + tetracycline

1. pBR322 is cleaved in the Amp^R gene by PstI.
2. DNA fragments to be cloned are ligated to cleaved pBR322. Where ligation is successful, the Amp^R gene is disrupted. The Tet^R gene remains intact.
3. *E. coli* cells are transformed, then grown on agar plates containing tetracycline to select for those that have taken up plasmid.
4. Individual colonies are transferred to matching positions on additional plates. One plate contains tetracycline, the other tetracycline and ampicillin.
5. Cells that grow on tetracycline but not on tetracycline + ampicillin contain recombinant plasmids with disrupted ampicillin resistance, hence the foreign DNA. Cells with pBR322 without foreign DNA retain ampicillin resistance and grow on both plates.

FIGURE 9-4 Use of pBR322 to clone foreign DNA in *E. coli* and identify cells containing the DNA. [Courtesy Elizabeth A. Wood, University of Wisconsin-Madison, Department of Biochemistry.]

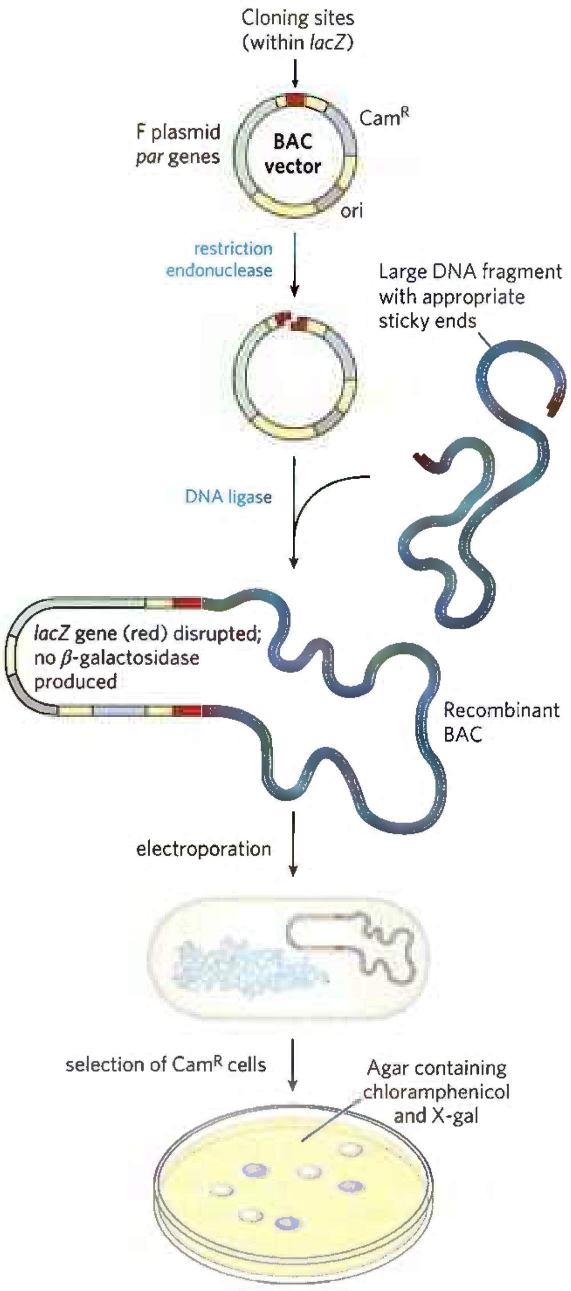
Bacterial Artificial Chromosomes Researchers sometimes want to clone much longer DNA segments than can typically be incorporated into standard plasmid cloning vectors such as pBR322. To meet this need, plasmid vectors have been developed with special features that allow the cloning of very long segments (typically 100,000 to 300,000 bp) of DNA. Once such large segments of cloned DNA have been added, these vectors are large enough to be thought of as chromosomes and are known as **bacterial artificial chromosomes**, or **BACs** (**Fig. 9-5**).

A BAC vector (without any cloned DNA inserted) is a relatively simple plasmid, generally not much larger than other plasmid vectors. To accommodate very long segments of cloned DNA, BAC vectors have stable origins of replication that maintain the plasmid at one or two copies per cell. The low copy number is useful in cloning large segments of DNA, because it limits the opportunities for unwanted recombination reactions that can unpredictably alter large cloned DNAs over time. BACs also include *par* genes, derived from a type of plasmid called an F plasmid. The *par* genes encode proteins that direct the reliable distribution of the recombinant chromosomes to daughter cells at cell division, thereby increasing the likelihood of each daughter cell carrying one copy, even when few copies are present.

The BAC vector includes both selectable and screenable markers. The BAC vector shown in Figure 9-5 contains a gene that confers resistance to the antibiotic chloramphenicol (Cam^R). Vector-containing cells can be selected by growing them on agar plates containing this antibiotic—a positive selection, as the cells with the vector survive. A *lacZ* gene, required for production of the enzyme β-galactosidase, is a screenable marker that can reveal which cells contain plasmids—now chromosomes—that incorporate the cloned DNA segments. The β-galactosidase catalyzes conversion of the colorless molecule 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (more simply, X-gal) to a blue product. If the gene is intact and expressed, the colony containing it is blue. If gene expression is disrupted by the introduction of a cloned DNA segment, the colony is white.

Yeast Artificial Chromosomes As with *E. coli*, yeast genetics is a well-developed discipline. Research on large genomes and the associated need for high-capacity cloning vectors led to the development of **yeast artificial chromosomes**, or **YACs** (**Fig. 9-6**). As with BACs, YAC vectors can be used to clone very long segments of DNA. In addition, **P3** the DNA cloned in a YAC can be altered to study the function of specialized sequences in chromosome metabolism, mechanisms of gene regulation and expression, and many other aspects of eukaryotic molecular biology.

The genome of *Saccharomyces cerevisiae* contains only 14×10^6 bp (less than four times the size of the *E. coli* chromosome), and its entire sequence is known.


Cloning sites
(within *lacZ*)
F plasmid
par genes
BAC
vector
Cam^R
ori
restriction
endonuclease
Large DNA fragment
with appropriate
sticky ends
DNA ligase
lacZ gene (red) disrupted;
no *β*-galactosidase
produced
Recombinant
BAC
electroporation
selection of Cam^R cells
Agar containing
chloramphenicol
and X-gal

Colonies with recombinant BACs are white.

FIGURE 9-5 Bacterial artificial chromosomes (BACs) as cloning vectors. The vector is a relatively simple plasmid, with a replication origin (ori) that directs replication. The *par* genes assist in the even distribution of plasmids to daughter cells at cell division. This increases the likelihood of each daughter cell carrying one copy of the plasmid, even when few copies are present. The low number of copies is useful in cloning large segments of DNA, because this limits the opportunities for unwanted recombination reactions that can unpredictably alter large cloned DNAs over time. The BAC includes selectable markers. A *lacZ* gene (required for the production of the enzyme *β*-galactosidase) is situated in the cloning region such that it is inactivated by cloned DNA inserts. Introduction of recombinant BACs into cells by electroporation is promoted by the use of cells with an altered (more porous) cell wall. Recombinant DNAs are screened for resistance to the antibiotic chloramphenicol (Cam^R). Plates also contain X-gal, a substrate for *β*-galactosidase that yields a blue product. Colonies with active *β*-galactosidase, and hence no DNA insert in the BAC vector, turn blue; colonies without *β*-galactosidase activity, and thus with the desired DNA inserts, are white.

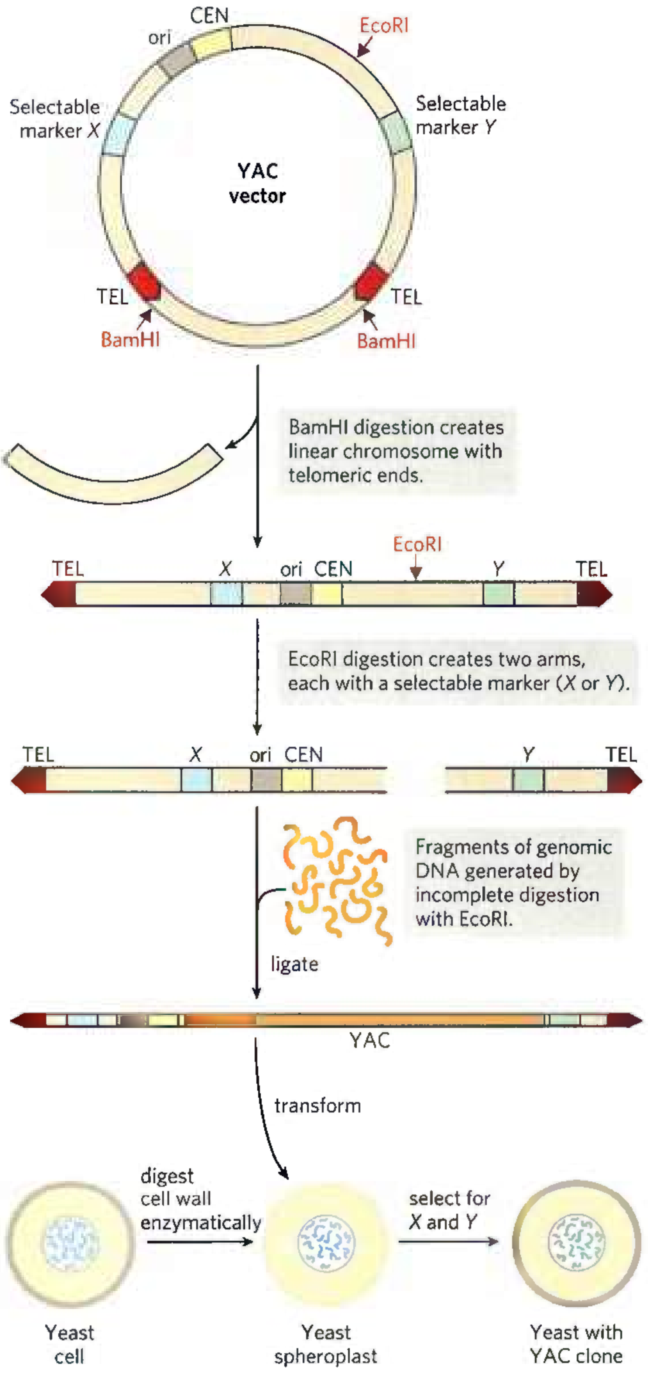

CEN
ori
EcoRI
Selectable
marker *X*
Selectable
marker *Y*
YAC
vector
TEL
TEL
BamHI
BamHI
BamHI digestion creates
linear chromosome with
telomeric ends.
EcoRI
TEL
X
ori
CEN
Y
TEL
EcoRI digestion creates two arms,
each with a selectable marker (*X* or *Y*).
TEL
X
ori
CEN
Y
TEL
Fragments of genomic
DNA generated by
incomplete digestion
with EcoRI.
ligate
YAC
transform
digest
cell wall
enzymatically
select for
X and *Y*
Yeast
cell
Yeast
spheroplast
Yeast with
YAC clone

FIGURE 9-6 Construction of a yeast artificial chromosome (YAC). A YAC vector includes an origin of replication (ori), a centromere (CEN), two telomeres (TEL), and selectable markers (*X* and *Y*). Digestion with BamHI and EcoRI generates two separate DNA arms, each with a telomeric end and one selectable marker. A large segment of DNA (e.g., up to 2×10^6 bp from the human genome) is ligated to the two arms to create a yeast artificial chromosome. The YAC transforms yeast cells (prepared by removal of the cell wall to form spheroplasts), and the cells are selected for *X* and *Y*; the surviving cells propagate the DNA insert.

Yeast is also very easy to maintain and grow on a large scale in the laboratory. Plasmid vectors have been constructed for insertions into yeast cells, employing the same principles that govern the use of *E. coli* vectors. Convenient methods for moving DNA into and out of yeast cells permit the study of many aspects of eukaryotic cell biochemistry. Some recombinant plasmids incorporate multiple replication origins and other elements that allow them to be used in more than one species (e.g., in yeast and in *E. coli*). Plasmids that can be propagated in cells of two or more species are called **shuttle vectors**.

YAC vectors contain all the elements needed to maintain a eukaryotic chromosome in the yeast nucleus: a yeast origin of replication, two selectable markers, and specialized sequences (derived from the telomeres and centromere) needed for stability and proper segregation of the chromosomes at cell division (see Chapter 24). In preparation for its use in cloning, the vector is propagated as a circular bacterial plasmid and then isolated and purified. Cleavage with a restriction endonuclease (BamHI in Fig. 9-6) removes a length of DNA between two telomere sequences (TEL), leaving the telomeres at the ends of the linearized DNA. Cleavage at another internal site (by EcoRI in Fig. 9-6) divides the vector into two DNA segments, referred to as vector arms, each with a different selectable marker.

Genomic DNA to be cloned is prepared by partial digestion with restriction endonucleases to obtain a suitable fragment size. Genomic fragments are then separated by **pulsed field gel electrophoresis**, a variation of gel electrophoresis (see Fig. 3-18) that segregates very large DNA segments. DNA fragments of appropriate size (up to about 2×10^6 bp) are mixed with the prepared vector arms and ligated. The ligation mixture is then used to transform yeast cells (pretreated to partially degrade their cell walls) with these very large DNA molecules—which now have the structure and size to be considered yeast chromosomes. Culture on a medium that requires the presence of both selectable marker genes ensures the growth of only those yeast cells that contain an artificial chromosome with a large insert sandwiched between the two vector arms (Fig. 9-6). The stability of YAC clones increases with the length of the cloned DNA segment (up to a point). Those with inserts of more than 150,000 bp are nearly as stable as normal cellular chromosomes, whereas those with inserts of fewer than 100,000 bp are gradually lost during mitosis (so, generally, there are no yeast cell clones carrying only the two vector ends ligated together or vectors with only short inserts). YACs that lack a telomere at either end are rapidly degraded.

Cloned Genes Can Be Expressed to Amplify Protein Production

P1 Frequently, the product of a cloned gene, rather than the gene itself, is of primary interest—particularly when the protein has commercial, therapeutic, or research value. Proteins are encoded by genes in DNA; alter the DNA in a gene, and one can alter the protein product of that gene. Biochemists use purified proteins for many purposes, including to elucidate protein function, study reaction mechanisms, generate antibodies to the proteins, reconstitute complex cellular activities in the test tube with purified components, and examine protein binding partners. With an increased understanding of the fundamentals of DNA, RNA, and protein metabolism and their regulation in a host organism such as *E. coli* or yeast, investigators can manipulate cells to express cloned genes in order to study their protein products. The general goal is to alter the sequences around a cloned gene to trick the host organism into producing the protein product of the gene, often at very high levels. This overexpression of a protein can make its subsequent purification much easier.

We'll use the expression of a eukaryotic protein in a bacterium as an example. Eukaryotic genes have surrounding sequences needed for their transcription and regulation in the cells they are derived from, but these sequences do not function in bacteria. Thus, eukaryotic genes lack the DNA sequence elements required for their controlled expression in bacterial cells: promoters (sequences that instruct RNA polymerase where to bind to initiate mRNA synthesis), ribosome-binding sites (sequences that allow translation of the mRNA to protein), and additional regulatory sequences. Appropriate bacterial regulatory sequences for transcription and translation must be inserted in the vector DNA at the correct positions relative to the eukaryotic gene.

Cloning vectors with the transcription and translation signals needed for the regulated expression of a cloned gene are called **expression vectors**. The rate of expression of the cloned gene is controlled by replacing the gene's normal promoter and regulatory sequences with more efficient and convenient versions supplied by the vector. Generally, a well-characterized promoter and its regulatory elements are positioned near several unique restriction sites for cloning, so that genes inserted at the restriction sites will be expressed from the regulated promoter elements (**Fig. 9-7**). Some of these vectors incorporate other features, such as a bacterial ribosome-binding site to enhance translation of the mRNA derived from the gene (Chapter 27) or a transcription termination sequence (Chapter 26). In some cases, cloned genes are so efficiently expressed that their protein product represents 10% or more of the cellular protein. At these concentrations, some foreign proteins can kill the host cell (usually *E. coli*), so expression of the cloned gene must be limited to the few hours before the planned harvesting of the cells.

Many Different Systems Are Used to Express Recombinant Proteins

Every living organism has the capacity to express genes in its genomic DNA; thus, in principle, any organism can serve as a host to express proteins from a different

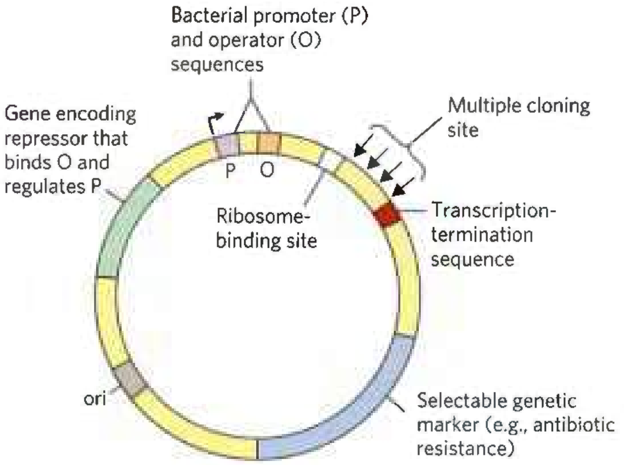

Bacterial promoter (P) and operator (O) sequences
Gene encoding repressor that binds O and regulates P
P
O
Multiple cloning site
Ribosome-binding site
Transcription-termination sequence
ori
Selectable genetic marker (e.g., antibiotic resistance)

FIGURE 9-7 DNA sequences in a typical *E. coli* expression vector. The gene to be expressed is inserted into one of the restriction sites in the MCS, near the promoter (P), with the end of the gene encoding the amino terminus of the protein positioned closest to the promoter. The promoter allows efficient transcription of the inserted gene, and the transcription-termination sequence sometimes improves the amount and stability of the mRNA produced. The operator (O) permits regulation by a repressor that binds to it. The ribosome-binding site provides sequence signals for the efficient translation of the mRNA derived from the gene. The selectable marker allows the selection of cells containing the recombinant DNA.

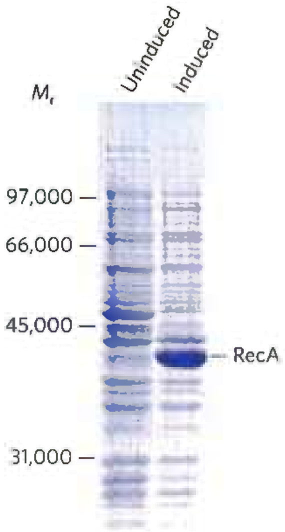


FIGURE 9-8 Regulated expression of RecA protein in a bacterial cell. The gene encoding the RecA protein, fused to a bacteriophage T7 promoter, is cloned into an expression vector. Under normal growth conditions (uninduced), no RecA protein appears. When the T7 RNA polymerase is induced in the cell, the *recA* gene is expressed, and large amounts of RecA protein are produced. The positions of standard molecular weight markers that were run on the same gel are indicated. [Courtesy Rachel Britt, University of Wisconsin–Madison, Department of Biochemistry.]

(heterologous) species. Almost every sort of organism has, indeed, been used for this purpose, and each host type has a particular set of advantages and disadvantages.

Bacteria Bacteria, especially *E. coli*, remain the most common hosts for protein expression. The regulatory sequences that govern gene expression in *E. coli* and many other bacteria are well understood and can be harnessed to express cloned proteins at high levels. Bacteria are easy to store and grow in the laboratory, on inexpensive growth media. Efficient methods also exist to get DNA into bacteria and extract DNA from them. Bacteria can be grown in huge amounts in commercial fermenters, providing a rich source of the cloned protein.

Problems do exist, however. When expressed in bacteria, some heterologous proteins do not fold correctly, and many do not undergo the posttranslational modifications or proteolytic cleavage that may be necessary for their activity. Certain features of a gene sequence also can make a particular gene difficult to express in bacteria. For example, intrinsically disordered regions are more common in eukaryotic proteins. When expressed in bacteria, many eukaryotic proteins aggregate into insoluble cellular precipitates called **inclusion bodies**. For these and many other reasons, some eukaryotic proteins are inactive when purified from bacteria or cannot be expressed at all. To help address some of these problems, researchers are regularly developing new bacterial host strains that include enhancements such as the engineered presence of eukaryotic protein chaperones or enzymes that modify eukaryotic proteins.

There are many specialized systems for expressing proteins in bacteria. The well-characterized promoter and regulatory sequences associated with the lactose operon (see Chapter 28) are often fused to the gene of interest to direct transcription. The cloned gene will be transcribed when lactose is added to the growth medium. However, regulation in the lactose system is "leaky": it is not turned off completely when lactose is absent — a potential problem if the product of the cloned gene is toxic to the host cells. Transcription from the Lac promoter is also not efficient enough for some applications.

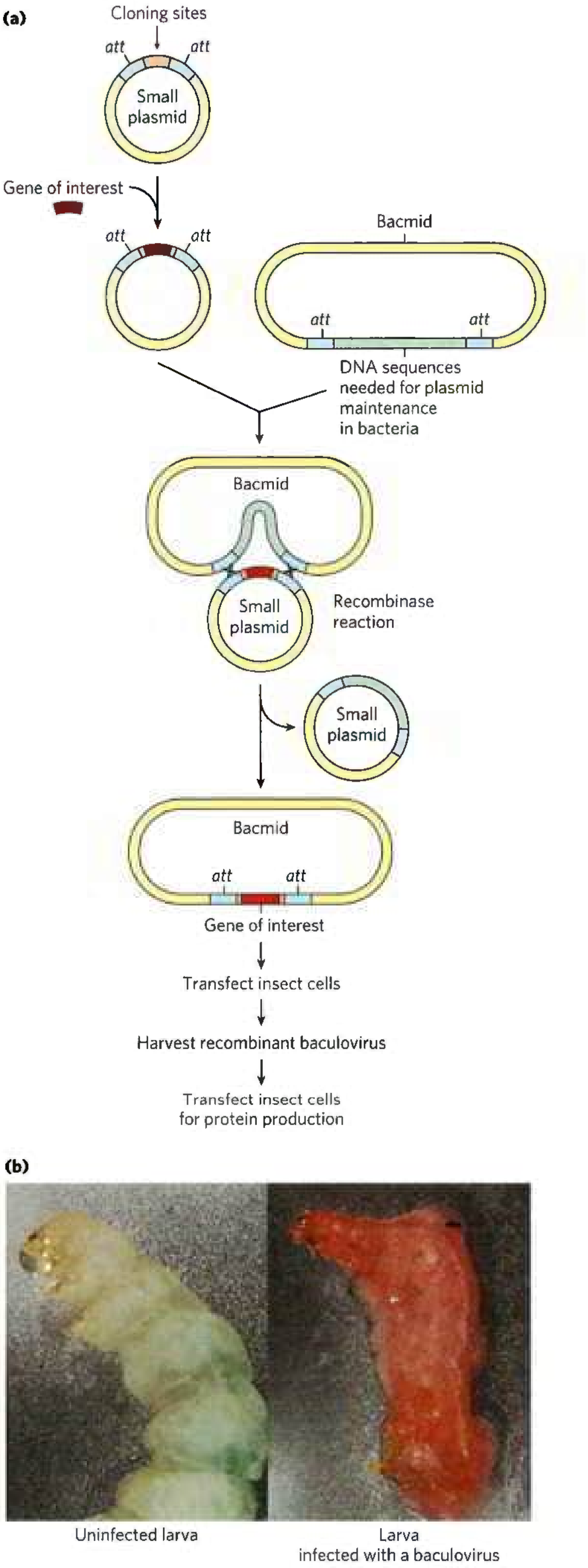
An alternative system uses the promoter and RNA polymerase of a bacterial virus called bacteriophage T7. If the cloned gene is fused to a T7 promoter, it is transcribed, not by the *E. coli* RNA polymerase, but by the T7 RNA polymerase. The gene encoding this polymerase is separately cloned into the same cell in a construct that affords tight regulation (allowing controlled production of the T7 RNA polymerase). The polymerase is also very efficient and directs high levels of expression of most genes fused to the T7 promoter. This system has been used to express the RecA protein in bacterial cells (**Fig. 9-8**).

Yeast *Saccharomyces cerevisiae* is probably the best understood eukaryotic organism. The principles underlying the expression of a protein in yeast are the same as those for bacteria. Cloned genes must be linked to promoters that can direct high-level expression in yeast cells. For example, the yeast *GAL1* and *GAL10* genes (encoding enzymes involved in galactose metabolism) are under cellular regulation such that they are expressed when yeast cells are grown in media with galactose but shut down when the cells are grown in glucose. Thus, if a heterologous gene is expressed using these same regulatory sequences, the expression of that gene can be controlled simply by choosing an appropriate medium for cell growth.

Some of the same problems that accompany protein expression in bacteria also occur with yeast. Heterologous proteins may not fold properly, yeast may lack the enzymes needed to modify the proteins to their active forms, or certain features of the gene sequence may hinder expression of a protein. However, because *S. cerevisiae* is a eukaryote, the expression of eukaryotic genes (especially yeast genes) is sometimes more efficient in this host than in bacteria. As yeast possess many of the same protein chaperones and modification systems of higher eukaryotes, protein products may also be folded and modified more accurately than are proteins expressed in bacteria.

Insects and Insect Viruses **Baculoviruses** are insect viruses with double-stranded DNA genomes. When baculoviruses infect their insect larval hosts, they act as parasites, killing the larvae and turning them into factories for virus production. Late in the infection process, the viruses produce large amounts of two proteins (p10 and polyhedrin), neither of which is needed for production of viruses in cultured insect cells. The genes for both of these proteins can be replaced with the gene for a heterologous protein. When the resulting recombinant virus is used to infect insect cells or larvae, the heterologous protein is often produced at very high levels—up to 25% of the total protein present at the end of the infection cycle.

Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV; *A. californica* is a moth species that it infects) is the baculovirus most often used for protein expression. It has a large genome (134,000 bp), too large for direct cloning. Virus purification is also cumbersome. These problems have been solved by the creation of **bacmids**, large circular DNAs that include the entire baculovirus genome along with sequences that allow replication of the bacmid in *E. coli* (**Fig. 9-9**). The gene of interest is cloned into a smaller plasmid and combined with the larger plasmid by site-specific recombination



Uninfected larva

Larva infected with a baculovirus

FIGURE 9-9 **Cloning with baculoviruses.** (a) Shown here is the construction of a typical vector used for protein expression in baculoviruses. The gene of interest is cloned into a small plasmid (top left) between two sites (*att*) recognized by a site-specific recombinase, then is introduced into the baculovirus vector by site-specific recombination. This generates a circular DNA product that is used to infect the cells of an insect larva. The gene of interest is expressed during the infection cycle, downstream of a promoter that normally expresses a baculovirus coat protein at very high levels. (b) The photographs show larvae of the cabbage looper moth. The larva on the left is uninfected; the larva on the right was infected with a recombinant baculovirus vector expressing a protein that produces a red color. [(b) USDA-ARS.]

in vivo (see Fig. 25-37). The recombinant bacmid is then isolated and transfected into insect cells (the term **transfection** is used when the DNA used for transformation includes viral sequences and leads to viral replication), followed by recovery of the protein once the infection cycle is finished. A wide range of bacmid systems are available commercially. Baculovirus systems are not successful with all proteins. However, with these systems, insect cells sometimes successfully replicate the protein-modification patterns of higher eukaryotes and produce active, correctly modified eukaryotic proteins.

Mammalian Cells in Culture The most convenient way to introduce cloned genes into a mammalian cell is with viruses. This method takes advantage of the natural capacity of a virus to insert its DNA or RNA into a cell, and sometimes into the cellular chromosome. A variety of engineered mammalian viruses are available as vectors, including human adenoviruses and retroviruses. The gene of interest is cloned so that its expression is controlled by a virus promoter. The virus uses its natural infection mechanisms to introduce the recombinant genome into cells, where the cloned protein is expressed. One advantage of these systems is that proteins can be expressed either transiently (if the viral DNA is maintained separately from the host cell genome and eventually degraded) or permanently (if the viral DNA is integrated into the host cell genome). With the correct choice of host cell, the proper posttranslational modification of the protein to its active form can be ensured. However, the growth of mammalian cells in tissue culture is very expensive, and this technology is generally used to test the function of a protein in vivo rather than to produce a protein in large amounts.

Alteration of Cloned Genes Produces Altered Proteins

P1 Cloning techniques can be used not only to overproduce proteins but also to produce proteins that are altered, subtly or dramatically, from their native forms. Specific amino acids may be replaced individually by **site-directed mutagenesis**. This approach has greatly enhanced research on proteins by allowing investigators to make specific changes in the primary structure and examine the effects of these changes on the protein's folding, three-dimensional structure, and activity. The amino acid sequence of the protein is changed by altering the DNA sequence of the cloned gene. If appropriate restriction sites flank the sequence to be altered, researchers can simply remove a DNA segment and replace it with a synthetic one, identical to the original except for the desired change (**Fig. 9-10a**).

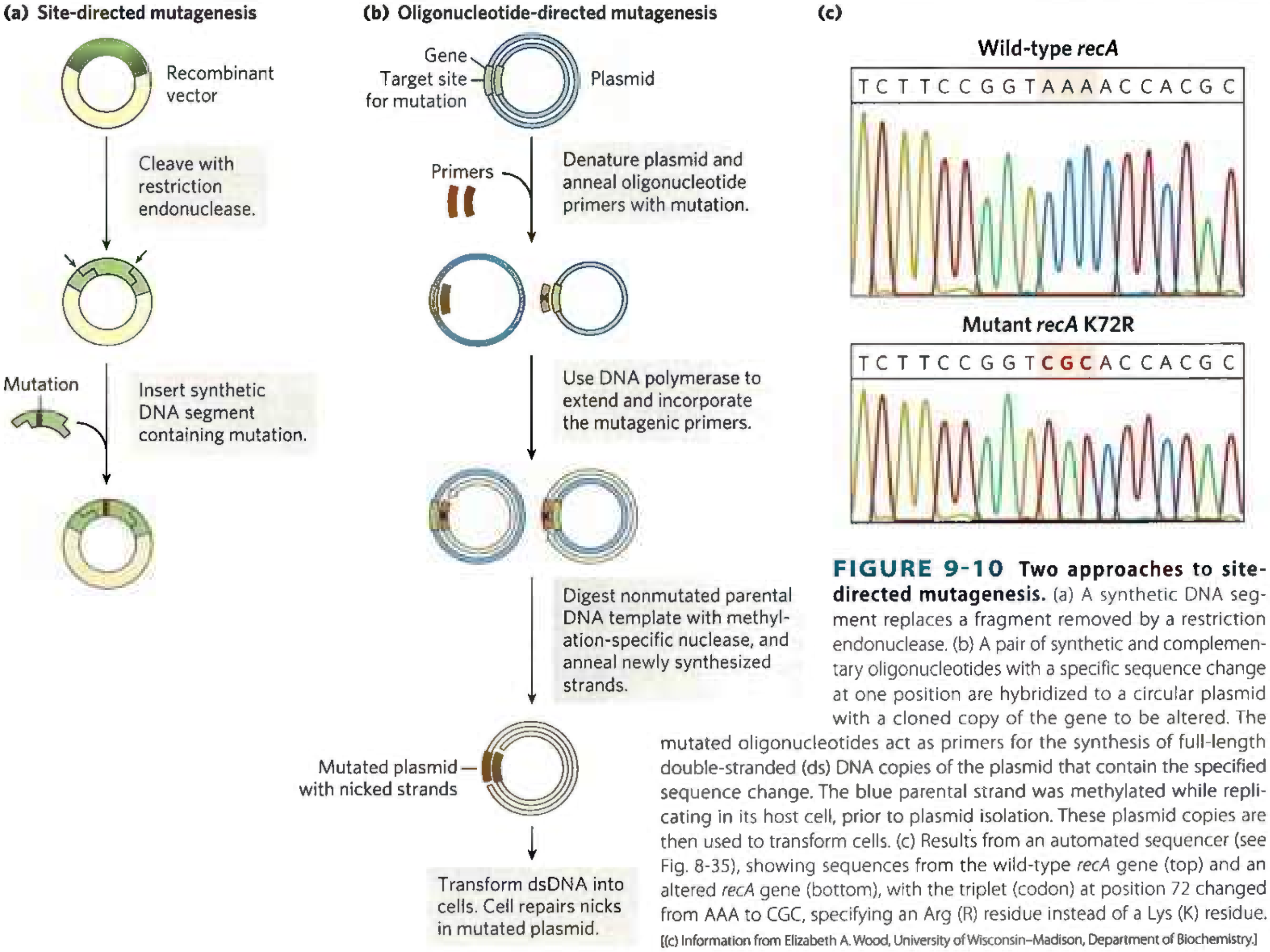

(a) Site-directed mutagenesis
Recombinant vector
Cleave with restriction endonuclease.
Mutation
Insert synthetic DNA segment containing mutation.
(b) Oligonucleotide-directed mutagenesis
Gene
Target site for mutation
Plasmid
Primers
Denature plasmid and anneal oligonucleotide primers with mutation.
Use DNA polymerase to extend and incorporate the mutagenic primers.
Digest nonmutated parental DNA template with methylation-specific nuclease, and anneal newly synthesized strands.
Mutated plasmid with nicked strands
Transform dsDNA into cells. Cell repairs nicks in mutated plasmid.
(c)
Wild-type *recA*
T C T T C C G G T A A A A C C A C G C
Mutant *recA* K72R
T C T T C C G G T C G C A C C A C G C

FIGURE 9-10 Two approaches to site-directed mutagenesis. (a) A synthetic DNA segment replaces a fragment removed by a restriction endonuclease. (b) A pair of synthetic and complementary oligonucleotides with a specific sequence change at one position are hybridized to a circular plasmid with a cloned copy of the gene to be altered. The mutated oligonucleotides act as primers for the synthesis of full-length double-stranded (ds) DNA copies of the plasmid that contain the specified sequence change. The blue parental strand was methylated while replicating in its host cell, prior to plasmid isolation. These plasmid copies are then used to transform cells. (c) Results from an automated sequencer (see Fig. 8-35), showing sequences from the wild-type *recA* gene (top) and an altered *recA* gene (bottom), with the triplet (codon) at position 72 changed from AAA to CGC, specifying an Arg (R) residue instead of a Lys (K) residue. [(c) Information from Elizabeth A. Wood, University of Wisconsin–Madison, Department of Biochemistry.]

When suitably located restriction sites are not present, **oligonucleotide-directed mutagenesis** can create a specific DNA sequence change (Fig. 9-10b). The cloned gene is denatured, separating the strands. Two short, complementary synthetic DNA strands, each with the desired base change, are annealed to opposite strands of the cloned gene within a suitable circular DNA vector. The mismatch of a single base pair in 30 to 40 bp does not prevent annealing. The two annealed oligonucleotides serve to prime DNA synthesis in both directions around the plasmid vector, creating two complementary strands that contain the mutation. After several cycles of selective amplification by the polymerase chain reaction (PCR; see Fig. 8-33), the mutation-containing DNA predominates in the population and can be used to transform bacteria. Most of the transformed bacteria will have plasmids carrying the mutation.

For an example, we go back to the bacterial *recA* gene. The product of this gene, the RecA protein, has several activities (see Section 25.3) including the hydrolysis of ATP. The Lys residue at position 72 in RecA (a 352 residue polypeptide) is involved in ATP hydrolysis. Changing Lys^{72} to an Arg creates a variant of RecA protein that will bind, but not hydrolyze, ATP (Fig. 9-10c). The engineering and purification of this variant RecA protein has facilitated research into the roles of ATP hydrolysis in the functioning of this protein.

Changes can be introduced into a gene that involve far more than one base pair. Large parts of a gene can be deleted by cutting out a segment with restriction endonucleases and ligating the remaining portions to form a smaller gene. For example, if a protein has two domains, the gene segment encoding one of the domains can be removed so that the gene now encodes a protein with only one of the original two domains. Parts of two different genes can be ligated to create new combinations; the product of such a fused gene is called a **fusion protein**. Researchers have ingenious methods to bring about virtually any genetic alteration in vitro. After reintroducing the altered DNA into the cell, they can investigate the consequences of the alteration.

Terminal Tags Provide Handles for Affinity Purification

Affinity chromatography is one of the most efficient methods for purifying proteins (see Fig. 3-17c). Unfortunately, many proteins do not bind a ligand that can be conveniently immobilized on a column matrix. However, the gene for almost any protein can be altered to express a fusion protein that can be purified by affinity chromatography. The gene encoding the target protein is fused to a gene encoding a peptide or protein that binds a simple, stable ligand with high affinity and specificity. The peptide or protein used for this purpose is referred to as a **tag**. Tag sequences can be added to genes such that the resulting proteins have tags at their amino terminus or carboxyl terminus. **Table 9-3** lists some of the peptides or proteins commonly used as tags.

TABLE 9-3 Commonly Used Protein Tags

Tag protein/peptide	Molecular mass (kDa)	Immobilized ligand
Protein A	59	Fc portion of IgG
$(His)_6$	0.8	Ni^{2+}
Glutathione-*S*-transferase (GST)	26	Glutathione
Maltose-binding protein	41	Maltose
β-Galactosidase	116	*p*-Aminophenyl-*β*-D-thiogalactoside (TPEG)
Chitin-binding domain	5.7	Chitin

The general procedure can be illustrated by focusing on a system that uses the glutathione-*S*-transferase (GST) tag (**Fig. 9-11**). GST is a small enzyme (M_r 26,000) that binds tightly and specifically to glutathione. When the GST gene sequence is fused to a target gene, the fusion protein acquires the capacity to bind glutathione. The fusion protein is expressed in a host organism such as a bacterium, and a crude extract is prepared. A column is filled with a porous matrix consisting of the ligand (glutathione) immobilized on microscopic beads of a stable polymer such as cross-linked agarose. As the crude extract percolates through this matrix, the fusion protein becomes immobilized by binding the glutathione. The other proteins in the extract are washed through the column and discarded. The interaction between GST and glutathione is tight but noncovalent, allowing the fusion protein to be gently eluted from the column with a solution containing either a higher concentration of salts or free glutathione to compete with the immobilized ligand for GST binding. The fusion protein is often obtained with good yield and high purity. In some commercially available systems, the tag can be entirely or largely removed from the purified fusion protein by a protease that cleaves a sequence near the junction between the target protein and its tag.

A shorter tag with widespread application consists of a simple sequence of six or more His residues. These histidine tags, or His tags, bind tightly and specifically to nickel ions. A chromatography matrix with immobilized Ni^{2+} can be used to quickly separate a His-tagged protein from other proteins in an extract. Some of the larger tags, such as maltose-binding protein, provide added stability and solubility, allowing the purification of cloned proteins that are otherwise inactive due to improper folding or insolubility.

Affinity chromatography using terminal tags is powerful and convenient. The tags have been successfully used in thousands of published studies; in many cases, the protein would be impossible to purify and study without the tag. However, even very small tags can affect the properties of the proteins they are attached to, thereby influencing the study results. For example, the tag may adversely affect protein folding. Even if the tag is removed by a protease, one or a few extra amino acid residues can remain behind on the target protein, which may or may not affect the protein's activity. The types of experiments to be carried out, and the results obtained from them, should always be evaluated with the aid of well-designed controls to assess any effect of a tag on protein function.

The Polymerase Chain Reaction Offers Many Options for Cloning Experiments

Many adaptations of PCR have increased its utility in cloning. P1 For example, sequences in RNA can be amplified if the first PCR cycle uses reverse transcriptase, an enzyme that works like DNA polymerase (see Fig. 8-33) but uses RNA as a template (**Fig. 9-12a**). After the DNA strand is made from the RNA template, the remaining cycles can be carried out with DNA polymerases, using standard PCR protocols. This **reverse transcriptase PCR (RT-PCR)** can be used, for example, to detect sequences derived from living cells (which are transcribing their DNA into RNA) as opposed to dead tissues.

PCR protocols can also be used to estimate the relative copy numbers of particular sequences in a sample, an approach called **quantitative PCR (qPCR)** or **real-time PCR**. If a DNA sequence is present in higher

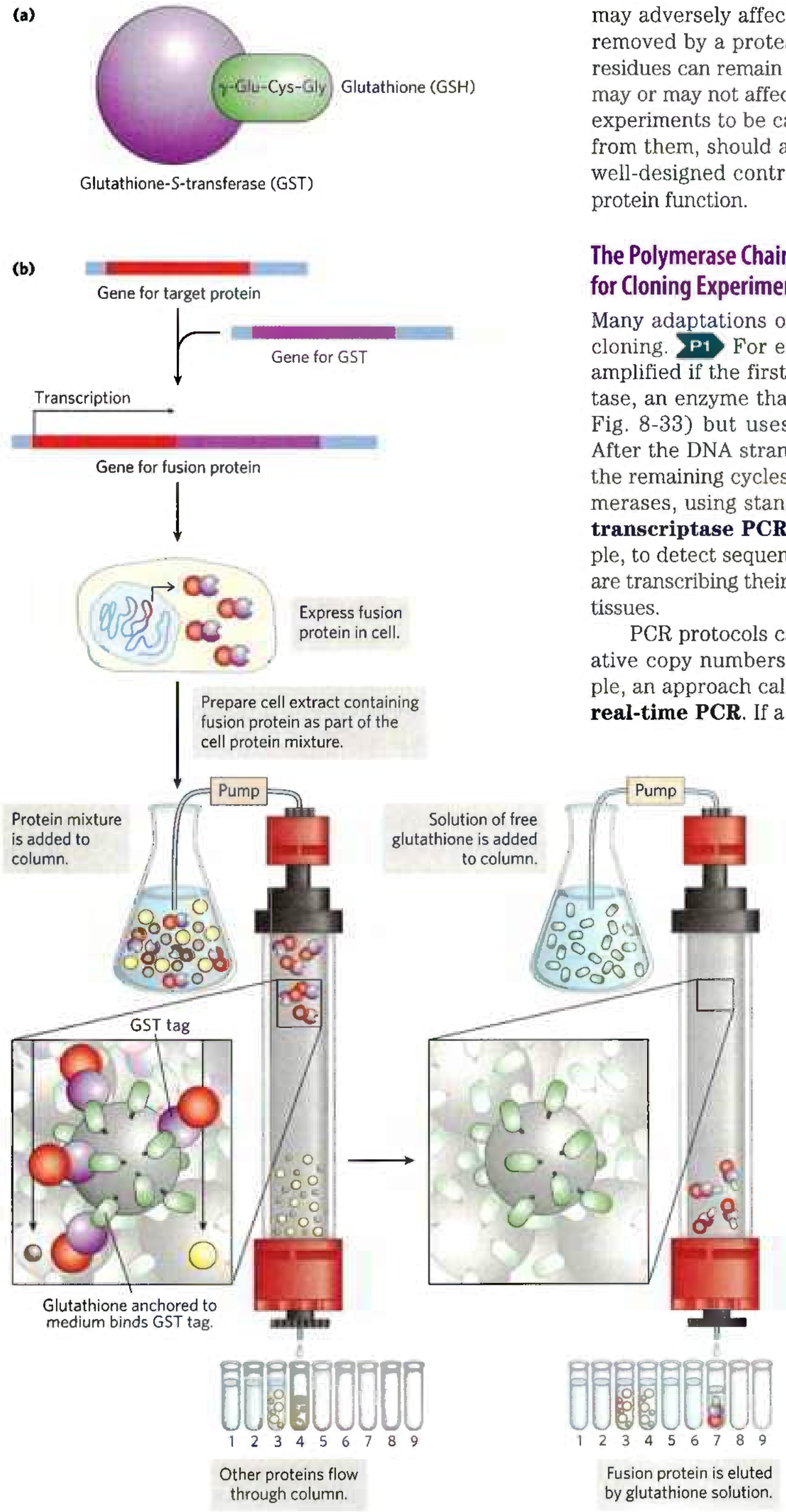
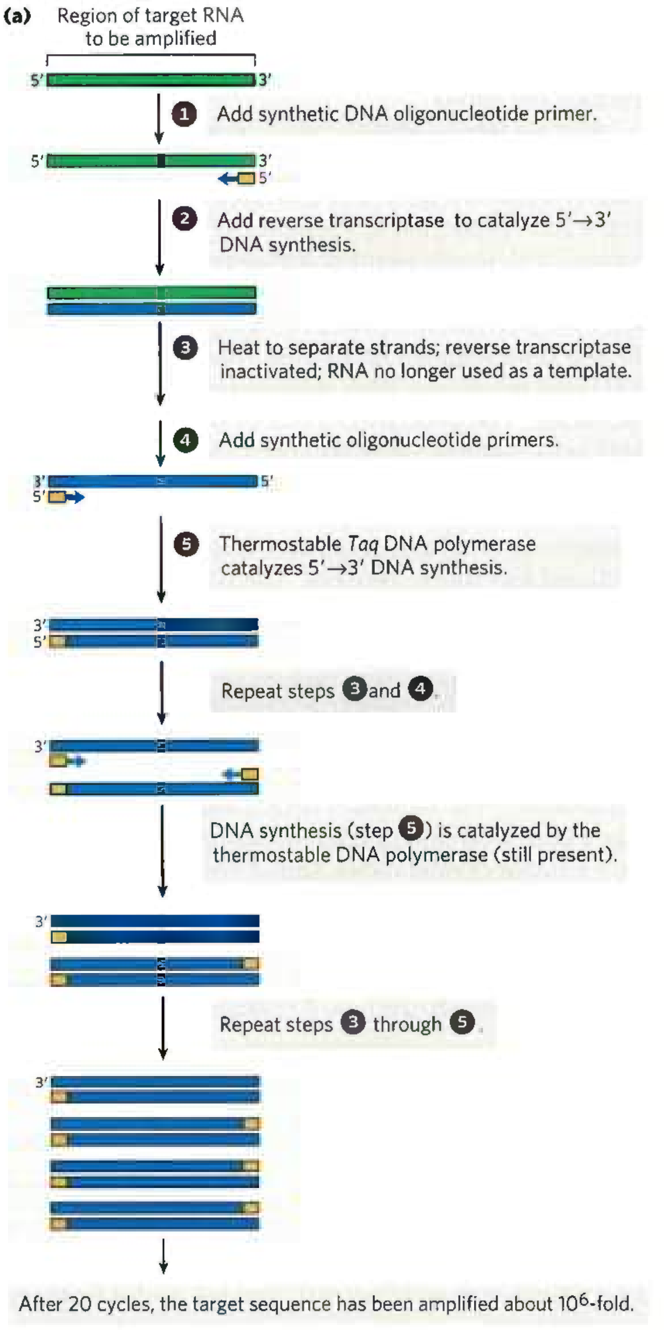

(a)
γ-Glu-Cys-Gly
Glutathione (GSH)
Glutathione-S-transferase (GST)
(b)
Gene for target protein
Gene for GST
Transcription
Gene for fusion protein
Express fusion protein in cell.
Prepare cell extract containing fusion protein as part of the cell protein mixture.
Protein mixture is added to column.
Pump
GST tag
Glutathione anchored to medium binds GST tag.
1 2 3 4 5 6 7 8 9
Other proteins flow through column.
Solution of free glutathione is added to column.
Pump
1 2 3 4 5 6 7 8 9
Fusion protein is eluted by glutathione solution.

FIGURE 9-11 Use of tagged proteins in protein purification. (a) Glutathione-S-transferase (GST) is a small enzyme that binds glutathione. (b) The GST tag is fused to the carboxyl terminus of the protein by genetic engineering. The tagged protein is expressed in the cell and is present in the crude extract when the cells are lysed. The extract is subjected to affinity chromatography through a matrix with immobilized glutathione.

than usual amounts in a sample—for example, if certain genes are amplified in tumor cells—qPCR can reveal the increased representation of that sequence. In brief, the PCR is carried out in the presence of a probe that emits a fluorescent signal when the PCR product is present (Fig. 9-12b). If the sequence of interest is present at higher levels than other sequences in the sample, the PCR signal will reach a predetermined threshold faster. Reverse transcriptase PCR and qPCR can be combined to determine the relative concentrations of a particular mRNA molecule in a cell, and thereby monitor gene expression under different environmental conditions.

DNA Libraries Are Specialized Catalogs of Genetic Information

In some instances, it is useful to clone many genes or genomic segments rather than a particular one. A **DNA library** is a collection of DNA clones, usually gathered for purposes of gene discovery or the determination of gene or protein function. The library can take a variety


(a)
Region of target RNA to be amplified
5'
3'
① Add synthetic DNA oligonucleotide primer.
5'
3'
5'
② Add reverse transcriptase to catalyze 5'→3' DNA synthesis.
③ Heat to separate strands; reverse transcriptase inactivated; RNA no longer used as a template.
④ Add synthetic oligonucleotide primers.
3'
5'
5'
⑤ Thermostable Taq DNA polymerase catalyzes 5'→3' DNA synthesis.
3'
5'
Repeat steps ③ and ④.
3'
DNA synthesis (step ⑤) is catalyzed by the thermostable DNA polymerase (still present).
3'
Repeat steps ③ through ⑤.
3'
After 20 cycles, the target sequence has been amplified about 10⁶-fold.

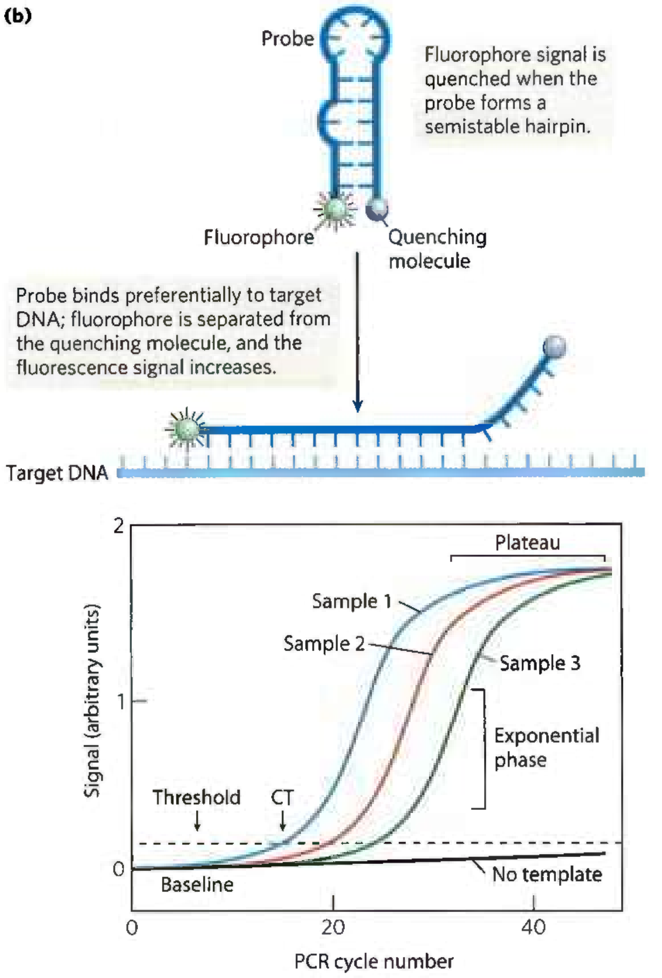


FIGURE 9-12 Some applications of PCR. (a) In reverse transcriptase PCR, or RT-PCR, RNA molecules are amplified by using reverse transcriptase in the first two cycles. (b) In quantitative PCR, or qPCR, careful monitoring of the progress of a PCR amplification allows one to determine when a DNA segment has been amplified to a specified threshold level. The amount of PCR product present is determined by measuring the level of a fluorescent probe attached to a reporter oligonucleotide complementary to the DNA segment that is being amplified. Probe fluorescence is not detectable initially, due to a fluorescence quencher attached to the same oligonucleotide. When the reporter oligonucleotide pairs with its complement in a copy of the amplified DNA segment, the fluorophore is separated from the quenching molecule and fluorescence results. As the PCR reaction proceeds, the amount of the targeted DNA segment increases exponentially, and the fluorescent signal also increases exponentially as the oligonucleotide probes anneal to the amplified segments. After many PCR cycles, the signal reaches a plateau as one or more reaction components become exhausted. When a segment is present in greater amounts in one sample than another, its amplification reaches a defined threshold level earlier. The "No template" line follows the slow increase in background signal observed in a control that does not include added sample DNA. CT is the cycle number at which the threshold is first surpassed.

of forms, depending on the source of the DNA and the ultimate purpose of the library.

An example is a library that includes only the genes that are transcribed into RNA—*expressed*—in a given organism or even just in certain cells or tissues. Such a library lacks any genomic DNA that is not transcribed. The researcher first extracts mRNA from an organism, or from specific cells of an organism, and then prepares the **complementary DNAs (cDNAs)**. Like RT-PCR, this multistep reaction (**Figure 9-13a**) relies on reverse transcriptase, which synthesizes DNA from a template RNA. The resulting double-stranded DNA fragments are inserted into a suitable vector and cloned, creating a population of clones called a **cDNA library**. If the library host is a bacterium like *E. coli*, each cell in the population will carry one particular cloned sequence. The library will encompass many millions of cells with millions of different cloned segments. The presence of a gene for a particular protein in such a library implies that this gene is expressed in the cells and under the conditions used to generate the library.

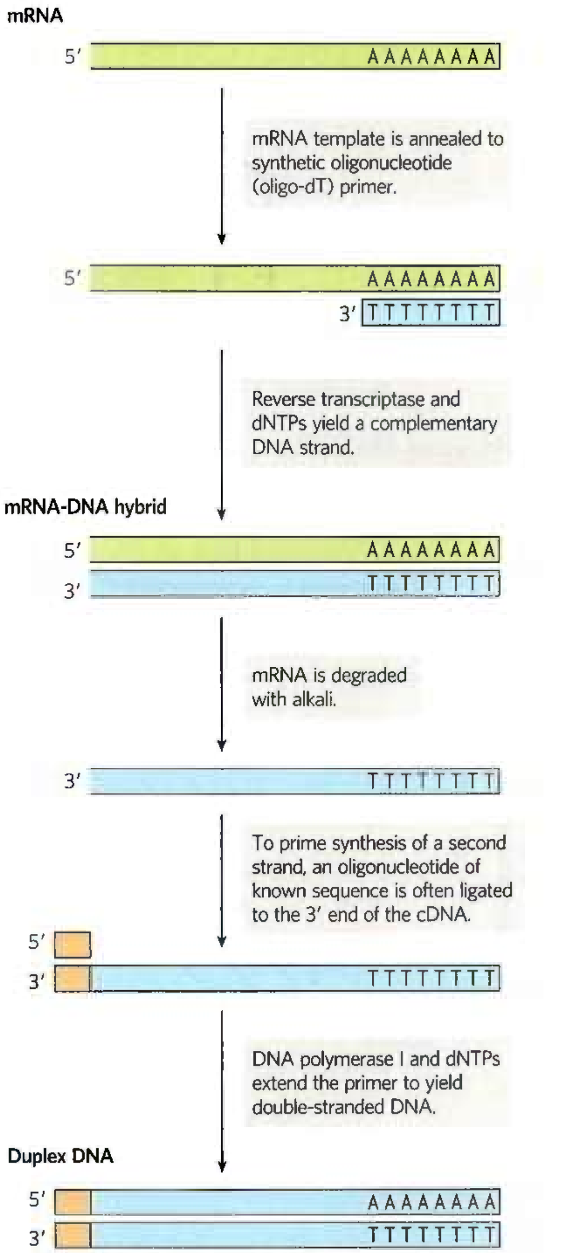

mRNA
5′
AAAAAAAA
mRNA template is annealed to synthetic oligonucleotide (oligo-dT) primer.
5′
AAAAAAAA
3′
TTTTTTTT
Reverse transcriptase and dNTPs yield a complementary DNA strand.
mRNA-DNA hybrid
5′
AAAAAAAA
3′
TTTTTTTT
mRNA is degraded with alkali.
3′
TTTTTTTT
To prime synthesis of a second strand, an oligonucleotide of known sequence is often ligated to the 3′ end of the cDNA.
5′
3′
TTTTTTTT
DNA polymerase I and dNTPs extend the primer to yield double-stranded DNA.
Duplex DNA
5′
AAAAAAAA
3′
TTTTTTTT

FIGURE 9-13 Building a cDNA library from mRNA. A cell's total mRNA content includes transcripts from thousands of genes, and the cDNAs generated from this mRNA are correspondingly heterogeneous. Reverse transcriptase can synthesize DNA on an RNA or a DNA template. To prime the synthesis of a second DNA strand, oligonucleotides of known sequence are ligated to the 3′ end of the first strand, and the double-stranded cDNA so produced is cloned into a plasmid.

Another type of library, called a **combinatorial gene library** or simply a **gene library**, focuses on sequence variants within one gene. For example, beginning with the cloned gene of enzyme X, a segment of the gene could be replaced with nearly identical fragments synthesized with a slight imprecision so that each clone had one or two random base pair changes relative to the original. For example, the gene segment of interest could be amplified by PCR using an altered DNA polymerase that was slightly inaccurate. The library of clones would then consist of many cells, many of which harbored a different variant of the gene for enzyme X. Investigators could use the library to select for variants of enzyme X with enhanced catalytic properties or could simply determine which changes were functional and which were not. The possibilities are limited only by the imagination of the researcher.

SUMMARY 9.1 *Studying Genes and Their Products*

- DNA cloning and genetic engineering involve the cleavage of DNA and assembly of DNA segments in new combinations—recombinant DNA. Cloning entails generating a DNA fragment of interest, inserting the fragment into a suitable cloning vector, transferring the vector with the DNA insert into a host cell for replication, and identifying and selecting cells that contain the DNA fragment.
- Key enzymes in gene cloning include restriction endonucleases (especially the type II enzymes) and DNA ligase.
- Cloning vectors include plasmids and, for the longest DNA inserts, bacterial artificial chromosomes (BACs) and yeast artificial chromosomes (YACs).
- Cloned genes can be expressed in a host cell by incorporating them into expression vectors that have the sequence signals needed for transcription and translation.
- Proteins can be expressed in different types of cells using expression systems with various useful features and advantages.
- Genetic engineering techniques can alter cloned genes as required by the investigator.
- Proteins or peptides can be attached to a protein of interest by altering its cloned gene, creating a fusion protein. The additional peptide segments can be used to detect the protein or to purify it, using convenient affinity chromatography methods.
- The polymerase chain reaction (PCR) permits the amplification of chosen segments of DNA or RNA for

cloning and can be adapted to determine gene copy number or to monitor gene expression quantitatively.

- DNA libraries consist of many clones, encompassing many genomic segments or many variants of a particular gene.

9.2 Exploring Protein Function on the Scale of Cells or Whole Organisms

Protein function can be described on three levels. **Phenotypic function** describes the effects of a protein on the entire organism. For example, loss of the protein may lead to slower growth of the organism, an altered development pattern, or even death. **Cellular function** is a description of the network of interactions a protein engages in at the cellular level. Identifying interactions with other proteins in the cell can help define the kinds of metabolic processes in which the protein participates. Finally, **molecular function** refers to the precise biochemical activity of a protein, including details such as the reactions that an enzyme catalyzes or the ligands that a receptor binds. In response to the challenge of understanding these functions of the thousands of proteins in a typical cell, scientists have developed a variety of techniques in the broader discipline of genomics. We can apply these techniques to determine when a particular protein is expressed, what other proteins it might be related to, where it is located in the cell, what other cellular components it interacts with, and what happens to the cell when the protein is missing.

A variety of interrelated methods broadly probe a cell's RNA or protein content. The entire complement of transcribed RNAs present at a given moment in a cell is defined as the cellular **transcriptome**. As introduced in Chapters 1 and 3, the entire complement of proteins present at a given moment in a cell is defined as that cell's **proteome**. Studies of transcriptomes and studies of proteomes are referred to as **transcriptomics** and **proteomics**, respectively. Changes in these cellular macromolecules that occur when a particular gene or its expression is altered can provide important additional clues about protein function, as we will see. The methods we cover here are summarized in **Table 9-4**. The list is by no means comprehensive, but it serves to illustrate important approaches.

TABLE 9-4 Methods for Discovering New Proteins and Exploring Their Functions

Clue	Method to Apply
What is a protein's function?	
What other proteins of known function have similar sequences?	Comparative genomics
What known sequence motifs does the protein possess?	Comparative genomics
Under what conditions is the gene encoding the protein expressed?	RNA-Seq
How much of the protein is present in the cell under different conditions?	Mass spectrometry
Where is the protein located in the cell?	Microscopy with fusion proteins and immunofluorescence
What does the protein interact with?	Immunoprecipitation; tandem affinity purification; yeast two-hybrid analysis
What happens to the cell when the protein is missing or altered?	CRISPR/Cas9 or other mutagenic methods
What genes (some unknown) are involved in a process?	Large-scale screening

Sequence or Structural Relationships Can Suggest Protein Function

P2 One important reason to sequence many genomes is to provide databases that can be used to assign gene functions by genome comparisons, an enterprise referred to as **comparative genomics**.

A genome sequence is simply a very long string of A, G, T, and C residues, all meaningless until interpreted. **Genome annotation** yields information about the location and function of genes and other critical sequences. Genome annotation converts the sequence into information that any researcher can use, and it is typically focused on genomic DNA encompassing genes that encode RNA and protein, the most common targets of scientific investigation. Every newly sequenced genome includes many genes—often 40% or more of the total—about which little or nothing is known.

Using online tools that apply computational power to comparative genomics, scientists can define gene locations and assign tentative gene functions (where possible) based on similarity to genes previously studied in other genomes. The classic BLAST (Basic Local Alignment Search Tool) algorithm allows a rapid search of all genome databases for sequences related to one that a researcher is exploring, and it is especially valuable for investigating the function of a particular gene. BLAST is one of many resources available at the NCBI (National Center for Biotechnology Information) site (www.ncbi.nlm.nih.gov), sponsored by the National Institutes of Health, and the Ensembl site (www.ensembl.org), cosponsored by the EMBL-EBI (European Molecular Biology Laboratory–European Bioinformatics Institute).

Comparative genomics is made possible by evolutionary biology. Sometimes a newly discovered gene is related by sequence homologies to a previously studied gene in another or the same species, and its function can be entirely or partly defined by that relationship. Genes that occur in different species but have a clear sequence and functional relationship to each other are called **orthologs**. Genes similarly related to each other within a single species are called **paralogs**. We introduced these terms in Chapter 3 in the context of proteins. As with proteins, information about the function of a gene in one species can be used to at least tentatively assign function to the orthologous gene found in a second species. The correlation

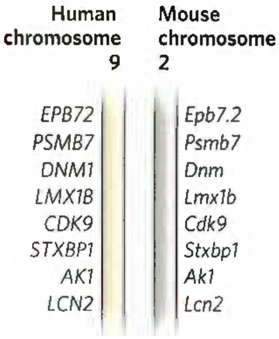


FIGURE 9-14 Synteny in the human and mouse genomes. Large segments of the two genomes have closely related genes aligned in the same order on the chromosomes. In these short segments of human chromosome 9 and mouse chromosome 2, the genes show a very high degree of homology, as well as the same gene order. The different lettering schemes for the gene names simply reflect the different naming conventions for the two species. [Information from T. G. Wolfsberg et al., *Nature* 409:824, 2001, Fig. 1.]

is easiest to make when comparing genomes from relatively closely related species, such as mouse and human, although many clearly orthologous genes have been identified in species as distant as bacteria and humans. Sometimes even the order of genes on a chromosome is conserved over large segments of the genomes of closely related species (**Fig. 9-14**). Conserved gene order, called **synteny**, provides additional evidence for an orthologous relationship between genes at identical locations within the related segments.

Alternatively, certain amino acid sequences associated with particular structural motifs (Chapter 4) may be identified within a protein. **P2** The presence of a structural motif may help to define molecular function by suggesting that a protein, say, catalyzes ATP hydrolysis, binds to DNA, or forms a complex with zinc ions. These relationships are determined with the aid of sophisticated computer programs, limited only by the current information on gene and protein structure and by our capacity to associate sequences with particular structural motifs. Sequences at an enzyme active site that have been highly conserved during evolution are typically associated with catalytic function, and their identification is often a key step in defining an enzyme's reaction mechanism. The reaction mechanism, in turn, provides information needed to develop new enzyme inhibitors that can be used as pharmaceutical agents.

When and Where a Protein Is Present in a Cell Can Suggest Protein Function

If a protein is involved in a reaction or process, it must be present at the location and at the moment that reaction or process occurs. This aspect of protein function can now be explored at multiple levels and with ever-increasing precision.

RNA-Seq and Transcriptomics The RNAs that are transcribed from a genome under a given set of conditions can be determined using DNA sequencing methods described in Chapter 8. The approach is called **RNA-Seq** (**Fig. 9-15**). RNA is first isolated from a tissue or a population of cells. The RNA is fragmented and converted to double-stranded DNA using reverse transcriptase (see Fig. 9-12a). This DNA is then subjected to deep DNA sequencing, which reveals both the RNAs that are present and the relative abundance of each (if more copies of one RNA are present, they will give rise to more DNA sequencing reads). This method is sensitive enough to apply to single cells, an approach called **single cell RNA-Seq**, or **scRNA-Seq**. It allows investigators to catalog the RNAs being transcribed in different parts of a tissue.

The transcriptional state of a human cell or tissue can be diagnostic of conditions ranging from diabetes to cancer. If a particular gene under study is expressed in a certain tissue or under particular metabolic conditions, the result provides a new functional clue. Detailed knowledge about what genes are expressed in a given tumor may eventually help guide treatment options. RNA-Seq can reveal patterns of gene regulation

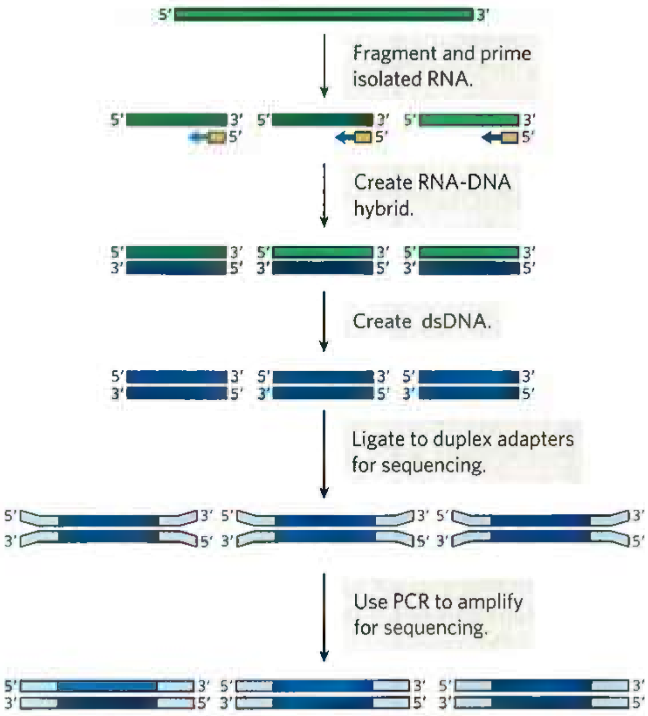

5'
3'
Fragment and prime isolated RNA.
Create RNA-DNA hybrid.
Create dsDNA.
Ligate to duplex adapters for sequencing.
Use PCR to amplify for sequencing.

FIGURE 9-15 RNA-Seq. To define a cellular transcriptome, the first step is to isolate cellular RNA. Because many RNAs, particularly mRNAs and rRNAs, are quite long, the RNA is then fragmented to an average size commensurate with the DNA sequencing platform to be used. The RNA is converted to DNA using reverse transcriptase. Hexameric DNA oligonucleotides of random sequence are used to prime the reverse transcriptase if all RNA is to be included in the transcriptome. RNA-DNA hybrids are more stable than DNA-DNA hybrids, so hexameric duplexes are sufficient for this task. If the transcriptome is focused on expression of protein-coding genes, beads coated with poly(dT) can be used to hybridize to the poly(A) tails of eukaryotic mRNAs, allowing their precipitation and enrichment relative to other RNAs. After reverse transcription, the DNA fragments are ligated to duplex adapters that provide a universal priming location for the DNA sequencing as well as sequences that allow annealing to anchors on the sequencing flow cell (see Fig. 8-36). This is followed by deep DNA sequencing and data analysis.

and expression. It has special importance in studying cancerous tumors, in which rapid evolution triggered by genome instability creates a range of cell types. The mRNAs that are present in tumor cells provide a clue to the proteins that may be present, although not all mRNAs are immediately translated into protein. RNA-Seq also reveals the presence of many types of noncoding RNAs (described in Chapter 26) that are now being defined. ■

Cellular Proteomes and Mass Spectrometry A more direct way to establish protein presence or absence is to assess the cellular proteome. Mass spectrometry (Chapter 3) can accurately catalog and quantify the thousands of proteins present in a typical cell. This approach is a complement to RNA-Seq, as it provides a comprehensive list of the genes that are both transcribed and translated into protein. Mass spectrometry also provides information about how those proteins are modified, in turn allowing an assessment of their regulatory state.

Fusion Proteins and Immunofluorescence P2 Often, an important clue to the function of a gene product comes from determining its location within the cell. For example, a protein found exclusively in the nucleus could be involved in processes that are unique to that organelle, such as transcription, replication, or chromatin condensation. Researchers often engineer fusion proteins for the purpose of locating a protein in the cell or organism. Some of the most useful fusions are the attachment of marker proteins that signal the location by direct visualization or by immunofluorescence.

A particularly useful marker is the **green fluorescent protein (GFP)** (**Fig. 9-16**), discovered by Osamu Shimomura. As subsequently shown by Martin Chalfie, a target gene (encoding the protein of interest) fused to the GFP gene generates a fusion protein that is highly fluorescent—it literally lights up when exposed to blue light—and can be visualized directly in a living cell. GFP is a protein derived from the jellyfish *Aequorea victoria* (Fig. 9-16a).

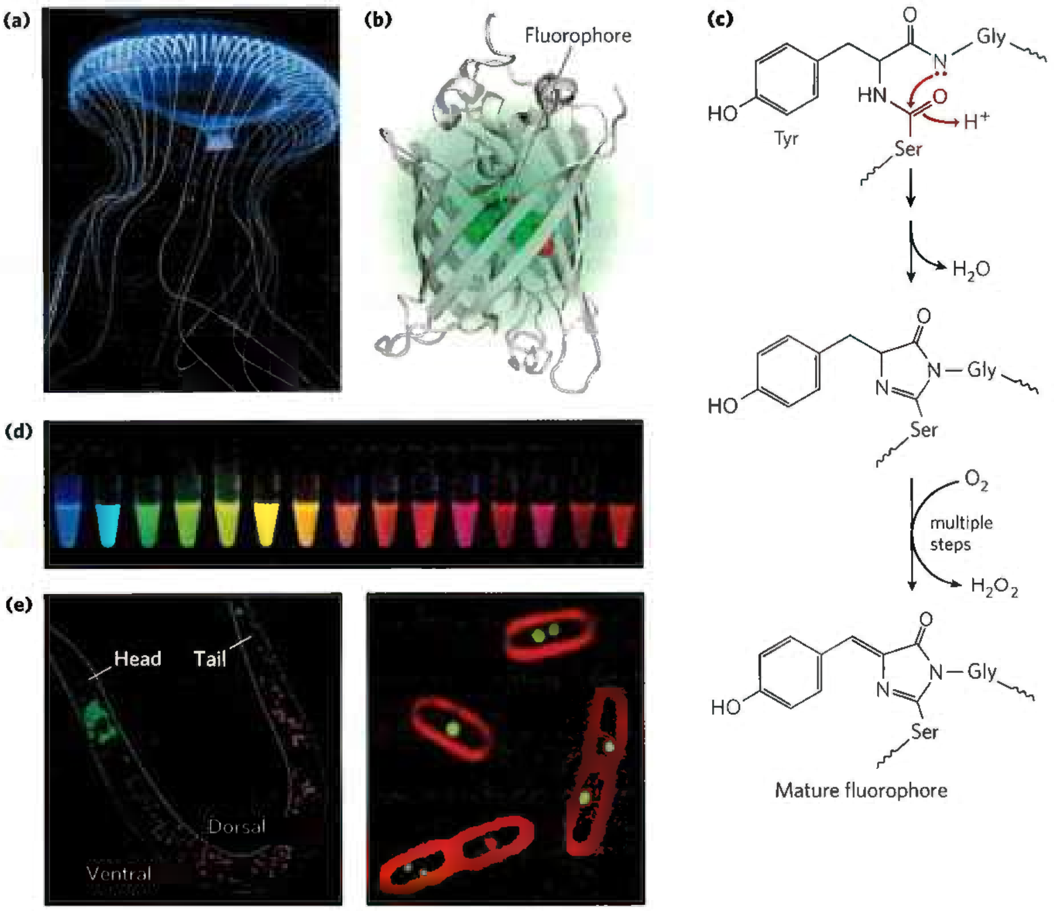


FIGURE 9-16 Green fluorescent protein (GFP). (a) GFP is derived from the jellyfish *Aequorea victoria*. (b) The protein has a β-barrel structure; the fluorophore is in the center of the barrel. (c) The fluorophore in GFP is derived from a sequence of three amino acids: $-\text{Ser}^{65}-\text{Tyr}^{66}-\text{Gly}^{67}-$. The fluorophore achieves its mature form through an internal rearrangement, coupled to a multistep oxidation reaction. An abbreviated mechanism is shown here. (d) Variants of GFP are now available in almost any color of the visible spectrum. (e) A GLR1-GFP fusion protein fluoresces bright green in *C. elegans*, a nematode worm (left). GLR1 is a glutamate receptor of nervous tissue. (In this photograph, autofluorescing fat droplets are false-colored in magenta.) The membranes of *E. coli* cells (right) are stained with a red fluorescent dye. The cells are expressing a protein that binds to a resident plasmid, fused to GFP. The green spots indicate the locations of plasmids. [(a) Chris Parks/ImageQuest Marine. (b) Data from PDB ID 1GFL, F. Yang et al., *Nature Biotechnol.* 14:1246, 1996. (c) Information from Roger Tsien, University of California, San Francisco, Department of Pharmacology, and Paul Steinbach. (d) Courtesy of Roger Tsien and Paul Steinbach, University of California, San Diego, Department of Pharmacology. (e) (left) Courtesy Penelope J. Brockie and Andres V. Maricq, Department of Biology, University of Utah; (right) Courtesy Joseph A. Pogliano, from J. Pogliano et al. (2001), Multicopy plasmids are clustered and localized in *Escherichia coli*, *Proc. Natl. Acad. Sci. USA* 98:4486–4491.]

The protein has a β-barrel structure with a fluorophore (the fluorescent component of the protein) in the center (Fig. 9-16b). The fluorophore is derived from a rearrangement and oxidation of three amino acid residues (Fig. 9-16c). Because this reaction is autocatalytic and requires no proteins or cofactors other than molecular oxygen, GFP is readily cloned in an active form in almost any cell. Just a few molecules of this protein can be observed microscopically, allowing the study of its location and movements in a cell.

Careful protein engineering by Roger Tsien, coupled with the isolation of related fluorescent proteins from other marine coelenterates, has made variants of these proteins available in an array of colors (Fig. 9-16d) and other characteristics (brightness, stability). If fusion to GFP does not impair the function or properties of a protein one wishes to study, the fusion protein can be used to reveal the protein's location in the cell under a range of conditions and to detect interactions with other labeled proteins. With this technology, for example, the protein GLR1 (a glutamate receptor of nervous tissue) has been visualized as a GLR1-GFP fusion protein in the nematode *Caenorhabditis elegans* (Fig. 9-16e).

In some cases, the GFP fusion protein may be inactive or may not be expressed at sufficient levels to allow visualization. **Immunofluorescence** is an alternative approach for visualizing the endogenous (unaltered) protein. This approach requires fixation (and thus death) of the cell. The protein of interest is sometimes expressed as a fusion protein with an **epitope tag**, a short protein sequence that is bound tightly by a well-characterized, commercially available antibody. Fluorescent molecules (fluorochromes) are attached to this antibody. More commonly, the target protein is unaltered and is bound by an antibody that is specific for the protein. Next, a second antibody is added that binds specifically to the first one, and it is the second antibody that has the attached fluorochrome(s) (**Fig. 9-17**). A variation of this indirect approach to visualization is to attach biotin molecules to the first antibody, then add streptavidin (a bacterial protein closely related to avidin, a protein that binds biotin; see Table 5-1) complexed with fluorochromes. The interaction between biotin and streptavidin is one of the strongest and most specific known, and the potential to add multiple fluorochromes to each target protein gives this method great sensitivity. In all of these cases, the end product is a microscopic view of a cell in which a spot of light (a focus) reveals the location of the protein.

Knowing What a Protein Interacts with Can Suggest Its Function

Another key to defining the function of a particular protein is to determine its biochemical playmates. **P2** In the case of protein-protein interactions, the association of a protein of unknown function with one whose function is known can compellingly imply a functional relationship. The techniques used in this effort are quite varied.

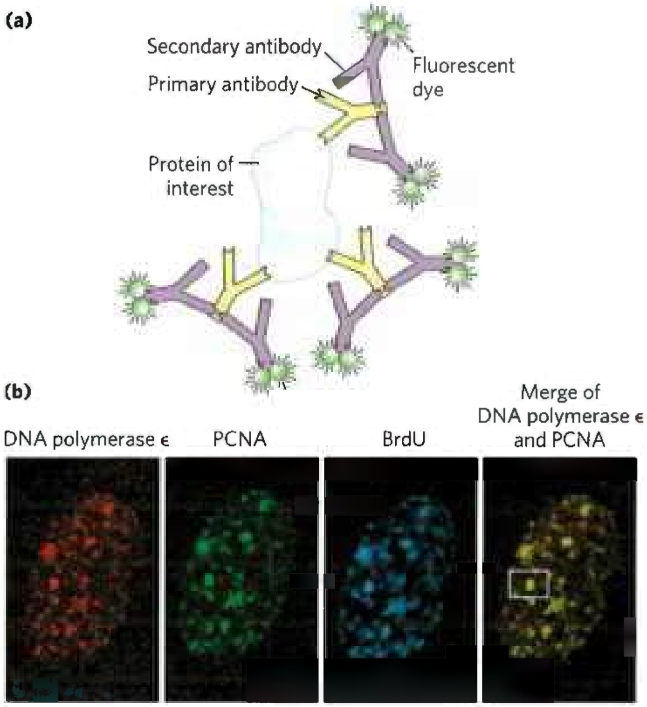


FIGURE 9-17 Indirect immunofluorescence. (a) The protein of interest is bound to a primary antibody, and a secondary antibody is added; this second antibody, with one or more attached fluorescent groups, binds to the first. Multiple secondary antibodies can bind the primary antibody, amplifying the signal. If the protein of interest is in the interior of the cell, the cell is fixed and permeabilized, and the two antibodies are added in succession. (b) The end result is an image in which bright spots indicate the location of the protein or proteins of interest in the cell. The images here show a nucleus from a human fibroblast, successively stained with antibodies and fluorescent labels for DNA polymerase ε; for PCNA, an important polymerase accessory protein; and for bromo-deoxyuridine (BrdU), a nucleotide analog. The BrdU, added as a brief pulse, identifies regions undergoing active DNA replication. The patterns of staining show that DNA polymerase ε and PCNA co-localize to regions of active DNA synthesis (rightmost image); one such region is visible in the white box. [(b) Fuss, J. and Linn, S., 2002, "Human DNA Polymerase ε Colocalizes with Proliferating Cell Nuclear Antigen and DNA Replication Late, but Not Early, in S Phase," *J. Biol. Chem.* 277:8658–8666. Courtesy Jill Fuss, University of California, Berkeley.]

Purification of Protein Complexes By fusing the gene encoding a protein under study with the gene for an epitope tag, investigators can precipitate the protein product of the fusion gene by complexing it with the antibody that binds the epitope. This process is called **immunoprecipitation** (**Fig. 9-18**). If the tagged protein is expressed in cells, other proteins that bind to it precipitate with it. Identifying the associated proteins reveals some of the intracellular protein-protein interactions of the tagged protein. There are many variations of this process. For example, a crude extract of cells that express a tagged protein is added to a column containing immobilized antibody (see Fig. 3-17c for a description of affinity chromatography). The tagged protein binds to the antibody, and proteins that interact with the tagged protein are sometimes also retained on the column. The connection between the protein and the tag is cleaved with a specific protease. The protein complexes are eluted from the column, and the proteins in them are

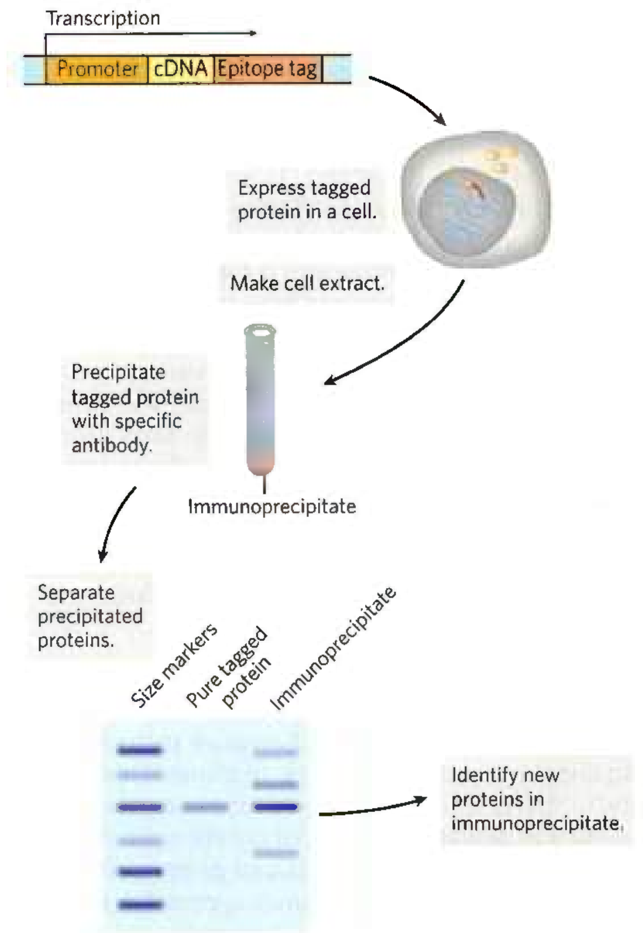

Transcription
Promoter
cDNA
Epitope tag
Express tagged protein in a cell.
Make cell extract.
Precipitate tagged protein with specific antibody.
Immunoprecipitate
Separate precipitated proteins.
Size markers
Pure tagged protein
Immunoprecipitate
Identify new proteins in immunoprecipitate.

FIGURE 9-18 The use of epitope tags to study protein-protein interactions. The gene of interest is cloned next to a gene for an epitope tag, and the resulting fusion protein is precipitated by antibodies to the epitope. Any other proteins that interact with the tagged protein also precipitate, thereby helping to elucidate protein-protein interactions.

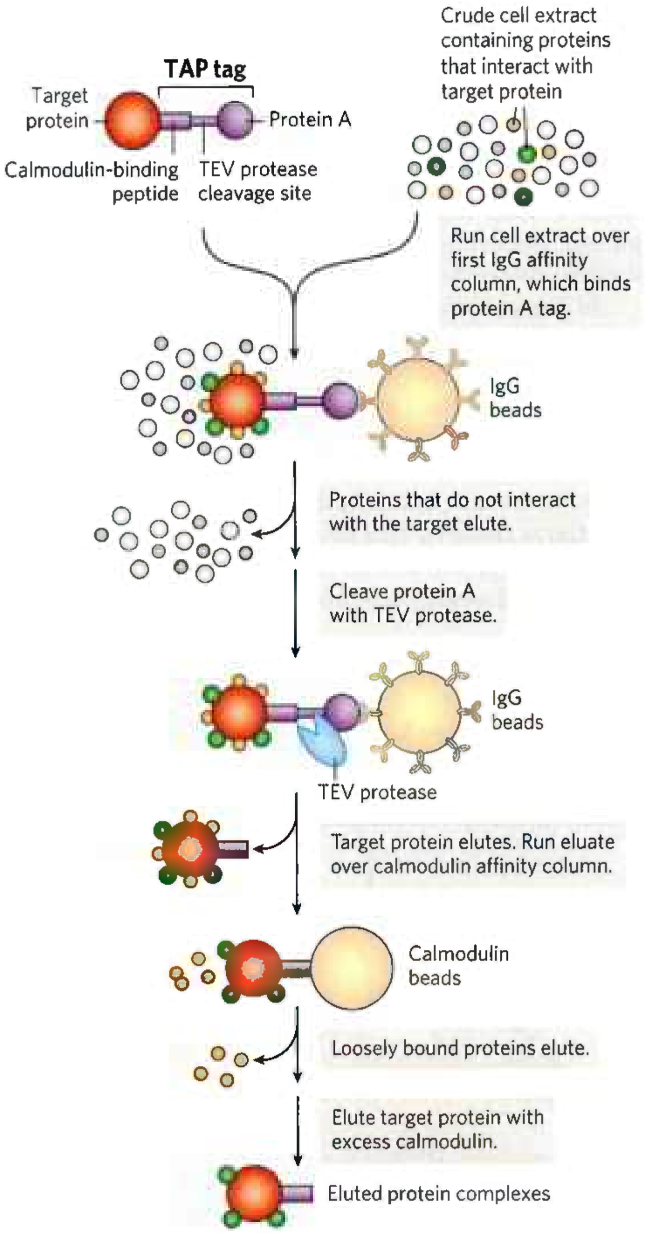

TAP tag
Target protein
Protein A
Calmodulin-binding peptide
TEV protease cleavage site
Crude cell extract containing proteins that interact with target protein
Run cell extract over first IgG affinity column, which binds protein A tag.
IgG beads
Proteins that do not interact with the target elute.
Cleave protein A with TEV protease.
IgG beads
TEV protease
Target protein elutes. Run eluate over calmodulin affinity column.
Calmodulin beads
Loosely bound proteins elute.
Elute target protein with excess calmodulin.
Eluted protein complexes

FIGURE 9-19 Tandem affinity purification (TAP) tags. A TAP-tagged protein and associated proteins are isolated by two consecutive affinity purifications, as described in the text.

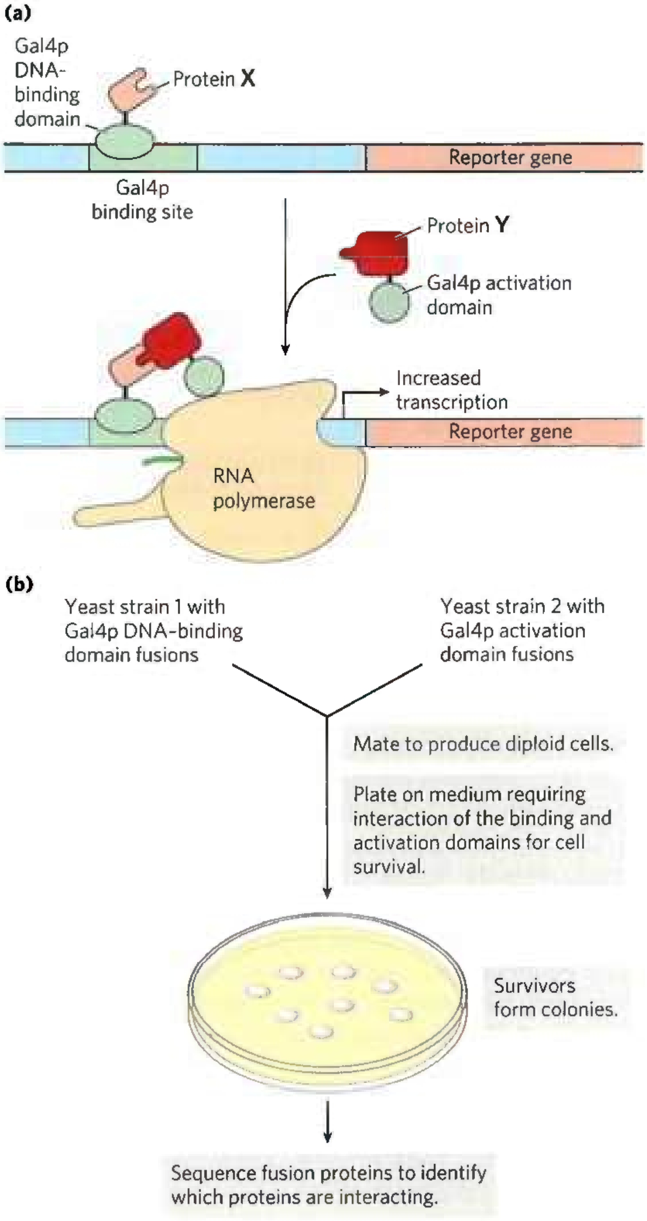
identified by mass spectrometry. Researchers can use these methods to define complex networks of interactions within a cell. In principle, the chromatographic approach to analyzing protein-protein interactions can be used with any type of protein tag (His tag, GST, etc.) that can be immobilized on a suitable chromatographic medium.

The selectivity of this approach can be enhanced with **tandem affinity purification (TAP) tags**. Two consecutive tags are fused to a target protein, and the fusion protein is expressed in a cell (**Fig. 9-19**). The first tag is protein A, a protein found at the surface of the bacterium *Staphylococcus aureus* that binds tightly to mammalian immunoglobulin G (IgG). The second tag is often a calmodulin-binding peptide. A crude extract containing the TAP-tagged fusion protein is passed through a column matrix with attached IgG antibodies that bind protein A. Most of the unbound cellular proteins are washed through the column, but proteins that normally interact with the target protein in the cell are retained. The first tag is then cleaved from the fusion protein with a highly specific protease, TEV protease, and the shortened fusion target protein and any proteins associated noncovalently with the target protein are eluted from the column. The eluate is then passed through a second column containing a matrix with attached calmodulin that binds the second tag. Loosely bound proteins are again washed from the column. After the second tag is cleaved, the target protein is eluted from the column with its associated proteins. The two consecutive purification steps eliminate most weakly bound contaminants. False positives are minimized, and protein interactions that persist through both steps are likely to be functionally significant.

Yeast Two-Hybrid Analysis A sophisticated genetic approach to defining protein-protein interactions is based on the properties of the Gal4 protein (Gal4p; see Fig. 28-32), which activates the transcription of *GAL* genes (encoding the enzymes of galactose metabolism) in yeast. Gal4p has two domains: one that binds a specific DNA sequence, and another that activates RNA polymerase to synthesize mRNA from an adjacent gene. The two domains of Gal4p are stable when separated, but activation of RNA polymerase requires interaction with the activation domain, which in turn requires positioning by the DNA-binding domain. Hence, the domains must be brought together to function correctly.

In **yeast two-hybrid analysis**, the protein-coding regions of the genes to be analyzed are fused to the yeast gene for either the DNA-binding domain or the activation domain of Gal4p, and the resulting genes express a series of fusion proteins (**Fig. 9-20**). If a protein fused to the DNA-binding domain interacts with a protein fused to the activation domain, transcription is activated. The reporter gene transcribed by this activation is generally one that yields a protein required for growth or an enzyme that catalyzes a reaction with a colored product. Thus, when grown on the proper medium, cells that contain a pair of interacting proteins are easily distinguished from those that do not.

A library can be set up with a particular yeast strain in which each cell in the library has a gene fused to the Gal4p DNA-binding domain gene, and many such genes are represented in the library. In a second yeast strain, a gene of interest is fused to the gene for the Gal4p activation domain. The yeast strains are mated, and individual diploid cells are grown into colonies. The only cells that grow on the selective medium, or that produce the appropriate color, are those in which the gene of interest is binding to a partner, allowing transcription of the reporter gene. This allows large-scale screening for cellular proteins that interact with the target protein. The interacting protein that is fused to the Gal4p DNA-binding domain present in a particular selected colony can be quickly identified by DNA sequencing of the fusion protein's gene. Some false positive results occur, due to the formation of multiprotein complexes.



Mate to produce diploid cells.

Plate on medium requiring interaction of the binding and activation domains for cell survival.

Survivors form colonies.

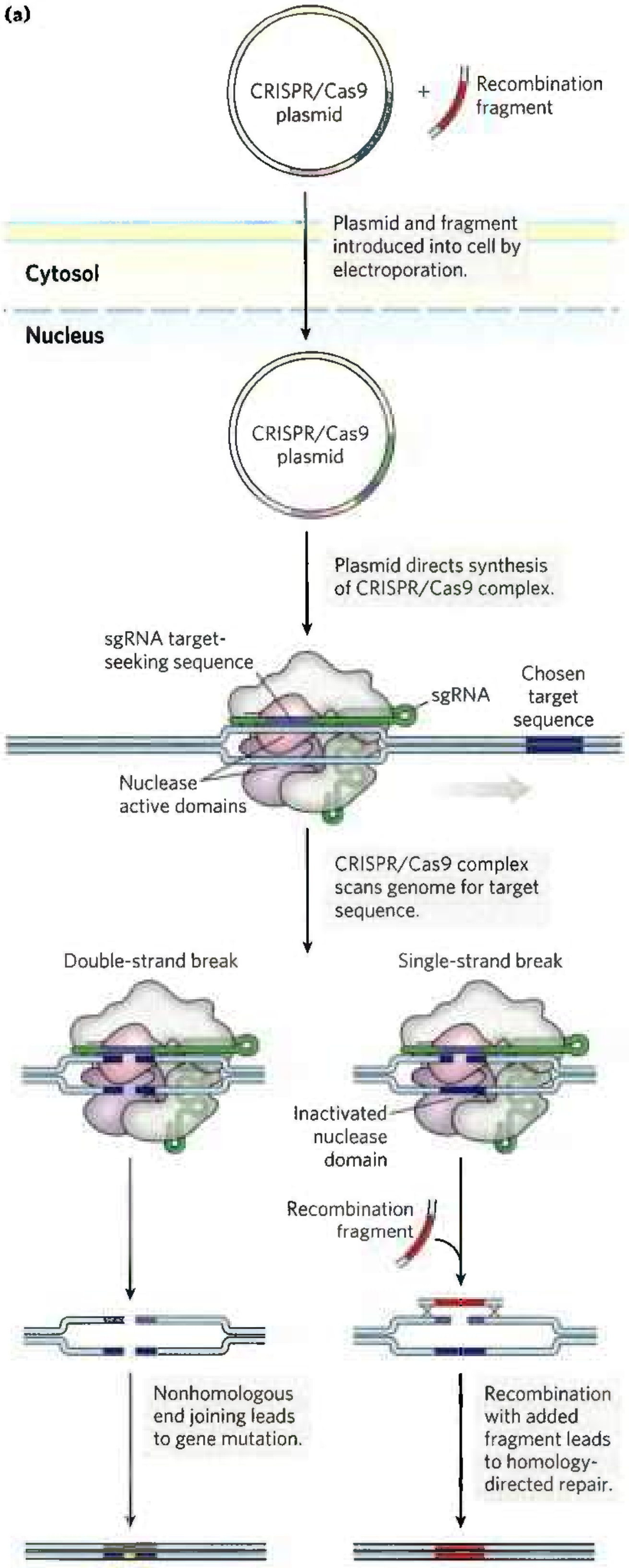
Sequence fusion proteins to identify which proteins are interacting.

FIGURE 9-20 Yeast two-hybrid analysis. (a) The goal is to bring together the DNA-binding domain and the activation domain of the yeast Gal4 protein (Gal4p) through the interaction of two proteins, X and Y, to which one or other of the domains is fused. This interaction is accompanied by the expression of a reporter gene. (b) The two gene fusions are created in separate yeast strains, which are then mated. The mated mixture is plated on a medium on which the yeast cannot survive unless the reporter gene is expressed. Thus, all surviving colonies have interacting fusion proteins. Sequencing of the fusion proteins in the survivors reveals which proteins are interacting.

The Effect of Deleting or Altering a Protein Can Suggest Its Function

P2 **P3** One of the most informative paths to understanding the function of a gene is to change (mutate) the gene or delete it. An investigator can then examine how the genomic alteration affects cell growth or function. The methods available to modify genomes grow more sophisticated every year. The most common approach is to cut the gene of interest at a site that is functionally critical, generating a double-strand break. In eukaryotes, such breaks are most commonly repaired by cellular systems that promote nonhomologous end joining (NHEJ), a process described in Chapter 25. NHEJ seals the double-strand break, but the process is imprecise. Nucleotides are often deleted or added during the repair, inactivating the gene. In bacteria, introduced double-strand breaks are usually repaired more accurately, by homologous recombination systems (Chapter 25), but inactivating mutations can appear. Many traditional approaches to targeting a gene in this way were supplanted by the advent of CRISPR/Cas systems in 2011.

CRISPR/Cas Systems "CRISPR" stands for *c*lustered, *r*egularly *i*nterspaced *s*hort *p*alindromic *r*epeats; as the name suggests, these consist of a series of regularly spaced short repeats in the bacterial genome. A Cas (*C*RISPR-*as*sociated) protein is a nuclease. The CRISPR sequences and Cas protein are components of a kind of immune system that evolved to allow bacteria to survive infection


(a)
CRISPR/Cas9 plasmid
+
Recombination fragment
Plasmid and fragment introduced into cell by electroporation.
Cytosol
Nucleus
CRISPR/Cas9 plasmid
Plasmid directs synthesis of CRISPR/Cas9 complex.
sgRNA target-seeking sequence
sgRNA
Chosen target sequence
Nuclease active domains
CRISPR/Cas9 complex scans genome for target sequence.
Double-strand break
Single-strand break
Inactivated nuclease domain
Recombination fragment
Nonhomologous end joining leads to gene mutation.
Recombination with added fragment leads to homology-directed repair.

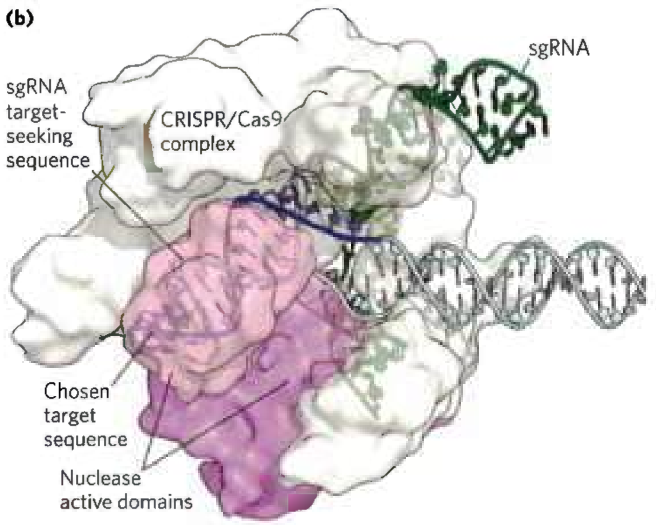

(b)
sgRNA
sgRNA target-seeking sequence
CRISPR/Cas9 complex
Chosen target sequence
Nuclease active domains

FIGURE 9-21 The CRISPR/Cas9 system for genomic engineering. (a) The genes encoding the Cas9 protein and sgRNA are introduced into a cell in which a targeted genomic change is planned. The sgRNA has a region complementary to the chosen genomic target sequence (purple); this region can be engineered to include any desired sequence. A complex consisting of the CRISPR sgRNA and the Cas9 protein forms within the cell and binds to the chosen target site in the DNA. The structure of the bound complex is shown in (b). In the pathway shown on the left in (a), two nuclease active sites in the Cas9 protein separately cleave each DNA strand in the target, producing a double-strand break. The double-strand break is usually repaired by nonhomologous end joining, which generally deletes or alters the nucleotides at the site where joining occurs. Alternatively, as shown in the pathway on the right, if one nuclease site is inactivated, Cas9 nuclease activity creates a single-strand break in the target sequence. In the presence of a recombination donor DNA fragment, identical to the target sequence but incorporating the desired sequence change (fragment shown in red), homologous DNA recombination will sometimes change the sequence at the site of the break to match that of the donor DNA. [Data from PDB ID 4UN3, C. Anders et al., *Nature* 513:569, 2014.]

by bacteriophages. CRISPR sequences are embedded in the bacterial genome, surrounding sequences derived from phage pathogens that previously infected the bacterium without killing it. The viral sequences are, in effect, spacer sequences separating the CRISPR sequences. When the same bacteriophage again attacks a bacterium that has the corresponding CRISPR/Cas system, the CRISPR sequence and Cas protein act together to destroy the viral DNA. First, the CRISPR sequences are transcribed to RNA, and individual viral spacer sequences are cleaved to form products called **guide RNAs (gRNAs)**, which include some adjacent repeat RNA. A gRNA forms a complex with one or more Cas proteins and, in some cases, with another RNA called a **trans-activating CRISPR RNA**, or **tracrRNA**. The resulting complex binds specifically to the invading bacteriophage DNA, cleaving and destroying it through the nuclease activities associated with the Cas proteins.

The current technology was made possible by discovery of a relatively simple CRISPR/Cas system in *Streptococcus pyogenes*. This system requires only a single Cas protein, Cas9, to cleave DNA. Work in many laboratories, particularly those of Jennifer Doudna and Emmanuelle Charpentier, has produced a streamlined CRISPR/Cas9 system composed of just one protein (Cas9) and one associated RNA, consisting of gRNA and tracrRNA fused into a **single guide RNA (sgRNA)**. P2 P3 The power of the system is embedded in this sgRNA, in which the guide sequence can be altered to specifically and efficiently target almost any genomic sequence (**Fig. 9-21**). Cas9 has two separate nuclease domains: one domain cleaves the DNA strand paired with the sgRNA, and the other cleaves the opposite DNA strand. Inactivating one domain creates an enzyme that cleaves just one strand, forming a single-strand break, or nick. The sgRNA is needed both to pair with the target sequence in the DNA and to activate the nuclease domains for cleavage.

Plasmids expressing the required protein and RNA components of CRISPR/Cas9 can be introduced into microbial cells by electroporation (p. 306). For mammalian cells, the genes encoding the CRISPR/Cas9 components can be incorporated into engineered viruses that subsequently deliver them to the cell nuclei. For many organisms, the targeted gene is inactivated in a high percentage of the treated cells. If a genomic change (mutation) rather than a simple gene inactivation is required, it can be introduced by recombination when a DNA fragment encompassing the cleavage site and including the desired change enters the cell with the CRISPR/Cas9 plasmids. This recombination is often inefficient, but success can be improved somewhat by introducing a nick rather than a double-strand break at the target site (Fig. 9-21).

CRISPR/Cas9 can be combined with other approaches to extract additional information. For example, a particular gene can be inactivated with CRISPR/Cas9. Then, the effect of that gene inactivation on the transcription of other genes can be probed with RNA-Seq at the level of tissues, cell populations, or single cells.

New applications for CRISPR/Cas9 are being developed rapidly, both for basic research and for medicine. Genetic screens based on CRISPR are described in the next section. CRISPR is being used to enhance food production, provide new approaches to combat bacterial infections, and eliminate nonnative pest species that can harbor diseases (**Box 9-1**). New CRISPR-based treatments for genetic diseases are being cautiously advanced to clinical trials for vision loss due to inherited retinal dystrophies, Duchenne muscular dystrophy, β-thalassaemia, and many other conditions. Uncertainties remain, particularly the potential for occasional cleavage at unintended chromosomal sites (off-target cleavage). The impact of CRISPR/Cas9 will continue to grow as problems are overcome, current applications mature, and new applications are imagined and created.

Many Proteins Are Still Undiscovered

For most biological processes, ranging from intermediary metabolism to neurological function to DNA metabolism, the list of known participating enzymes and proteins is far from complete. **P2** Genetic screening for new gene functions has been underway for many decades. The goal is to efficiently interrogate large numbers of genes, sometimes the entire genome, for genes that affect a particular cellular reaction or process. A gene perturbation—a treatment that inactivates a gene or activates its expression—is introduced under conditions in which just one gene is affected in each cell, but most or all of the genes are affected in one or more cells within the population (**Fig. 9-22**). The population is then subjected to a stress or selection. Cells in which a gene required to respond to the selection is altered may drop out of the population or be enriched in the population, depending upon the goals and design of the screen.

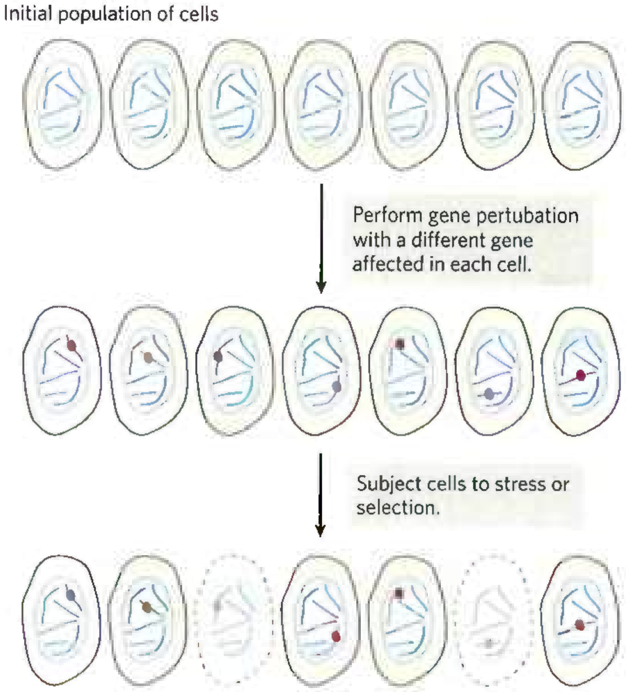

Initial population of cells
Perform gene pertubation with a different gene affected in each cell.
Subject cells to stress or selection.

FIGURE 9-22 High-throughput genetic screening. A gene perturbation, either inactivation or activation, is introduced to a population of cells such that only one gene in each cell is affected. However, all or most genes are affected in one cell or another within the population. The population is then subjected to a selection that requires a response from some cellular genes. Cells that lack the required genes or that have those genes activated will drop out or be enriched within the population, respectively. In the example shown, two cells drop out.

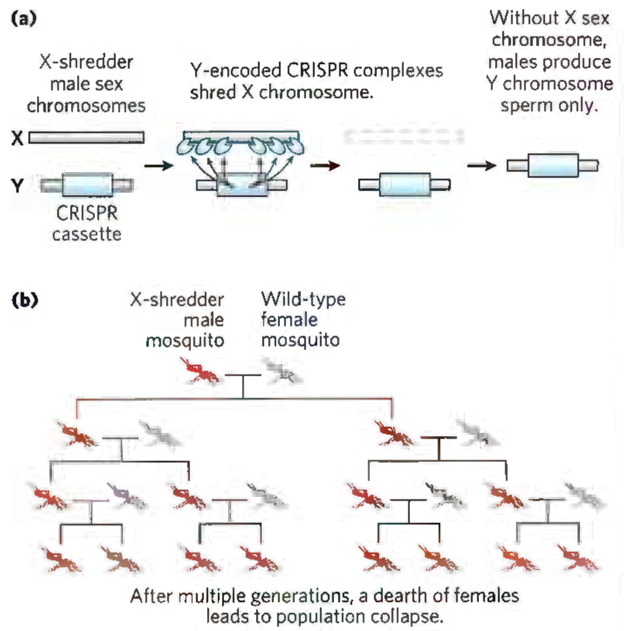
CRISPR-based technologies increasingly play a central role in large-scale screening protocols (**Fig. 9-23**). Libraries of sgRNAs have been generated to target virtually all genes in a mammalian genome, or specialized subsets of them. The targeting sequence in each sgRNA is 20 bp long. In addition to targeting a particular gene, each targeting sequence acts as a kind of unique bar code identifier that is readily recognized by computer programs after sequencing. The sgRNAs are packaged in a DNA cassette set up to also express the Cas9 protein or a Cas9 variant. The cassettes are incorporated into carefully engineered lentiviral vectors derived from HIV (with genes required for HIV multiplication eliminated). The viral vectors deliver the cassette to the nucleus as a single-stranded RNA, convert it to double-stranded

BOX 9-1

Getting Rid of Pests with Gene Drives

Invasive introduced plant and animal species can wreak havoc on any natural environment, and can also spread human disease. Mosquitoes that harbor Zika virus and other diseases in many parts of the world, introduced rats on almost every continent, cane toads and rabbits in Australia, the kudzu vine in the southern United States—represent just a few examples of invasive species that cause human misery and annual financial damage totaling billions of dollars. Traditional methods of control such as poisoning or trapping are often unsuccessful and can have detrimental effects on native species that become unintended targets.

The discovery of selfish DNA elements such as homing endonucleases and transposons that can spread through a population gave rise to the concept of **gene drives** as a new approach to the control of invasive species. The most recent and promising iteration of this idea involves synthetic gene drives based on CRISPR/Cas9. The overall idea is to set up a system that skews the male to female ratio in a target species far away from the favored 1:1, resulting in population collapse. A strategy called X shredder, already proven in the laboratory with mosquitoes, is highlighted in Figure 1. A cassette that includes genes expressing Cas9, as well as several sgRNAs targeted to different unique sites on the X chromosome, is inserted into an intergenic region on the Y chromosome. The cassette is engineered into males, and is controlled by a gene regulatory system that is expressed only during spermatogenesis. Thus, during spermatogenesis, the cassette is expressed so that the X chromosome is cleaved at multiple locations, basically destroying it. This ensures that the only viable sperm have Y chromosomes. All the offspring of any cross with a female are all males, and all of them possess Y chromosomes containing the X-shredder cassette. When those male offspring mate with other females later, the same result ensues. As these males mate and spread the cassette through the population, a dearth of females occurs and the population collapses. In principle, this same strategy could be applied to rats, cane toads, and many other invasive species.


(a)
X-shredder male sex chromosomes
X
Y
CRISPR cassette
Y-encoded CRISPR complexes shred X chromosome.
Without X sex chromosome, males produce Y chromosome sperm only.
(b)
X-shredder male mosquito
Wild-type female mosquito

After multiple generations, a dearth of females leads to population collapse.

FIGURE 1 The X-shredder gene drive concept.

To date, gene drives have been restricted to the laboratory. The potential for a gene drive escaping to species that are not intended targets is not yet clear. Resistance in the target species could occur by mutating the sgRNA target sites, although the use of multiple sites makes this less likely. The gene drive approach is a good illustration of the power and potential of CRISPR. However, once males with a gene drive are released, it would be essentially impossible to call a halt to the effects. Nature has a way of imposing consequences, both unintended and unexpected. The potential positive effects on health and agriculture continue to drive research to improve the technology and address potential problems.

DNA with the viral-encoded reverse transcriptase, and integrate the DNA into a chromosome. The CRISPR/Cas9 components are expressed to perturb the target gene specified by the particular sgRNA delivered to that cell. The effect produced depends upon the Cas9 variant used. The unmodified Cas9 nuclease will create a double-strand break that inactivates the gene. A modified Cas9 that lacks the nuclease activity will simply bind to its target and block transcription. Cas9 fused to a protein transcription inhibitor or activator may more effectively block or activate transcription, respectively (Fig. 9-23b).

Whatever strategy is used, a different gene is affected in each cell. Once the population has been treated with a stress or selection, cells in which genes required to survive the treatment are inactivated or activated by the CRISPR/Cas9 variant will die or thrive. The decreased or increased presence of the relevant bar code sequences can be detected by deep DNA sequencing, using a universal priming sequence incorporated into the cassette near the sgRNA sequence. The strategies outlined here only hint at the variety of protocols in use, limited only by the imagination of the investigators.

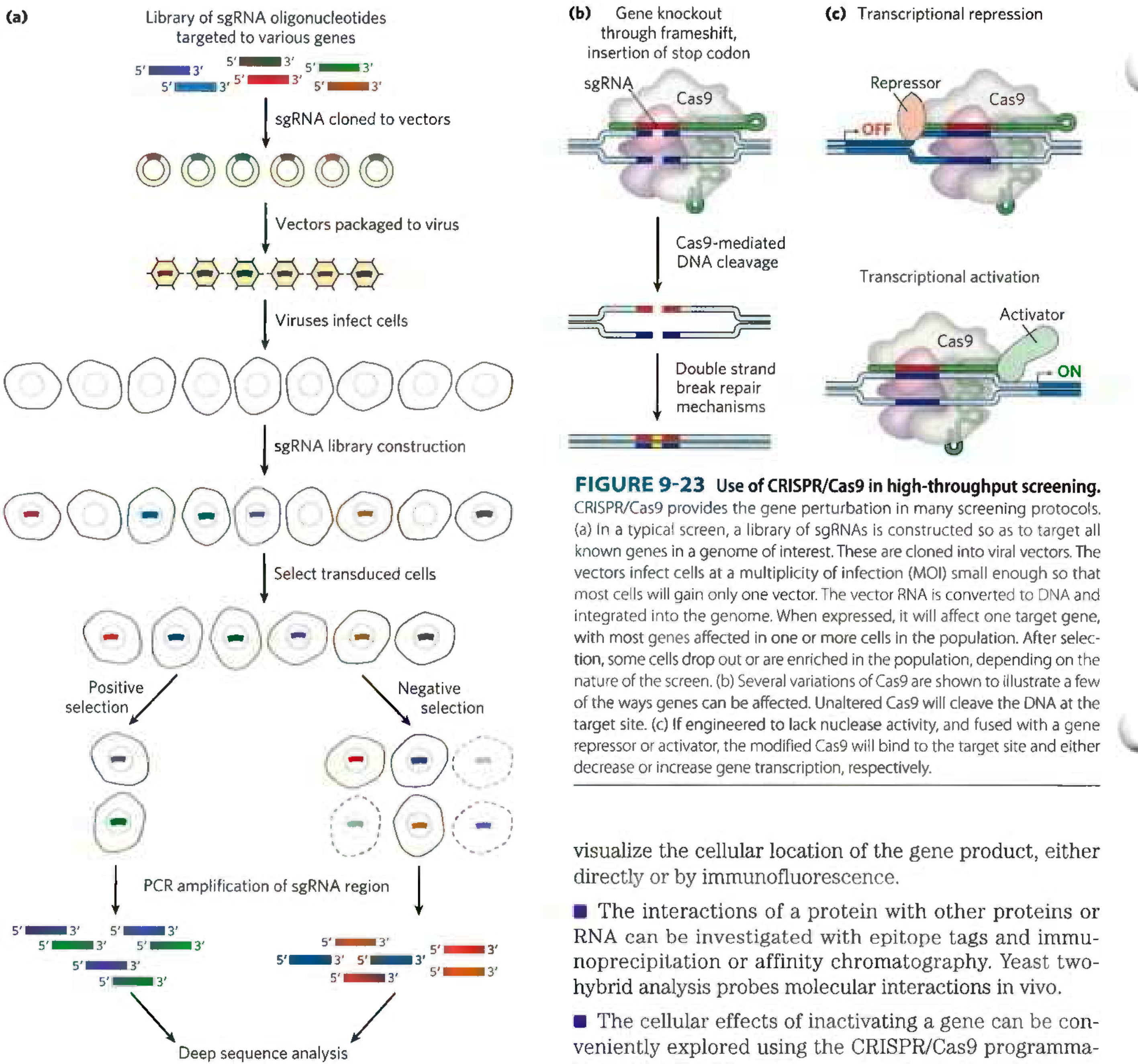

(a)
Library of sgRNA oligonucleotides targeted to various genes
5′ 3′
sgRNA cloned to vectors
Vectors packaged to virus
Viruses infect cells
sgRNA library construction
Select transduced cells
Positive selection
Negative selection
PCR amplification of sgRNA region
Deep sequence analysis
(b) Gene knockout through frameshift, insertion of stop codon
sgRNA
Cas9
Cas9-mediated DNA cleavage
Double strand break repair mechanisms
(c) Transcriptional repression
Repressor
Cas9
OFF
Transcriptional activation
Activator
Cas9
ON

FIGURE 9-23 Use of CRISPR/Cas9 in high-throughput screening. CRISPR/Cas9 provides the gene perturbation in many screening protocols. (a) In a typical screen, a library of sgRNAs is constructed so as to target all known genes in a genome of interest. These are cloned into viral vectors. The vectors infect cells at a multiplicity of infection (MOI) small enough so that most cells will gain only one vector. The vector RNA is converted to DNA and integrated into the genome. When expressed, it will affect one target gene, with most genes affected in one or more cells in the population. After selection, some cells drop out or are enriched in the population, depending on the nature of the screen. (b) Several variations of Cas9 are shown to illustrate a few of the ways genes can be affected. Unaltered Cas9 will cleave the DNA at the target site. (c) If engineered to lack nuclease activity, and fused with a gene repressor or activator, the modified Cas9 will bind to the target site and either decrease or increase gene transcription, respectively.

SUMMARY 9.2 *Exploring Protein Function on the Scale of Cells or Whole Organisms*

- Proteins can be studied at the level of phenotypic, cellular, or molecular function.
- Comparative genomics can elucidate protein function by identifying structural motifs within the encoded protein and comparing gene sequences from different organisms.
- A determination of when and where a protein appears in a cell can offer functional clues. RNA-Seq provides information on what genes are being expressed in a cell. Mass spectrometry can define cellular proteomes. By fusing a gene of interest with genes that encode green fluorescent protein or epitope tags, researchers can visualize the cellular location of the gene product, either directly or by immunofluorescence.
- The interactions of a protein with other proteins or RNA can be investigated with epitope tags and immunoprecipitation or affinity chromatography. Yeast two-hybrid analysis probes molecular interactions in vivo.
- The cellular effects of inactivating a gene can be conveniently explored using the CRISPR/Cas9 programmable nuclease. CRISPR/Cas9 can also be used to alter gene sequences in a targeted manner.
- Screens for new genes increasingly employ variants of the CRISPR/Cas9 system.

9.3 Genomics and the Human Story

Since the report of the first complete human genomes in 2001, human genome sequencing has become routine. The genomes of tens of thousands of other species have now been sequenced and made publicly available, providing a look at genomic complexity throughout the three domains of living organisms: Bacteria, Archaea, and Eukarya. Whereas many early sequencing projects focused on species commonly used in research laboratories, the projects now include species of practical, medical, agricultural, and evolutionary interest. Genomes from every known bacterial family have been sequenced. Completed eukaryotic genome sequences number in the tens of thousands. Genomes of extinct species such as *Homo neanderthalensis* and of humans who died in past millennia have also been sequenced. Personal genomes are playing an ever-increasing role in medicine.

Each genome sequence becomes an international resource for researchers. Collectively, the sequences provide a source for broad comparisons that help pinpoint both variable and highly conserved gene segments, and they allow the identification of genes that are unique to a species or group of species. Efforts to map genes, identify new proteins and disease-related genes, elucidate genetic patterns of medical interest, and trace our evolutionary history are among the many initiatives under way.

The Human Genome Contains Many Types of Sequences

The rapidly growing genome databases have the potential not only to fuel advances in all realms of biochemistry but also to change the way we think about ourselves. What does our own genome, and comparisons with those of other organisms, tell us?

In some ways, we are not as complicated as we once imagined. Humans have only about 20,000 protein-coding genes—less than twice the number in a fruit fly (13,600 genes), not many more than in a nematode worm (19,700 genes), and fewer than in a rice plant (38,000 genes).

In other ways, we are more complex than we previously realized. Many, if not most, eukaryotic genes contain one or more segments of DNA that do not code for the amino acid sequence of a polypeptide product. These nontranslated segments interrupt the otherwise colinear relationship between the gene's nucleotide sequence and the amino acid sequence of the encoded polypeptide. Such nontranslated DNA segments are called **introns**, and the coding segments are called **exons** (**Fig. 9-24**). Few bacterial genes contain introns. The introns are spliced from a precursor RNA transcript to generate a transcript that can be translated contiguously into a protein product (see Chapter 26). An exon often (but not always) encodes a single domain of a larger, multidomain protein. Humans share many protein domain types with plants, worms, and flies, but the domains in the human genome are mixed and matched in more complex ways, increasing the variety of proteins found in our proteome. Alternative modes of gene expression and RNA splicing permit alternative combinations of exons, leading to the production of more than one protein from a single gene. Alternative splicing (Chapter 26) is far more common in humans and other vertebrates than in worms or bacteria, allowing greater complexity in the number and kinds of proteins generated.

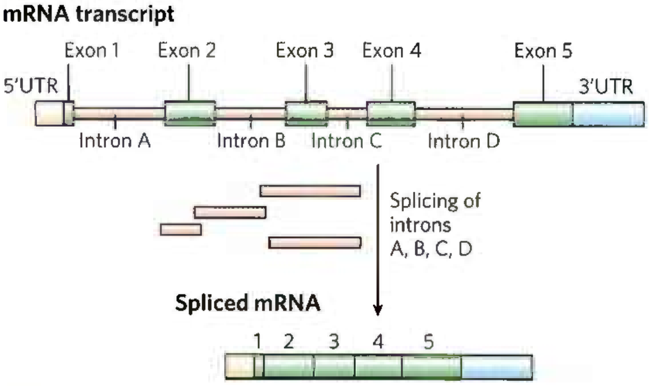


FIGURE 9-24 Introns and exons. This gene transcript contains five exons and four introns, along with 5′ and 3′ untranslated regions (5′UTR and 3′UTR). Splicing removes the introns to create an mRNA product for translation into protein.

In mammals and some other eukaryotes, the typical gene has a much higher proportion of intron DNA than exon DNA; in most cases, the function of introns is not clear. Less than 1.5% of human DNA is "protein-coding" or exon DNA, carrying information for protein products (**Fig. 9-25a**). However, when introns are included in the accounting, as much as 30% of the human genome consists of genes that encode proteins. Several efforts are

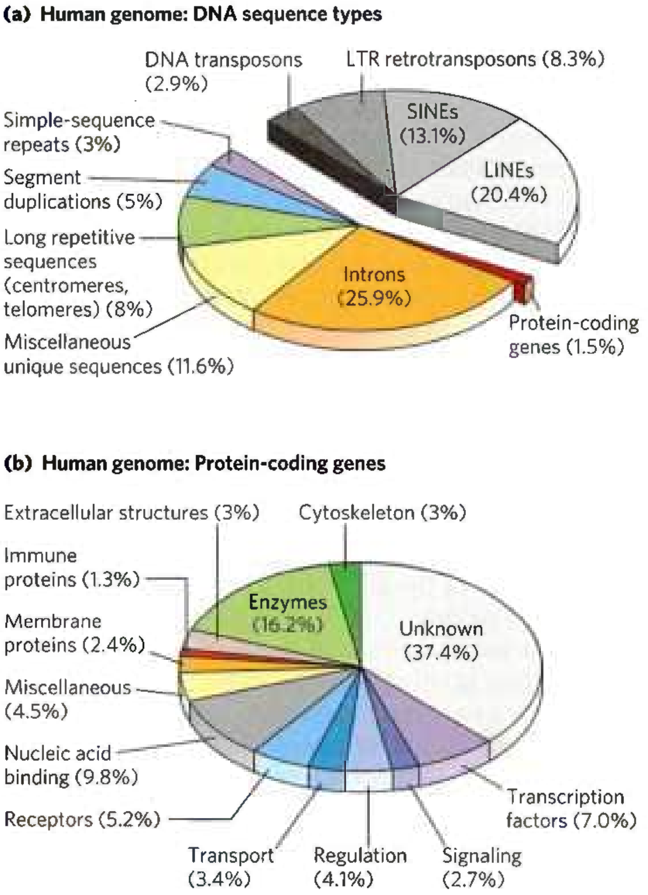


FIGURE 9-25 A snapshot of the human genome. (a) This pie chart shows the proportions of various types of sequences in our genome. The classes of transposons that represent nearly half of the total genomic DNA are indicated in shades of gray. LTR retrotransposons are retrotransposons with long terminal repeats (see Fig. 26-33). Long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) are special classes of particularly common DNA transposons. (b) The approximately 20,000 protein-coding genes in the human genome can be classified by the type of protein encoded. [Information from (a) T. R. Gregory, *Nature Rev. Genet.* 6:699, 2005; (b) www.pantherdb.org.]

under way to categorize protein-coding genes by type of function (Fig. 9-25b).

The relative paucity of protein-coding genes in the human genome leaves a lot of DNA unaccounted for. Much of the DNA that does not encode proteins (exons or introns) is in the form of repeated sequences of several kinds. Perhaps most surprising is that about half the human genome is made up of moderately repeated sequences that are derived from **transposons**, segments of DNA, ranging from a few hundred to several thousand base pairs long, that can move from one location to another in the genome. Originally discovered in corn by Barbara McClintock, who called them transposable elements, transposons are a kind of molecular parasite. They make their home in the genomes of essentially every organism. Many transposons contain genes encoding the proteins that catalyze the transposition process itself, as described in more detail in Chapters 25 and 26. There are several classes of transposons in the human genome. Many are strictly DNA segments, which have slowly increased in number over the millennia as a result of replication events coupled to the transposition process. Some, called retrotransposons, are closely related to retroviruses, transposing from one genomic location to another through RNA intermediates that are reconverted to DNA by reverse transcription. Some transposons in the human genome are active elements, moving at a low frequency, but most are inactive, evolutionary relics altered by mutations. Transposon movement can lead to the redistribution of other genomic sequences, and this has played a major role in human evolution.

Once the protein-coding genes (including exons and introns) and transposons are accounted for, perhaps 25% of the total DNA remains. As a follow-up to the Human Genome Project, the ENCODE initiative was launched by the National Human Genome Research Institute in 2003 to identify functional elements in the human genome. P1 The work of the worldwide consortium of research groups engaged in the ENCODE initiative has revealed that the vast majority (>80%, including protein-coding genes, most transposons, and more) of the DNA in the human genome is either transcribed into RNA in at least one type of cell or tissue or is involved in some functional aspect of chromatin structure. Much of the noncoding (nontranscribed) DNA in the remaining 20% contains regulatory elements that affect the expression of the 20,000 protein-coding genes and the many additional genes encoding functional RNAs. Many mutations (SNPs; described below) associated with human genetic diseases lie in this noncoding DNA, probably affecting regulation of one or more genes. As described in Chapters 26 and 27, new classes of functional RNAs are being discovered at a rapid pace. P1 Many of these functional RNAs, now being identified by a variety of screening methods, are produced by RNA-coding genes whose existence was previously unsuspected.

About 3% or so of the human genome consists of highly repetitive sequences referred to as **simple-sequence repeats (SSRs)**. Generally less than 10 bp long, an SSR is sometimes repeated millions of times per cell, distributed in short segments of tandem repeats. The most prominent examples of SSR DNA are found in centromeres and telomeres (see Chapter 24). Human telomeres, for example, consist of up to 2,000 contiguous repeats of the sequence GGTTAG. Additional, shorter repeats of simple sequences also occur throughout the genome. These isolated segments of repeated sequences, often containing up to a few dozen tandem repeats of a simple sequence, are called **short tandem repeats (STRs)**. Such sequences are the targets of the technologies used in forensic DNA analysis (see Box 8-1).

What does all this information tell us about the similarities and differences among individual humans? Within the human population there are millions of single-base variations, called **single nucleotide polymorphisms**, or **SNPs** (pronounced "snips"). Each person differs from the next by, on average, 1 in every 1,000 bp. Many of these variations are in the form of SNPs, but the human population also has a wide range of larger deletions, insertions, and small rearrangements. From these often subtle genetic differences comes the human variety we are all aware of—such as differences in hair color, stature, foot size, eyesight, allergies to medication, and (to some unknown degree) behavior.

The process of genetic recombination during meiosis tends to mix and match these small genetic variations so that different combinations of genes are inherited (see Chapter 25). However, groups of SNPs and other genetic differences that are close together on a chromosome are rarely affected by recombination and are usually inherited together; such a grouping of multiple SNPs is known as a **haplotype**. Haplotypes provide convenient markers for certain human populations and for individuals within populations.

Defining a haplotype requires several steps. First, positions that contain SNPs in the human population are identified in genomic DNA samples from multiple individuals (**Fig. 9-26a**). Each SNP in a prospective haplotype may be separated from the next SNP by several thousand base pairs and still be regarded as "nearby" in the context of chromosomes that extend for millions of base pairs. Second, a set of SNPs typically inherited together is chosen as a defined haplotype (Fig. 9-26b); each haplotype consists of the particular bases found at the various SNP positions within the defined set. Finally, tag SNPs—a subset of SNPs that define an entire haplotype—are chosen to uniquely identify each haplotype (Fig. 9-26c). By sequencing just these tag positions in genomic samples from human populations, researchers can quickly identify which of the haplotypes are present in each individual. Especially stable haplotypes exist in the mitochondrial genome (which does not undergo meiotic recombination) and on the Y chromosome (only 3%

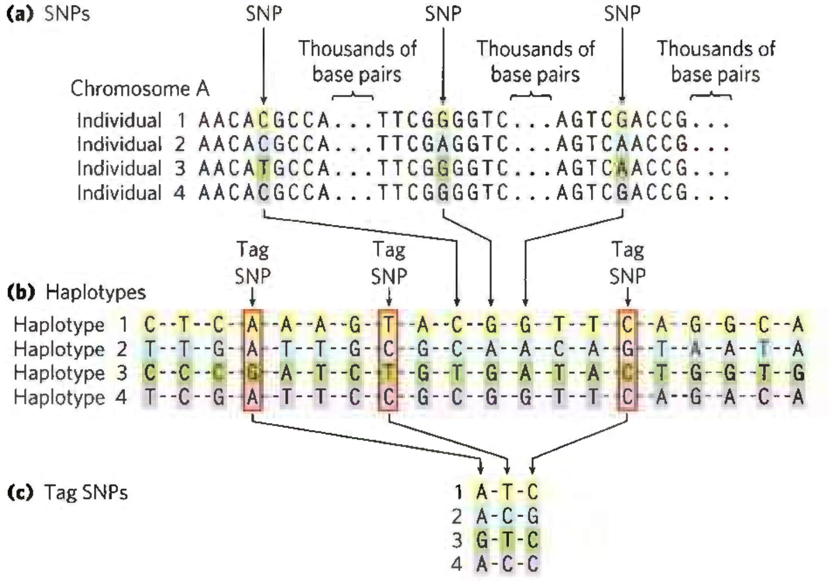

(a) SNPs
SNP
SNP
SNP
Thousands of base pairs
Thousands of base pairs
Thousands of base pairs
Chromosome A
Individual 1 AACACGCCA...TTCGGGGTC...AGTCGACCG...
Individual 2 AACACGCCA...TTCGAGGTC...AGTCAACCG...
Individual 3 AACATGCCA...TTCGGGGTC...AGTCAACCG...
Individual 4 AACACGCCA...TTCGGGGTC...AGTCGACCG...
Tag SNP
Tag SNP
Tag SNP
(b) Haplotypes
Haplotype 1 C--T--C--A--A--A--G--T--A--C--G--G--T--T--C--A--G--G--C--A
Haplotype 2 T--T--G--A--T--T--G--C--G--C--A--A--C--A--G--T--A--A--T--A
Haplotype 3 C--C--C--G--A--T--C--T--G--T--G--A--T--A--C--T--G--G--T--G
Haplotype 4 T--C--G--A--T--T--C--C--G--C--G--G--T--T--C--A--G--A--C--A
(c) Tag SNPs
1 A-T-C
2 A-C-G
3 G-T-C
4 A-C-C

FIGURE 9-26 Haplotype identification. (a) The positions of SNPs in the human genome can be identified in genomic samples. The SNPs can be in any part of the genome, whether or not it is part of a known gene. (b) Groups of SNPs are compiled into a haplotype. The SNPs vary in the overall human population, as in the four fictitious individuals shown here, but the SNPs chosen to define a haplotype are often the same in most individuals of a particular population. (c) A few SNPs are chosen as haplotype-defining (tag SNPs, outlined in red), and these are used to simplify the process of identifying an individual's haplotype (by sequencing 3 instead of 20 loci). (c) For example, if the positions shown here were sequenced, an A–T–C haplotype might be characteristic of a population native to one location in northern Europe, whereas G–T–C might be the prevailing sequence in a population in Asia. Multiple haplotypes of this kind are used to trace prehistoric human migrations. [Information from International HapMap Consortium, *Nature* 426:789, 2003, Fig. 1.]

of which is homologous to the X chromosome and thus subject to recombination). As we will see, haplotypes can be used as markers to trace human migrations.

Genome Sequencing Informs Us about Our Humanity

The human genome is very closely related to other mammalian genomes over large segments of every chromosome. However, for a genome measured in billions of base pairs, differences of just a few percent can add up to millions of genetic distinctions. Searching among these, and making use of comparative genomics techniques, researchers can begin to explore the molecular basis of definably human characteristics.

The genome sequences of our closest biological relatives, the chimpanzee (*Pan troglodytes*) and bonobo (*Pan paniscus*), offer some important clues, and we can use them to illustrate the comparative process. Human and chimpanzee shared a common ancestor about 7 million years ago. Genomic differences between the species, including SNPs and larger genomic rearrangements such as inversions, deletions, and fusions, can be used to construct a phylogenetic tree (**Fig. 9-27a**). Over the course of evolution, segments of chromosomes may become inverted as a result of a segmental duplication, transposition of one copy to another arm of the same chromosome, and recombination between them (Fig. 9-27b); such inversions have occurred in the human lineage on chromosomes 1, 12, 15, 16, and 18. Two chromosomes found in other primate lineages have been fused to form human chromosome 2 (Fig. 9-27c). The human lineage thus has 23 chromosome pairs rather than the 24 pairs typical of simians. Once this fusion appeared in the line leading to humans, it would have represented a major barrier to interbreeding with other primates that lacked it.

If we look only at base-pair changes, the published human and chimpanzee genomes differ by only 1.23% (compared with the 0.1% variance from one human to another). Some variations are at positions where there is a known polymorphism in either the human population or the chimpanzee population, and these are unlikely to reflect a species-defining evolutionary change. When we ignore these positions, the differences amount to about 1.06%, or about 1 in 100 bp. This small fraction translates into more than 30 million base-pair differences, some of which affect protein function and gene regulation. Humans are approximately as closely related to bonobos as to chimpanzees.

The genomic rearrangements that help distinguish chimpanzee and human include 5 million short insertions or deletions involving a few base pairs each, as well as a substantial number of larger insertions, deletions, inversions, and duplications that can involve many thousands of base pairs. When transposon insertions — a major source of genomic variation — are added to the list, the differences between the human and chimpanzee genomes increase. The chimpanzee genome has two classes of retrotransposons that are not present in the human genome (see Chapter 26). Other types of rearrangements, especially segmental duplications, are also common in primate

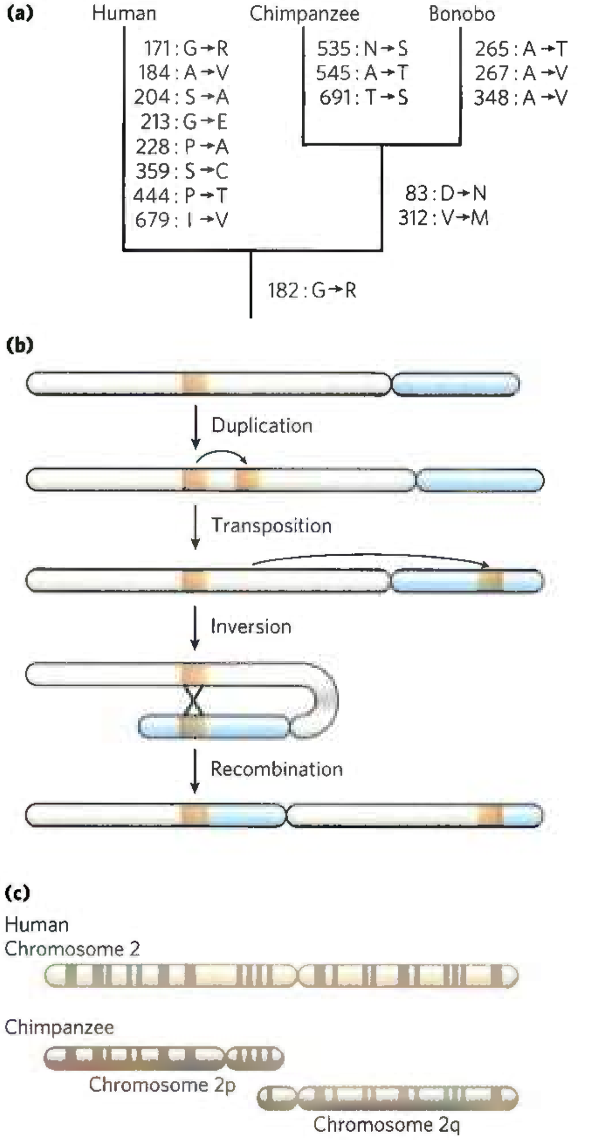

(a)
Human
Chimpanzee
Bonobo
171:G→R
184:A→V
204:S→A
213:G→E
228:P→A
359:S→C
444:P→T
679:I→V
535:N→S
545:A→T
691:T→S
265:A→T
267:A→V
348:A→V
83:D→N
312:V→M
182:G→R
(b)
Duplication
Transposition
Inversion
Recombination
(c)
Human
Chromosome 2
Chimpanzee
Chromosome 2p
Chromosome 2q

FIGURE 9-27 Genomic alterations in the human lineage. (a) This evolutionary tree is for the progesterone receptor, which helps regulate many events in reproduction. The gene encoding this protein has undergone more evolutionary alterations than most. Amino acid changes associated uniquely with human, chimpanzee, and bonobo are listed beside each branch (with the residue number). (b) One of the multistep processes that can lead to the inversion of a chromosome segment. A gene or a chromosome segment is duplicated, then moved to another chromosomal location by transposition. Recombination of the two segments may result in inversion of the DNA between them. (c) The genes on chimpanzee chromosomes 2p and 2q are homologous to those on human chromosome 2, implying that two chromosomes fused at some point in the line leading to humans. Homologous regions can be visualized as bands created in metaphase by certain dyes, as shown here. [(a) Information from C. Chen, *Mol. Phylogenet. Evol.* 47:637, 2008.]

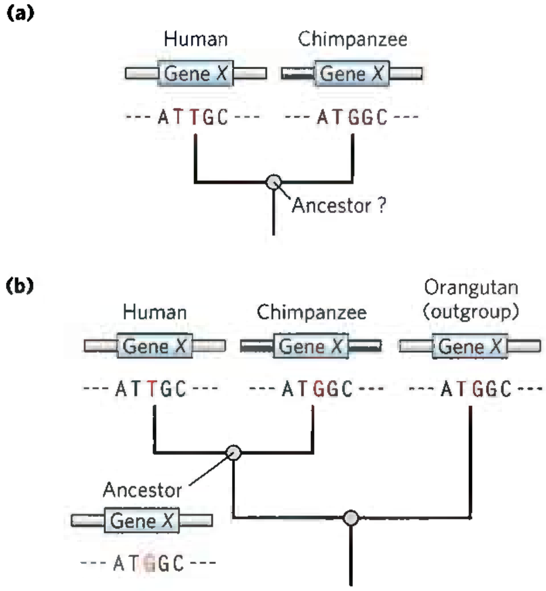

(a)
Human
Chimpanzee
Gene X
Gene X
--- ATTGC ---
--- ATGGC ---
Ancestor ?
(b)
Human
Chimpanzee
Orangutan (outgroup)
Gene X
Gene X
Gene X
--- ATTGC ---
--- ATGGC ---
--- ATGGC ---
Ancestor
Gene X
--- ATGGC ---

FIGURE 9-28 Determination of sequence alterations unique to one ancestral line. (a) Sequences from the same hypothetical gene in human and chimpanzee are compared. The sequence of this gene in the two species' last common ancestor is unknown. (b) The orangutan genome is used as an outgroup. Because the sequence of the orangutan gene is identical to that of the chimpanzee gene, the mutation causing the difference between human and chimpanzee almost certainly occurred in the line leading to modern humans, and the common ancestor of human and chimpanzee (and orangutan) had the variant now found in chimpanzees.

lineages. Duplications of chromosomal segments can lead to changes in the expression of genes contained in these segments. There are about 90 million bp of such differences between human and chimpanzee, representing another 3% of these genomes. Each species has segments of DNA, constituting 40 million to 45 million bp, that are entirely unique to that particular genome, with larger chromosomal insertions, duplications, and other rearrangements affecting more base pairs than do single-nucleotide changes. Thus, in all, chimpanzee and human differ over about 4% of their genomes.

Sorting out which genomic distinctions are relevant to features that are uniquely human is a daunting task. If one assumes a similar rate of evolution in the chimpanzee and human lines after they diverged from their common ancestor, half the changes represent chimpanzee lineage changes and half represent human lineage changes. By comparing both genome sequences with those of more distantly related species referred to as **outgroups**, we can determine which variant was present in the common ancestor. Consider a locus, X, where there is a difference between the human and chimpanzee genomes (**Fig. 9-28a**). The lineage of the orangutan, an outgroup, diverged from that of chimpanzee and human prior to the common ancestor of chimpanzee and human (Fig. 9-28b). If the sequence at locus X is identical in orangutan and chimpanzee, this sequence was probably present in the chimpanzee and human ancestor, and the sequence seen in humans is specific to the human lineage. Sequences that are identical in human and orangutan can be eliminated as candidates for human-specific genomic features. The importance of comparisons with closely related outgroups has given rise to new efforts to sequence the genomes of orangutan, macaque, and many other primate species. Comparison of the human and bonobo genomes is refining the analysis of genes and alleles of special significance to humans.

The search for the genetic underpinnings of special human characteristics, such as our enhanced brain function, can benefit from two complementary approaches. The first approach searches for genomic regions where extreme changes have occurred, such as genes that have been duplicated many times or large genomic segments not present in other primates. The second approach looks at genes known to be involved in relevant human disease conditions. For brain function, for example, one would examine genes that, when mutated, contribute to cognitive or mental disorders.

Notably, analyses of the human lineage have not detected an increased rate of genetic change in protein-coding genes involved in brain development or size. In primates, most genes that function uniquely in the brain are even more highly conserved than genes functioning in other tissues, perhaps due to some special constraints related to brain biochemistry. However, there are some differences in gene expression patterns between humans and other primates that may affect brain function. For example, the gene encoding the enzyme glutamate dehydrogenase, which plays an important role in neurotransmitter synthesis, has been subjected to gene duplication events, so that there are now multiple copies of it. Genomic regions related to gene regulation have disproportionately high numbers of changes in genes involved in neural development and nutrition. Our brains have become larger as a result, and additional functional effects may eventually be defined. A variety of RNA-coding genes, some with expression concentrated in the brain, also show evidence of accelerated evolution (**Fig. 9-29**). Many of these are probably involved in regulating the expression of other genes. As we continue to discover many new classes of RNA (see Chapter 26), we are likely to radically change our perspective on how evolution alters the workings of living systems.

Genome Comparisons Help Locate Genes Involved in Disease

One of the motivations for the Human Genome Project was its potential for accelerating the discovery of genes underlying genetic diseases. That promise has been fulfilled: **P1** more than 6,000 human mutation phenotypes, mostly associated with genetic diseases, have been mapped to particular genes or groups of genes.

For the last two decades, the main approach to gene mapping has been **linkage analysis**, yet another approach derived from evolutionary biology. In brief, the gene involved in a disease condition is mapped relative to well-characterized genetic polymorphisms that occur throughout the human genome. We can illustrate this approach by describing the search for one gene involved in early-onset Alzheimer disease.

About 10% of all cases of Alzheimer disease in the United States result from an inherited predisposition. Several different genes have been discovered that, when mutated, can lead to early onset of the disease. One such gene, *PS1*, encodes the protein presenilin-1, and its discovery made heavy use of linkage analysis. The search begins with large families having multiple individuals affected by a particular disease — in this case, Alzheimer disease. Two of the many family pedigrees used to search for this gene in the early 1990s are shown in **Figure 9-30a**. In studies of this type, DNA samples are collected from both affected and unaffected family members. Researchers first localize the region associated

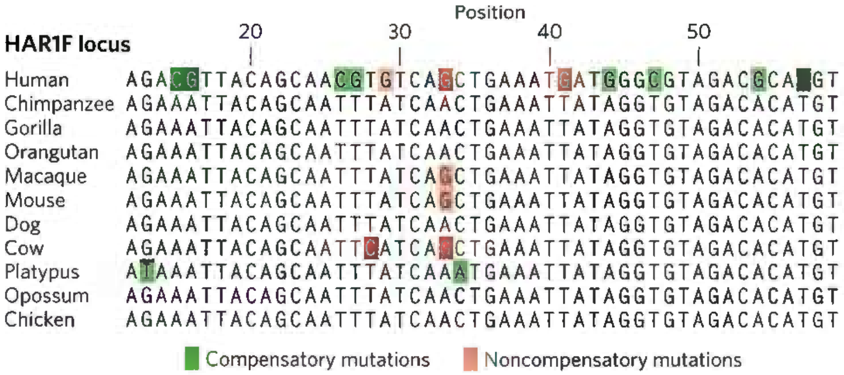

HAR1F locus
Position
20
30
40
50
Human AGACGTTACAGCAACGTGTCAGCTGAAATGATGGGCGTAGACGCA[illegible]GT
Chimpanzee AGAAATTACAGCAATTTATCAACTGAAATTATAGGTGTAGACACATGT
Gorilla AGAAATTACAGCAATTTATCAACTGAAATTATAGGTGTAGACACATGT
Orangutan AGAAATTACAGCAATTTATCAACTGAAATTATAGGTGTAGACACATGT
Macaque AGAAATTACAGCAATTTATCAGCTGAAATTATAGGTGTAGACACATGT
Mouse AGAAATTACAGCAATTTATCAGCTGAAATTATAGGTGTAGACACATGT
Dog AGAAATTACAGCAATTTATCAACTGAAATTATAGGTGTAGACACATGT
Cow AGAAATTACAGCAATTCATCAGCTGAAATTATAGGTGTAGACACATGT
Platypus ATAAATTACAGCAATTTATCAAATGAAATTATAGGTGTAGACACATGT
Opossum AGAAATTACAGCAATTTATCAACTGAAATTATAGGTGTAGACACATGT
Chicken AGAAATTACAGCAATTTATCAACTGAAATTATAGGTGTAGACACATGT
🟩 Compensatory mutations
🟥 Noncompensatory mutations

FIGURE 9-29 Accelerated evolution in some human genes. The HAR1F locus specifies a noncoding RNA that is highly conserved in vertebrates. The human HAR1F gene has an unusual number of substitutions (highlighted by color shading), providing evidence of accelerated evolution. HAR1F RNA functions in the brain during neurodevelopment. Compensatory substitutions are those that retain complementarity where strand segments are paired. [Information from T. Marques-Bonet, *Annu. Rev. Genomics Hum. Genet.* 10:355, 2009.]

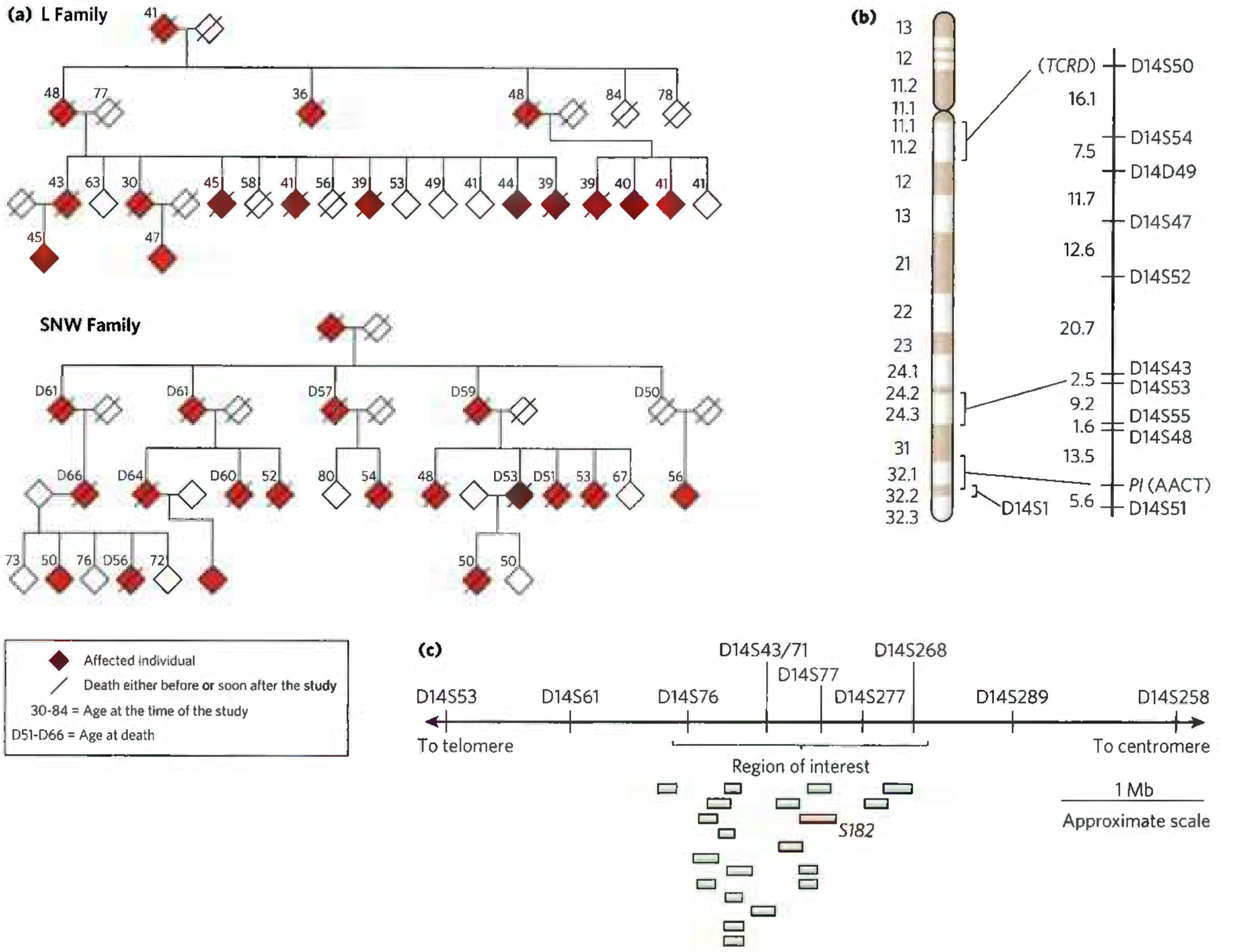

(a) L Family
SNW Family
(b)
13
12
11.2
11.1
11.1
11.2
12
13
21
22
23
24.1
24.2
24.3
31
32.1
32.2
32.3
(TCRD)
D14S50
16.1
D14S54
7.5
D14D49
11.7
D14S47
12.6
D14S52
20.7
D14S43
2.5
D14S53
9.2
D14S55
1.6
D14S48
13.5
PI (AACT)
5.6
D14S51
D14S1
Affected individual
Death either before or soon after the study
30-84 = Age at the time of the study
D51-D66 = Age at death
(c)
D14S43/71
D14S268
D14S77
D14S53
D14S61
D14S76
D14S277
D14S289
D14S258
To telomere
To centromere
Region of interest
S182
1 Mb
Approximate scale

FIGURE 9-30 Linkage analysis in the discovery of disease genes. (a) These pedigrees for two families affected by early-onset Alzheimer disease are based on the data available at the time of the study. To protect family privacy, gender is not indicated. (b) Chromosome 14, with bands created by certain dyes. Chromosome marker positions are shown at the right, with the genetic distance between them in centimorgans, a genetic distance measurement that reflects the frequency of recombination between markers. *TCRD* (T-cell receptor delta) and *PI* (AACT (α1-antichymotrypsin)), two genes with alterations in the human population, were used along with SNPs as markers in chromosome mapping. (c) By comparing DNA from affected and unaffected family members, researchers eventually defined a region of interest near marker D14S43 that contains 19 expressed genes. The gene labeled *S182* (red) encodes presenilin-1. (1 Mb = 10^6 base pairs.) [Information from (a, b) G. D. Schellenberg et al., *Science* 258:668, 1992; (c) R. Sherrington et al., *Nature* 375:754, 1995.]

with a disease to a specific chromosome by comparing the genotypes of individuals with and without the disease, focusing especially on close family members. The specific points of comparison are sets of well-characterized SNP loci mapped to each chromosome, as identified by the Human Genome Project. By identifying the SNPs that are most often inherited with the disease-causing gene, investigators can gradually localize the responsible gene to a single chromosome. In the case of the *PS1* gene, co-inheritance was strongest with markers on chromosome 14 (Fig. 9-30b).

Chromosomes are very large DNA molecules, and localizing the gene to one chromosome is only a small part of the battle. Localizing the gene to one chromosome (in this case, chromosome 14) is only the beginning of narrowing the search for a gene. Chromosomes are very large molecules; each one houses thousands of SNPs and other changes. Simply sequencing the entire chromosome would be unlikely to reveal the SNP or other change associated with the disease. Instead, investigators rely on statistical methods that correlate the inheritance of additional, more closely spaced polymorphisms with the occurrence of the disease, focusing on a denser panel of polymorphisms known to occur on the chromosome of interest. The more closely a marker is located to a disease gene, the more likely it is to be inherited along with that gene. This process can pinpoint a region of the chromosome that contains the gene. However, the region may still encompass many genes. In our example, linkage analysis indicated that the disease-causing gene, *PS1*, was somewhere near the SNP locus D14S43 (Fig. 9-30c).

The final steps in identifying the gene use the human genome databases. The local region containing the gene is examined, and the genes within it are identified. DNA from many individuals, some who have the disease and some who do not, is sequenced over this region. As the DNA in this region is sequenced from increasing numbers of individuals, gene variants that are consistently present in individuals with the disease and absent in unaffected individuals can be identified. An understanding of the function of the genes in the target region can aid the search, because particular metabolic pathways may be more likely than others to produce the disease state. In 1995, the chromosome 14 gene associated with Alzheimer disease was identified as *S182*. The product of this gene was given the name presenilin-1, and the gene was subsequently renamed *PS1*.

Many human genetic diseases are caused by mutations in a single gene or in sequences involved in regulation of that gene. Several different mutations in a particular gene, all leading to the same or related genetic condition, may be present in the human population. For example, there are several variants of *PS1*, all giving rise to a much-increased risk of early-onset Alzheimer disease. Another, more extreme example is the several genes encoding different hemoglobins: more than 1,000 known mutational variants are present in the human population. Some of these variants are innocuous; some cause diseases ranging from sickle cell disease to thalassaemias. The inheritance of particular mutant genes may be concentrated in families or in isolated populations.

More complex are cases in which a disease condition is caused by mutations in two different genes (neither of which, alone, causes the disease), or in which a particular condition is enhanced by an otherwise innocuous mutation in another gene. Identifying the genes and mutations responsible for these digenic diseases is exceedingly difficult, and sometimes such diseases can be documented only within small, isolated, and highly inbred populations.

Genome databases provide alternative paths to the identification of disease genes. In many cases, we already have biochemical information about the disease. In the case of early-onset Alzheimer disease, an accumulation of the amyloid-β protein in limbic and association cortices of the brain is at least partly responsible for the symptoms. Defects in presenilin-1 (and in a related protein, presenilin-2, encoded by a gene on chromosome 1) lead to the elevated cortical levels of amyloid-β protein. Focused databases are being developed that catalog such functional information on the protein products of genes and on protein-interaction networks and SNP locations, along with other data. The result is a streamlined path to the identification of candidate genes for a particular disease. If a researcher knows a little about the kinds of enzymes or other proteins likely to contribute to disease symptoms, these databases can quickly generate a list of genes known to encode proteins with relevant functions, a list of additional uncharacterized genes with orthologous or paralogous relationships to these genes, a list of proteins known to interact with the target proteins or orthologs in other organisms, and a map of gene positions. Often, with the aid of data from some selected family pedigrees, a short list of potentially relevant genes can be rapidly determined.

These approaches are not limited to human diseases. The same methods can be used to identify the genes involved in diseases—or genes that produce desirable characteristics—in other animals and in plants. Of course, they can also be used to track down genes involved in any observable trait that a researcher might be interested in. ■

Genome Sequences Inform Us about Our Past and Provide Opportunities for the Future

Anatomically modern humans arose in Africa between 250,000 and 350,000 years ago. About 100,000 to 120,000 years ago, humans in Africa looked out across the Red Sea to Asia. Perhaps encouraged by some innovation in small boat construction, or driven by conflict or famine, or simply curious, they crossed the water barrier. That initial colonization began a journey that did not stop until humans reached Tierra del Fuego (at the southern tip of South America), many thousands of years later. As *Homo sapiens* populations moved into more northern parts of Europe and Asia about 45,000 years ago, established populations from previous hominid expansions into Eurasia, including *Homo neanderthalensis* and a group now called the Denisovans, were displaced. The Neanderthals and Denisovans disappeared, just as other hominid lines had disappeared before them.

The story of how modern humans first appeared in Africa a few hundred thousand years ago, and their migrations as they eventually radiated out of Africa, is written in our DNA. Genomic sequences from multiple species have brought both primate and hominid evolution into sharper focus. Using haplotypes present in extant human populations, we can trace the migrations of our intrepid ancestors across the planet (**Fig. 9-31a**). The Neanderthals were not simply displaced. Some mingling occurred (Fig. 9-31b). Using sensitive PCR-based methods, we now possess multiple complete sequences of the Neanderthal genome (**Box 9-2**). We know that up to 5% of the genome of most

BOX 9-2

Getting to Know Humanity's Next of Kin

Modern humans and Neanderthals coexisted in Europe and Asia as recently as 30,000 years ago. The human and Neanderthal ancestral populations diverged about 370,000 years ago, somewhat before the appearance of anatomically modern humans. Neanderthals used tools, lived in small groups, and buried their dead. Of the known hominid relatives of modern humans, Neanderthals are the closest. For hundreds of millennia, they inhabited large parts of Europe and western Asia (Fig. 1). If the chimpanzee genome can tell us something about what it is to be human, the Neanderthal genome can tell us more. Buried in the bones and other remains taken from burial sites are fragments of Neanderthal genomic DNA. Technologies developed for use in forensic science (see Box 8-1) and studies of ancient DNA have been combined in the Neanderthal genome project.

This endeavor is unlike the genome projects aimed at extant species. The Neanderthal DNA is present in small amounts, and it is contaminated with DNA from other animals and bacteria. How does one get at it, and how can one be certain that the sequences really came from Neanderthals? The answers have been revealed by

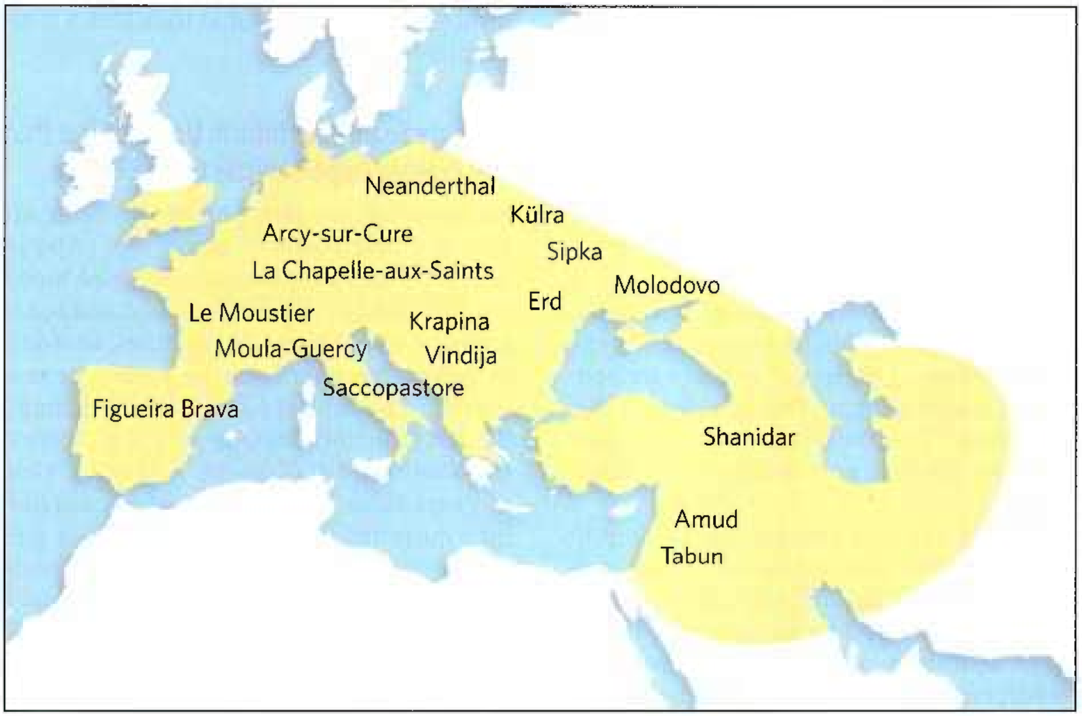


FIGURE 1 Neanderthals occupied much of Europe and western Asia until about 30,000 years ago. Major Neanderthal archaeological sites are shown here. (Note that the group was named for the site at Neanderthal in Germany.)

non-African humans is derived from Neanderthals. Anatomically modern human remains up to 45,000 years old have been sequenced, beginning an effort to pinpoint the period of interbreeding. Human populations native to Melanesia and Australia acquired up to 6% of their genomic DNA from the Denisovans. Neanderthal DNA gave humans a more complex immune system, making us more resistant to infection but also a little more susceptible to autoimmune diseases. The story of our past is gradually taking shape as more genomes, of humans alive today and those who lived in past millennia, are being assembled.

innovative applications of biotechnology. In essence, the small quantities of DNA fragments found in Neanderthal bone or other remains are cloned into a library, and the cloned DNA segments are sequenced at random, contaminants and all. The sequencing results are compared with the existing human genome and chimpanzee genome databases. Segments derived from Neanderthal DNA have sequences closely related to human DNA and chimpanzee DNA and thus are readily distinguished by computerized analysis from segments derived from bacteria or insects. Once these segments are sequenced, they can be used as probes to identify sequence fragments in ancient samples that overlap with these known fragments. The potential problem of contamination with the closely related modern human DNA can be controlled for by examining mitochondrial DNA. Human populations have readily identifiable haplotypes (distinctive sets of genomic differences; see Fig. 9-26) in their mitochondrial DNA, and analysis of Neanderthal samples has shown that Neanderthals' mitochondrial DNA has its own distinct haplotypes. The presence in the Neanderthal samples of some base-pair differences that are found in the chimpanzee database but not in the human database is more evidence that nonhuman hominid sequences are being found.

Multiple high-quality Neanderthal genomic sequences have been completed. The data provide evidence that modern humans and the Neanderthals who were the source of this DNA shared a common ancestor about 700,000 years ago (Fig. 2). Analysis of mitochondrial DNA suggests that the two groups continued on the same track, with some gene flow between them, for about 300,000 more years. The lines split with the appearance of anatomically modern humans, although evidence now exists for some intermingling of the lines somewhat later as humans spread through Eurasia.

Expanded libraries of Neanderthal DNA from different sets of remains are revealing Neanderthal genetic diversity, and may eventually tell us about Neanderthal migrations, providing a fascinating look at our hominid past.

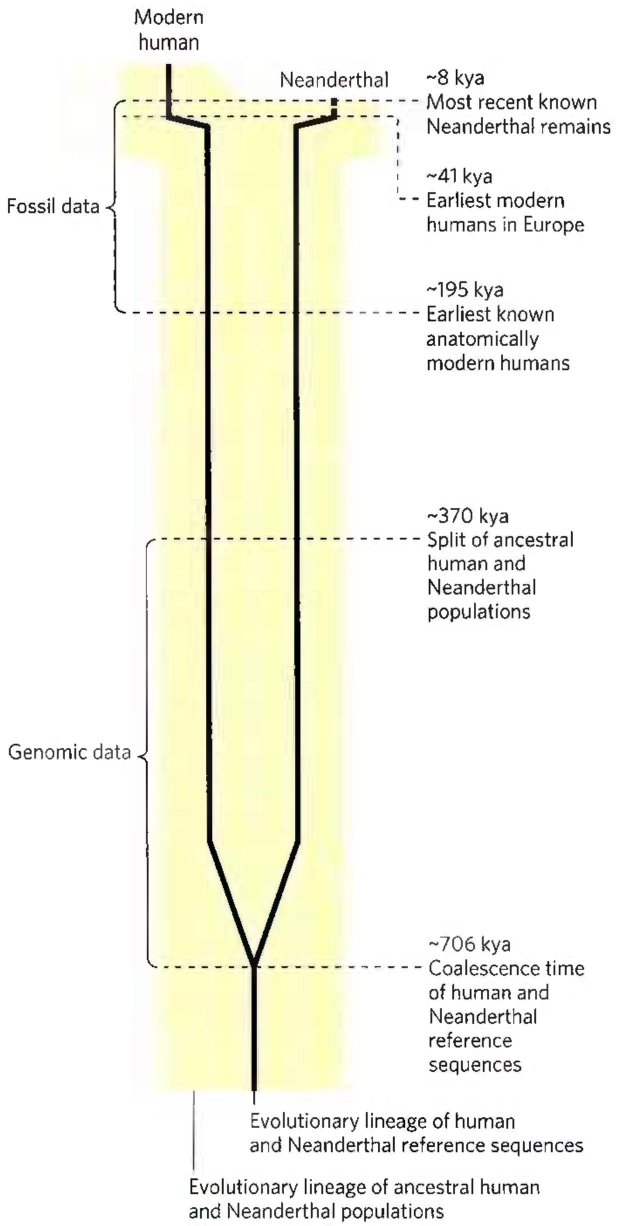

Modern human
Neanderthal
~8 kya
Most recent known Neanderthal remains
~41 kya
Earliest modern humans in Europe
~195 kya
Earliest known anatomically modern humans
Fossil data
~370 kya
Split of ancestral human and Neanderthal populations
Genomic data
~706 kya
Coalescence time of human and Neanderthal reference sequences
Evolutionary lineage of human and Neanderthal reference sequences
Evolutionary lineage of ancestral human and Neanderthal populations

FIGURE 2 This timeline shows the divergence of human and Neanderthal genome sequences (black lines) and of ancestral human and Neanderthal populations (yellow screen). Genomic data provide evidence for some intermingling of the populations up to about 45,000 years ago. Key events in human evolution are noted. [Information from J. P. Noonan et al., *Science* 314:1113, 2006.]

The medical promise of personal genomic sequences grows as sequencing costs continue to decline and more genes underlying inherited diseases are defined. **P3** Knowledge of genomic sequences also provides the prospect of altering them. It is now commonplace to engineer the DNA sequences of organisms ranging from bacteria and yeast to plants and mammals, for research and commercial purposes. Efforts to cure inherited human diseases by human gene therapy have not yet lived up to their potential, but technologies for gene delivery are constantly being improved. Few scientific disciplines will affect the future of our species more than modern genomics.

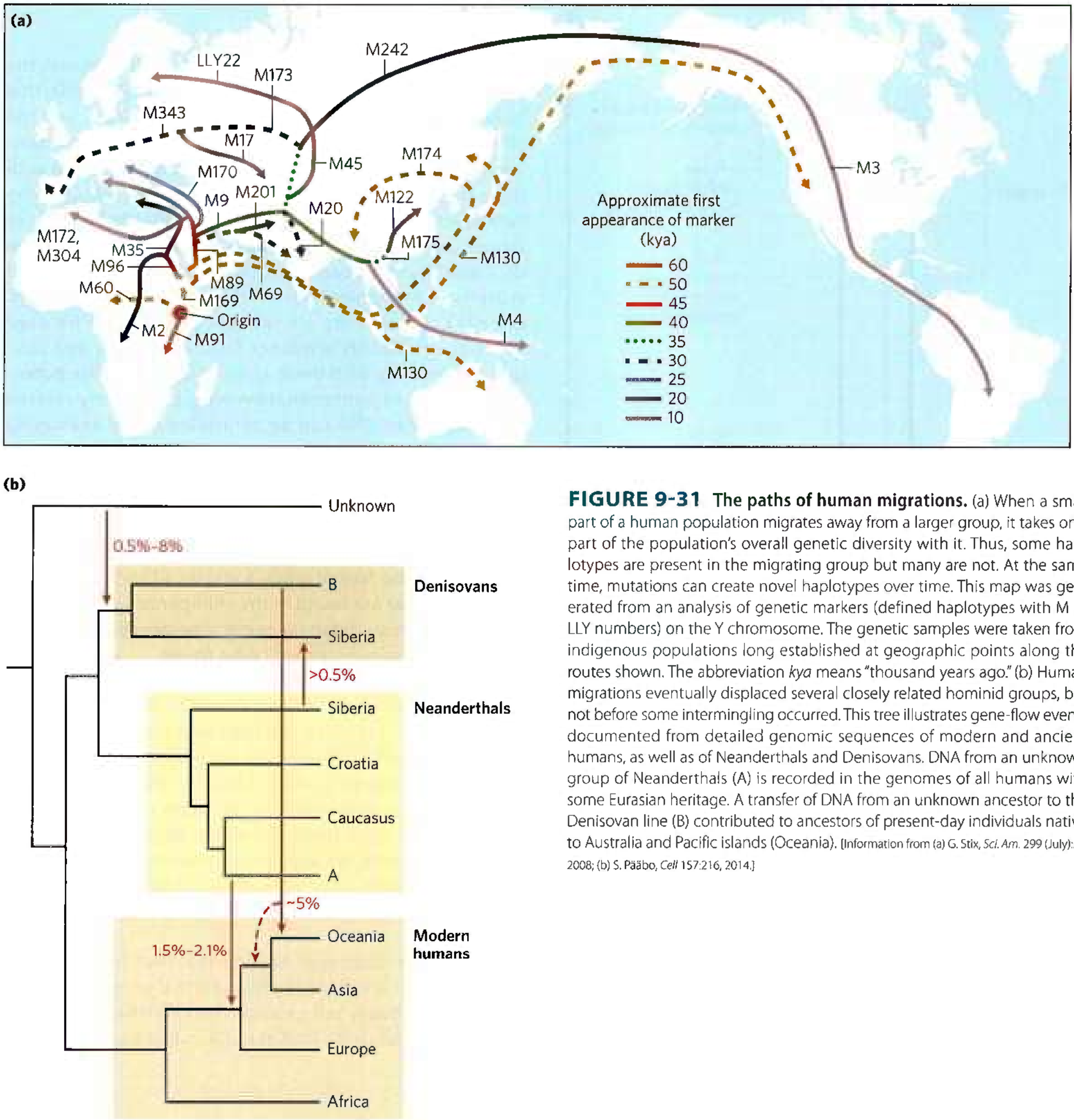

(a)
LLY22
M173
M242
M343
M17
M45
M174
M170
M9
M201
M20
M122
M3
M172,
M304
M35
M96
M175
M130
M60
M89
M169
M69
Origin
M2
M91
M4
M130
Approximate first appearance of marker (kya)
60
50
45
40
35
30
25
20
10
(b)
Unknown
0.5%–8%
B
Denisovans
Siberia
>0.5%
Siberia
Neanderthals
Croatia
Caucasus
A
~5%
1.5%–2.1%
Oceania
Modern humans
Asia
Europe
Africa

FIGURE 9-31 The paths of human migrations. (a) When a small part of a human population migrates away from a larger group, it takes only part of the population's overall genetic diversity with it. Thus, some haplotypes are present in the migrating group but many are not. At the same time, mutations can create novel haplotypes over time. This map was generated from an analysis of genetic markers (defined haplotypes with M or LLY numbers) on the Y chromosome. The genetic samples were taken from indigenous populations long established at geographic points along the routes shown. The abbreviation *kya* means "thousand years ago." (b) Human migrations eventually displaced several closely related hominid groups, but not before some intermingling occurred. This tree illustrates gene-flow events documented from detailed genomic sequences of modern and ancient humans, as well as of Neanderthals and Denisovans. DNA from an unknown group of Neanderthals (A) is recorded in the genomes of all humans with some Eurasian heritage. A transfer of DNA from an unknown ancestor to the Denisovan line (B) contributed to ancestors of present-day individuals native to Australia and Pacific islands (Oceania). [Information from (a) G. Stix, *Sci. Am.* 299 (July):56, 2008; (b) S. Pääbo, *Cell* 157:216, 2014.]

SUMMARY 9.3 *Genomics and the Human Story*

- About 30% of the DNA in the human genome is in the exons and introns of genes that encode proteins. Nearly half of the DNA is derived from parasitic transposons. Much of the rest encodes RNAs of many types. Simple-sequence repeats make up the centromere and telomeres.
- The gene alterations that define humanity can be discerned in part through comparative genomics, using other primates.
- Comparative genomics is also used to locate the gene alterations that define inherited diseases.
- Human genomics can be used to study the evolution and migration of our human ancestors over many millennia.

KEY TERMS

Terms in bold are defined in the glossary.

genome	301
genomics	301
systems biology	301
cloning	302
vector	302
recombinant DNA	302
recombinant DNA technology	302
genetic engineering	302
restriction endonucleases	302
DNA ligases	303
restriction-modification system	303
multiple cloning site (MCS)	304
plasmid	305
bacterial artificial chromosome (BAC)	307
yeast artificial chromosome (YAC)	307
expression vector	309
baculovirus	311
bacmid	311
site-directed mutagenesis	312
fusion protein	313
tag	313
reverse transcriptase PCR (RT-PCR)	314
quantitative PCR (qPCR)	314
DNA library	315
complementary DNA (cDNA)	316
cDNA library	316
transcriptome	317
proteome	317
transcriptomics	317
proteomics	317
comparative genomics	317
genome annotation	317
orthologs	317
paralogs	317
synteny	318
RNA-Seq	318
single cell RNA-Seq (scRNA-Seq)	318
green fluorescent protein (GFP)	319
epitope tag	320
yeast two-hybrid analysis	322
CRISPR/Cas	322
guide RNA (gRNA)	323
trans-activating CRISPR RNA (tracrRNA)	323
single guide RNA (sgRNA)	323
single nucleotide polymorphism (SNP)	328
haplotype	328

PROBLEMS

1. Engineering Cloned DNA When joining two or more DNA fragments, a researcher can adjust the sequence at the junction in a variety of subtle ways, as seen in these exercises.

(a) Write the sequence of each end of a linear DNA fragment produced by an EcoRI restriction digest (include those sequences remaining from the EcoRI recognition sequence).

(b) Write the sequence resulting from the reaction of this end sequence with DNA polymerase I and the four deoxynucleoside triphosphates (see Fig. 8-34).

(c) Write the sequence produced at the junction that arises if two ends with the structure derived in (b) are ligated (see Fig. 25-15).

(d) Write the sequence produced if the structure derived in (a) is treated with a nuclease that degrades only single-stranded DNA.

(e) Write the sequence of the junction produced if an end with structure (b) is ligated to an end with structure (d).

(f) Write the sequence of the end of a linear DNA fragment that was produced by a PvuII restriction digest (include those sequences remaining from the PvuII recognition sequence).

(g) Write the sequence of the junction produced if an end with structure (b) is ligated to an end with structure (f).

(h) Suppose you can synthesize a short duplex DNA fragment with any sequence you desire. With this synthetic fragment and the procedures described in (a) through (g), design a protocol that would remove an EcoRI restriction site from a DNA molecule and incorporate a new BamHI restriction site at approximately the same location. (See Fig. 9-2.)

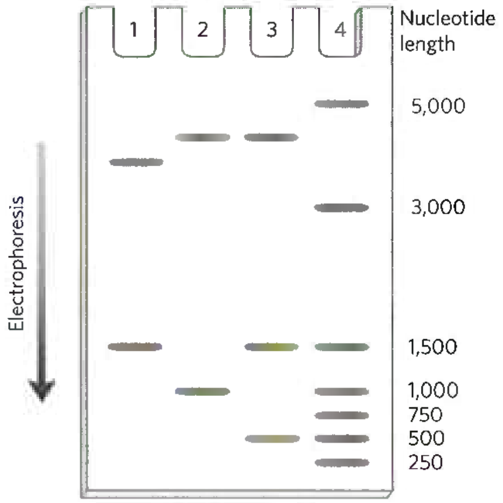
(i) Design four different short synthetic double-stranded DNA fragments that would permit ligation of structure (a) with a DNA fragment produced by a PstI restriction digest. In one of these fragments, design the sequence so that the final junction contains the recognition sequences for both EcoRI and PstI. In the second and third fragments, design the sequence so that the junction contains only the EcoRI and only the PstI recognition sequence, respectively. Design the sequence of the fourth fragment so that neither the EcoRI nor the PstI sequence appears in the junction.

2. Selecting for Recombinant Plasmids When cloning a foreign DNA fragment into a plasmid, it is often useful to insert the fragment at a site that interrupts a selectable marker (such as the tetracycline-resistance gene of pBR322). The loss of function of the interrupted gene can be used to identify clones containing recombinant plasmids with foreign DNA. With a yeast artificial chromosome (YAC) vector, a researcher can distinguish vectors that incorporate large foreign DNA fragments from those that do not, without interrupting gene function. How are these recombinant vectors identified?

3. DNA Cloning The restriction endonuclease PstI cleaves the plasmid cloning vector pBR322 (see Fig. 9-3). A researcher ligates an isolated DNA fragment from a eukaryotic genome (also produced by PstI cleavage) to the prepared vector. She then uses the mixture of ligated DNAs to transform bacteria and selects plasmid-containing bacteria by growth in the presence of tetracycline.

(a) In addition to the desired recombinant plasmid, what other types of plasmids might be found among the transformed bacteria that are tetracycline-resistant? How can the types be distinguished?

(b) The cloned DNA fragment is 1,000 bp long and has an EcoRI site 250 bp from one end. The researcher cleaves three different recombinant plasmids with EcoRI and analyzes them by gel electrophoresis, with the results shown in the image. What does each pattern say about the cloned DNA? Note that in pBR322, the PstI and EcoRI restriction sites are about 750 bp apart. The entire plasmid with no cloned insert is 4,361 bp. Size markers in lane 4 have the number of nucleotides noted.



4. Restriction Enzymes The partial sequence of one strand of a double-stranded DNA molecule is

5′ – – – GACGAAGTGCTGCAGAAAGTCCGCGTTATAGGCAT GAATTCCTGAGG – – – 3′

The cleavage sites for the restriction enzymes EcoRI and PstI are shown below.

```
EcoRI     ↓ *                  PstI            * ↓
     (5′) G A A T T C (3′)         (5′) C T G C A G (3′)
          C T T A A G                   G A C G T C
              *   ↑                     ↑   *
```

Write the sequence of *both strands* of the DNA fragment created when this DNA is cleaved with both EcoRI and PstI. The top strand of your duplex DNA fragment should be derived from the strand sequence given.

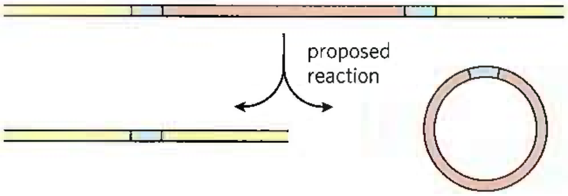
5. Designing a Diagnostic Test for a Genetic Disease Huntington disease (HD) is an inherited neurodegenerative disorder, characterized by the gradual, irreversible impairment of psychological, motor, and cognitive functions. Symptoms typically appear in middle age, but onset can occur at almost any age. The course of the disease can be 15 to 20 years. Biomedical research is improving our understanding of the molecular basis of the disease. The genetic mutation underlying HD has been traced to a gene encoding a protein (M_r 350,000) of unknown function. The region of the gene that encodes the amino terminus of the protein has a repeated sequence of CAG codons (for glutamine). The length of this simple trinucleotide repeat indicates whether an individual will develop HD, and at approximately what age the first symptoms will occur. The sequence is repeated 6 to 39 times in individuals who will not develop HD, 40 to 55 times in those with adult-onset HD, and more than 70 times in individuals with childhood-onset HD.

A small portion of the amino-terminal coding sequence of the 3,143-codon HD gene is shown. The nucleotide sequence of the DNA is given in black, the amino acid sequence corresponding to the gene is given in blue, and the CAG repeat is shaded. Using Figure 27-7 to translate the genetic code, outline a PCR-based test for HD that could be carried out using a blood sample. Assume the PCR primer must be 25 nucleotides long. By convention, unless otherwise specified, a DNA sequence encoding a protein is displayed with the coding strand—the sequence identical to the mRNA transcribed from the gene (except for U replacing T)—on top, such that it reads 5′ to 3′, left to right.

```
307 ATGGCGACCCTGGAAAAGCTGATGAAGGCCTTCGAGTCCCTCAAGTCCTTC
1   M  A  T  L  E  K  L  M  K  A  F  E  S  L  K  S  F

358 CAGCAGTTCCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
18  Q  Q  F  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q

409 CAGCAGCAGCAGCAGCAGCAGCAACAGCCGCCACCGCCGCCGCCGCCGCCG
35  Q  Q  Q  Q  Q  Q  Q  Q  Q  P  P  P  P  P  P  P  P

460 CCGCCTCCTCAGCTTCCTCAGCCGCCGCCG
52  P  P  P  Q  L  P  Q  P  P  P
```

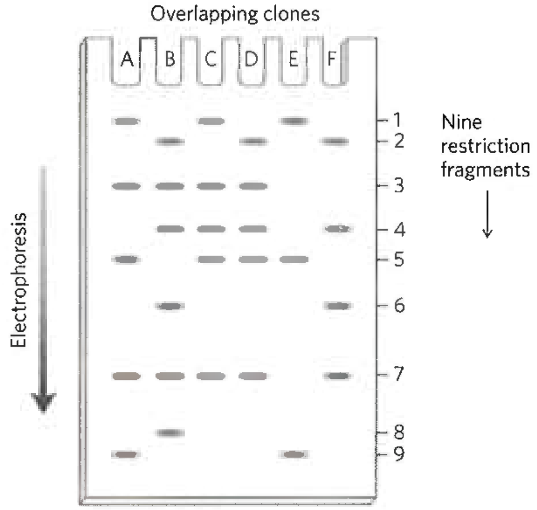
Information from The Huntington's Disease Collaborative Research Group, *Cell* 72:971, 1993.

6. Using PCR to Detect Circular DNA Molecules In a species of ciliated protist, a segment of genomic DNA is sometimes deleted. The deletion is a genetically programmed reaction associated with cellular mating. A researcher proposes that the DNA is deleted in a type of recombination called site-specific recombination, with the DNA at either end of the segment joined together and the deleted DNA ending up as a circular DNA reaction product. Suggest how the researcher might use the polymerase chain reaction (PCR) to detect the presence of the circular form of the deleted DNA in an extract of the protist.



7. Protein Dynamics within Cells In a bacterial cell, two proteins, X and Y, are thought to have similar functions. Researchers genetically engineered each protein to fuse with a variant of the green fluorescent protein, one that glows red (X) and the other yellow (Y). Controls showed that both fusion proteins retained their activity, and both produced visible spots of light (foci) when expressed. To better understand the biological functions of the two proteins, the researchers expressed the fusion proteins in the same bacterial cell under two different conditions. Under nutrient-rich conditions, distinct red and yellow puncta (well-defined clustering of foci) were distributed throughout the cell. One or two red puncta were typically found within the nucleoid (chromosomal DNA), whereas the multiple yellow puncta were distributed throughout the cell. However, under nutrient starvation, the yellow puncta migrated and co-localized (overlapped) with the red puncta. What might be concluded from these observations?

8. Mapping a Chromosome Segment Researchers isolated a group of overlapping clones, designated A through F, from one region of a chromosome. They then separately cleaved each of the clones using a restriction enzyme and resolved the pieces by agarose gel electrophoresis. The image shows the electrophoresis results. There are nine different restriction fragments in this chromosomal region, with a subset appearing in each clone. Using this information, deduce the order of the restriction fragments in the chromosome.



9. Immunofluorescence In a common protocol for immunofluorescence detection of cellular proteins, an investigator uses two antibodies. The first binds specifically to the protein of interest. The second is labeled with fluorochromes for easy visualization, and it binds to the first antibody. In principle, one could simply label the first antibody and skip one step. Why use two successive antibodies?

10. Yeast Two-Hybrid Analysis You are a researcher who has just discovered a new protein in a fungus. Design a yeast two-hybrid experiment to identify the other proteins in the fungal cell with which your protein interacts, and explain how this could help you determine the function of your protein.

11. RNA-Seq RNA-Seq is a next-generation sequencing method used to quantitatively profile the cellular transcriptome. Researchers use RNA-Seq to compare the expression of genes under different environmental conditions or between different types of cells. There are three general steps in an RNA-Seq workflow:

1. Generate a cDNA library from cellular RNA.
2. Add oligonucleotide adapters to the fragments of the cDNA library.
3. Use next-generation sequencing to identify transcriptionally active genes from the cDNA library.

What is the role of the enzyme reverse transcriptase in an RNA-Seq workflow?

12. Cellular RNA Suppose that an investigative team conducted an RNA-Seq experiment on mouse liver cells. The team found many sequences that contained no open reading frames (Chapter 27)—long stretches of consecutive triplet codons that could be translated into a protein and therefore suggest the presence of a gene. Suggest a reason for this observed lack of ORFs.

13. Use of Outgroups in Comparative Genomics A hypothetical protein found in human, orangutan, and chimpanzee has the following sequences (red indicates amino acid residue M differences; dashes indicate a deletion, meaning the residues are missing in that sequence):

Human: ATSAAG**Y**DEWEGGK**V**LIHL – – KLQNRGALLELDIGAV

Orangutan: ATSAAG**W**DEWEGGK**V**LIHL**DG**KLQNRGALLELDIGAV

Chimpanzee: ATSAAG**W**DEWEGGK**I**LIHL**DG**KLQNRGALLELDIGAV

What is the most likely sequence of the protein present in the last common ancestor of human and chimpanzee?

14. Human Migrations I Native American populations in North America and South America have mitochondrial DNA haplotypes that can be traced to populations in northeast Asia. The Aleut and Eskimo populations in the far northern parts of North America possess a subset of the same haplotypes that link other Native Americans to Asia, and the Aleut and Eskimo populations also have several additional haplotypes that can be traced to Asian origins but are not found in native populations in other parts of the Americas. Provide a possible explanation.

15. Human Migrations II DNA (haplotypes) originating from the Denisovans can be found in the genomes of Indigenous Australians and Melanesian Islanders. However, the same DNA markers are not found in the genomes of people native to Africa. Explain.

16. Finding Disease Genes You are a gene hunter, trying to find the genetic basis for a rare inherited disease. Examination of six pedigrees of families affected by the disease provides inconsistent results. For two of the families, the disease is co-inherited with markers on chromosome 7. For the other four families, the disease is co-inherited with markers on chromosome 12. Explain how this difference might have arisen.

17. RT-PCR Primer Design Investigators can use sequences of transcribed mRNA as a PCR template to produce a corresponding DNA sequence. Reverse transcriptase, an enzyme that works like DNA polymerase, amplifies the mRNA template as DNA in the first PCR cycle. After making the DNA strands from the RNA template, the investigator can carry out the remaining cycles with DNA polymerase, using standard PCR protocols. She can then compare the detected amplified sequences to the genome to analyze transcriptional activity. Thus, reverse transcriptase PCR (RT-PCR) is a powerful experimental technique used to detect RNA from living cells, which transcribe their DNA into RNA, as opposed to dead tissues, which do not. Consider the mRNA transcript shown.

5′–AUAUCGCUCCACGUAACUGAAAGAAAAGUGUGGAGCUAGCAGUCGAGA–3′

Which DNA oligonucleotide pair could serve as a suitable primer in an RT-PCR amplification of this transcript? The oligonucleotides are written in the 5′ to 3′ direction.

(a) Primer 1: GGAGACCTTGACT; Primer 2: AGTCAAGGTCTCC
(b) Primer 1: GACTGCTAGCTCC; Primer 2: GTTACGTGGAGCG
(c) Primer 1: GCCGCGCGCGCGC; Primer 2: CCCCGCCGCGCCG
(d) Primer 1: CACGATTCAACGTG; Primer 2: TTCGCATTGCCGAA

DATA ANALYSIS PROBLEM

18. HincII: The First Restriction Endonuclease Discovery of the first restriction endonuclease to be of practical use was reported in two papers published in 1970. In the first paper, Smith and Wilcox described the isolation of an enzyme that cleaved double-stranded DNA. They initially demonstrated the enzyme's nuclease activity by measuring the decrease in viscosity of DNA samples treated with the enzyme.

(a) Why does treatment with a nuclease decrease the viscosity of a solution of DNA?

The authors determined whether the enzyme was an endonuclease or an exonuclease by treating ^{32}P-labeled DNA with the enzyme, then adding trichloroacetic acid (TCA). Under the conditions used in their experiment, single nucleotides would be TCA-soluble and oligonucleotides would precipitate.

(b) No TCA-soluble ^{32}P-labeled material formed upon treatment of the ^{32}P-labeled DNA with the nuclease. Based on this finding, is the enzyme an endonuclease or is it an exonuclease? Explain your reasoning.

When a polynucleotide is cleaved, the phosphate usually is not removed but remains attached to the 5′ or 3′ end of the resulting DNA fragment. Smith and Wilcox determined the location of the phosphate on the fragment formed by the nuclease in three steps:

1. Treat unlabeled DNA with the nuclease.
2. Treat a sample (A) of the product with γ-^{32}P-labeled ATP and polynucleotide kinase (which can attach the γ-phosphate of ATP to a 5′ OH but not to a 5′ phosphate or to a 3′ OH or 3′ phosphate). Measure the amount of ^{32}P incorporated into the DNA.

3. Treat another sample (B) of the product of step 1 with alkaline phosphatase (which removes phosphate groups from free 5′ and 3′ ends), followed by polynucleotide kinase and γ-^{32}P-labeled ATP. Measure the amount of ^{32}P incorporated into the DNA.

(c) Smith and Wilcox found that sample A had 136 counts/min of ^{32}P; sample B had 3,740 counts/min. Did the nuclease cleavage leave the phosphate on the 5′ end or the 3′ end of the DNA fragments? Explain your reasoning.

(d) Treatment of bacteriophage T7 DNA with the nuclease gave approximately 40 specific fragments of various lengths. How is this result consistent with the enzyme's recognizing a specific sequence in the DNA as opposed to making random double-strand breaks?

At this point, there were two possibilities for the site-specific cleavage: the cleavage occurred either (1) at the site of recognition or (2) near the site of recognition but not within the sequence recognized. To address this issue, Kelly and Smith determined the sequence of the 5′ ends of the DNA fragments generated by the nuclease, in five steps:

1. Treat phage T7 DNA with the enzyme.
2. Treat the resulting fragments with alkaline phosphatase to remove the 5′ phosphates.
3. Treat the dephosphorylated fragments with polynucleotide kinase and γ-^{32}P-labeled ATP to label the 5′ ends.
4. Treat the labeled molecules with DNases to break them into a mixture of mono-, di-, and trinucleotides.
5. Determine the sequence of the labeled mono-, di-, and trinucleotides by comparing them with oligonucleotides of known sequence on thin-layer chromatography.

The labeled products were identified as follows: mononucleotides—A and G; dinucleotides—(5′)ApA(3′) and (5′)GpA(3′); trinucleotides—(5′)ApApC(3′) and (5′)GpApC(3′).

(e) Which model of cleavage is consistent with these results? Explain your reasoning.

Kelly and Smith went on to determine the sequence of the 3′ ends of the fragments. They found a mixture of (5′)TpC(3′) and (5′)TpT(3′). They did not determine the sequence of any trinucleotides at the 3′ end.

(f) Based on these data, what is the recognition sequence for the nuclease, and where in the sequence is the DNA backbone cleaved? Use Table 9-2 as a model for your answer.

References

Kelly, T. J., and H. O. Smith. 1970. A restriction enzyme from *Haemophilus influenzae*: II. Base sequence of the recognition site. *J. Mol. Biol.* 51:393–409.

Smith, H. O., and K. W. Wilcox. 1970. A restriction enzyme from *Haemophilus influenzae*: I. Purification and general properties. *J. Mol. Biol.* 51:379–391.

Chapter 10

LIPIDS

10.1 Storage Lipids *341*

10.2 Structural Lipids in Membranes *346*

10.3 Lipids as Signals, Cofactors, and Pigments *354*

10.4 Working with Lipids *361*

Biological lipids are a chemically diverse group of compounds, the common and defining feature of which is their insolubility in water. The biological functions of the lipids are as diverse as their chemistry. Fats and oils are the principal stored forms of energy in many organisms. Phospholipids and sterols are major structural elements of biological membranes. Other lipids, although present in relatively small quantities, play crucial roles as enzyme cofactors, electron carriers, light-absorbing pigments, hydrophobic anchors for proteins, "chaperones" to help membrane proteins fold, emulsifying agents in the digestive tract, hormones, and intracellular messengers. This chapter introduces representative lipids of each type, organized according to their functional roles, with emphasis on their chemical structure and physical properties. Although we follow a functional organization for our discussion, the thousands of different lipids can also be organized into eight general categories of chemical structure (listed in Table 10-2, at the end of this chapter). We discuss the energy-yielding oxidation of lipids in Chapter 17 and their synthesis in Chapter 21. Here, we will focus on four principles of cellular lipid function:

P1 **Fatty acids are water-insoluble hydrocarbons used for cellular energy storage.** Fatty acids are highly reduced and thus provide a rich source of stored chemical energy for cells. Storage of hydrophobic fats as triacylglycerols is also highly efficient because water is not needed to hydrate the stored fats.

P2 **Membrane lipids are composed of hydrophobic tails attached to polar head groups.** Cellular membranes are composed of a variety of lipids, including glycerophospholipids and sterols. These lipids are used for structuring membranes as well as for displaying molecules on the membrane surfaces for signaling and molecular recognition.

P3 **Lipids have uses in the cell beyond energy storage and construction of membranes.** Many lipids are present in the cell at smaller amounts than those making up membranes or being stored as fat. These lipids can function as cellular messengers, hormones, electron carriers, or pigments.

P4 **The chemical properties of lipids are related to their structure and composition.** As in studies of other biomolecules, methods such as enzymatic, chromatographic, and mass spectrometry can all be used to identify lipids and determine their atomic structure.

10.1 Storage Lipids

P1 The fats and oils almost universally used as stored forms of energy in living organisms are derivatives of **fatty acids**. The fatty acids are hydrocarbon derivatives, at about the same low oxidation state (that is, as highly reduced) as the hydrocarbons in fossil fuels. The cellular oxidation of fatty acids (to CO_2 and H_2O), like the controlled, rapid burning of fossil fuels in internal combustion engines, is highly exergonic.

We introduce here the structures and nomenclature of the fatty acids most commonly found in living organisms. We describe two types of fatty acid–containing compounds, triacylglycerols and waxes, to illustrate the diversity of structures and physical properties in this family of compounds.

Fatty Acids Are Hydrocarbon Derivatives

Fatty acids are carboxylic acids with hydrocarbon chains ranging from 4 to 36 carbons long (C_4 to C_{36}). In some fatty acids, this chain is unbranched and fully saturated (contains no double bonds); in others, the chain contains one or more double bonds (**Table 10-1**). A few contain three-carbon rings, hydroxyl groups, or methyl-group branches.

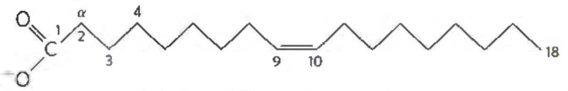
TABLE 10-1 Some Naturally Occurring Fatty Acids: Structure, Properties, and Nomenclature

Carbon skeleton	Structure[a]	Systematic name[b]	Common name (derivation)	Melting point (°C)	Solubility at 30 °C (mg/g solvent)	
					Water	Benzene
12:0	$CH_3(CH_2)_{10}COOH$	*n*-Dodecanoic acid	Lauric acid (Latin *laurus*, "laurel plant")	44.2	0.063	2,600
14:0	$CH_3(CH_2)_{12}COOH$	*n*-Tetradecanoic acid	Myristic acid (Latin *Myristica*, nutmeg genus)	53.9	0.024	874
16:0	$CH_3(CH_2)_{14}COOH$	*n*-Hexadecanoic acid	Palmitic acid (Latin *palma* "palm tree")	63.1	0.0083	348
18:0	$CH_3(CH_2)_{16}COOH$	*n*-Octadecanoic acid	Stearic acid (Greek *stear*, "hard fat")	69.6	0.0034	124
20:0	$CH_3(CH_2)_{18}COOH$	*n*-Eicosanoic acid	Arachidic acid (Latin *Arachis*, legume genus)	76.5		
24:0	$CH_3(CH_2)_{22}COOH$	*n*-Tetracosanoic acid	Lignoceric acid (Latin *lignum*, "wood" + *cera*, "wax")	86.0		
16:1(Δ^9)	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$	*cis*-9-Hexadecenoic acid	Palmitoleic acid	1 to –0.5		
18:1(Δ^9)	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	*cis*-9-Octadecenoic acid	Oleic acid (Latin *oleum*, "oil")	13.4		
18:2($\Delta^{9,12}$)	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	*cis*-,*cis*-9,12-Octadecadienoic acid	Linoleic acid (Greek *linon*, "flax")	1–5		
18:3($\Delta^{9,12,15}$)	$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	*cis*-,*cis*-,*cis*-9,12,15-Octadecatrienoic acid	α-Linolenic acid	–11		
20:4($\Delta^{5,8,11,14}$)	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$	*cis*-,*cis*-,*cis*-,*cis*-5,8,11,14-Icosatetraenoic acid	Arachidonic acid	–49.5		

[a] All acids are shown in their nonionized form. At pH 7, all free fatty acids have an ionized carboxylate. Note that numbering of carbon atoms begins at the carboxyl carbon.

[b] The prefix *n*- indicates the "normal" unbranched structure. For instance, "dodecanoic" simply indicates 12 carbon atoms, which could be arranged in a variety of branched forms; "*n*-dodecanic" specifies the linear unbranched form. For unsaturated fatty acids, the configuration of each double bond is indicated; in biological fatty acids the configuration is almost always cis.

KEY CONVENTION A simplified nomenclature for unbranched fatty acids specifies the chain length and number of double bonds, separated by a colon. For example, the 16-carbon saturated palmitic acid is abbreviated 16:0, and the 18-carbon oleic (octadecenoic) acid, with one double bond (shown below), is 18:1. Each line segment of the zigzag in the structure represents a single bond between adjacent carbons. The carboxyl carbon is assigned the number 1 (C-1), and the α carbon next to it is C-2. The positions of any double bonds, designated Δ (delta), are specified relative to C-1 by a superscript number indicating the lower-numbered carbon in the double bond. By this convention, oleic acid, with a double bond between C-9 and C-10, is designated 18:1(Δ^9); a 20-carbon fatty acid with one double bond between C-9 and C-10 and another between C-12 and C-13 is designated 20:2($\Delta^{9,12}$).

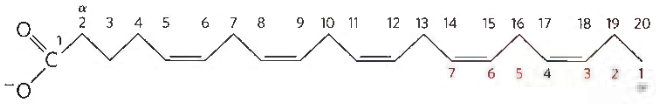


18:1(Δ^9) *cis*-9-Octadecenoic acid ■

The most commonly occurring fatty acids have even numbers of carbon atoms in an unbranched chain of 12 to 24 carbons (Table 10-1). As we shall see in Chapter 21, the even number of carbons results from the mode of synthesis of these compounds, which involves successive condensations of two-carbon acetyl units.

There is also a common pattern in the location of double bonds; in most monounsaturated fatty acids the double bond is between C-9 and C-10 (Δ^9), and the other double bonds of polyunsaturated fatty acids are generally Δ^{12} and Δ^{15}. (Arachidonic acid is an exception to this generalization; see Table 10-1.) The double bonds of polyunsaturated fatty acids are almost never conjugated (alternating single and double bonds, as in $—CH{=}CH—CH{=}CH—$), but are separated by a methylene group: $—CH{=}CH—CH_2—CH{=}CH—$. In nearly all naturally occurring unsaturated fatty acids, the double bonds are in the cis configuration. Notable exceptions include the trans fatty acids produced by fermentation in the rumen of dairy animals. Humans can ingest these trans fats from dairy products and meat.

KEY CONVENTION The family of **polyunsaturated fatty acids (PUFAs)** with a double bond between the third carbon and the fourth carbon from the methyl end of the chain are of special importance in human nutrition. Because the physiological role of PUFAs is related more to the position of the first double bond near the *methyl* end of the chain than to that near the carboxyl end, an alternative nomenclature is sometimes used for these fatty acids. The carbon of the methyl group—that is, the carbon most distant from the carboxyl group—is called the ω (omega; the last letter in the Greek alphabet) carbon and is given the number 1 (C-1); the carboxyl carbon in this convention has the highest number. The positions of the double bonds are indicated relative to the ω carbon. In this convention, PUFAs with a double bond between C-3 and C-4 are called **omega-3 (ω-3) fatty acids**, and those with a double bond between C-6 and C-7 are **omega-6 (ω-6) fatty acids**. Shown below is eicosapentaenoic acid, which can be designated as $20{:}5(\Delta^{5,8,11,14,17})$ by the standard nomenclature but is also referred to as an omega-3 fatty acid, emphasizing the biologically important double bond in the omega-3 position.



$20{:}5(\Delta^{5,8,11,14,17})$ Eicosapentaenoic acid (EPA) ■

Humans require the omega-3 PUFA α-linolenic acid (ALA; $18{:}3(\Delta^{9,12,15})$, in the standard convention), but do not have the enzymatic capacity to synthesize it and must therefore obtain ALA from the diet. Humans also use ALA to synthesize two other omega-3 PUFAs important in cellular function: eicosapentaenoic acid (EPA; $20{:}5(\Delta^{5,8,11,14,17})$, shown in the Key Convention above) and docosahexaenoic acid (DHA; $22{:}6(\Delta^{4,7,10,13,16,19})$). An imbalance of omega-6 and omega-3 PUFAs in the diet is associated with an increased risk of cardiovascular disease. The optimal dietary ratio of omega-6 to omega-3 PUFAs is between 1:1 and 4:1, but the ratio in the diets of most North Americans is closer to 10:1 to 30:1. The "Mediterranean diet," which has been associated with lowered cardiovascular risk, is richer in omega-3 PUFAs, obtained in leafy vegetables (salads) and fish oils. Fish oils especially rich in EPA and DHA are a common dietary supplement even though their exact role in preventing cardiovascular disease is controversial. ■

P1 The physical properties of the fatty acids, and of compounds that contain them, are largely determined by the length and degree of unsaturation of the hydrocarbon chain. The nonpolar hydrocarbon chain accounts for the poor solubility of fatty acids in water. Lauric acid (12:0, M_r 200), for example, has a solubility in water of 0.063 mg/g—much less than that of glucose (M_r 180), which is 1,100 mg/g. The longer the fatty acyl chain and the fewer the double bonds, the lower the solubility in water. The carboxylic acid group is polar (and ionized at neutral pH) and accounts for the slight solubility of short-chain fatty acids in water.

Melting points are also strongly influenced by the length and degree of unsaturation of the hydrocarbon chain. At room temperature (25 °C), the saturated fatty acids from 12:0 to 24:0 have a waxy consistency, whereas unsaturated fatty acids of these lengths are oily liquids. This difference in melting points is due to different degrees of packing of the fatty acid molecules (**Fig. 10-1**). In the fully saturated compounds, free rotation around each carbon–carbon bond gives the hydrocarbon chain great flexibility; the most stable conformation is the fully extended form, in which the steric hindrance of neighboring atoms is minimized. These molecules can pack together tightly in nearly crystalline arrays, with atoms along their lengths in van der Waals contact with the atoms of neighboring molecules. In unsaturated fatty acids, a cis double bond forces a kink in the hydrocarbon chain. Fatty acids with one or several such kinks cannot pack together as tightly as fully saturated fatty acids, and their interactions with each other are therefore weaker. Because less thermal energy is needed to disorder these poorly ordered arrays of unsaturated fatty acids, they have markedly lower melting points than saturated fatty acids of the same chain length (Table 10-1).

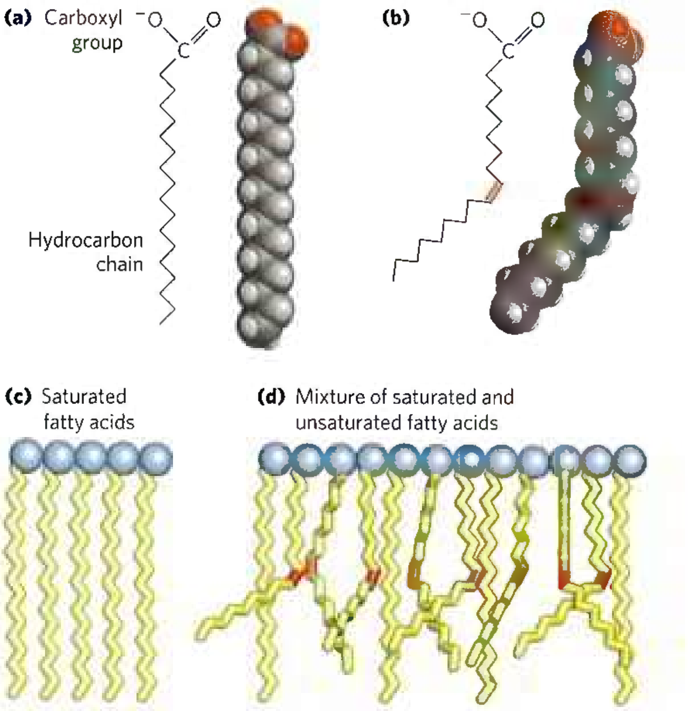

(a) Carboxyl group
⁻O
O
C
Hydrocarbon chain
(b)
⁻O
O
C
(c) Saturated fatty acids
(d) Mixture of saturated and unsaturated fatty acids

FIGURE 10-1 The packing of fatty acids into stable aggregates. The extent of packing depends on the degree of saturation. (a) Two representations of the fully saturated acid stearic acid, 18:0 (stearate at pH 7), in its usual extended conformation. (b) The cis double bond (red) in oleic acid, $18{:}1(\Delta^9)$ (oleate), restricts rotation and introduces a rigid bend in the hydrocarbon tail. All other bonds in the chain are free to rotate. (c) Fully saturated fatty acids in the extended form pack into nearly crystalline arrays, stabilized by extensive hydrophobic interaction. (d) The presence of one or more fatty acids with cis double bonds (red) interferes with this tight packing and results in less stable aggregates.

In vertebrates, free fatty acids (unesterified fatty acids, with a free carboxylate group) can circulate in the blood bound noncovalently to carrier proteins such as serum albumin. However, fatty acids are present in blood plasma mostly as carboxylic acid derivatives such as esters or amides. Lacking the charged carboxylate group, these fatty acid derivatives are generally even less soluble in water than are the free fatty acids and are transported through the blood primarily as lipoprotein particles (see Chapter 21).

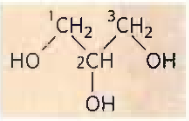
Triacylglycerols Are Fatty Acid Esters of Glycerol

The simplest lipids constructed from fatty acids are the **triacylglycerols**, also referred to as triglycerides, fats, or neutral fats. Triacylglycerols are composed of three fatty acids, each in ester linkage with a single glycerol (**Fig. 10-2**). Those containing the same kind of fatty acid in all three positions are called simple triacylglycerols and are named after the fatty acid they contain. Simple triacylglycerols of 16:0, 18:0, and 18:1, for example, are tripalmitin, tristearin, and triolein, respectively. Most naturally occurring triacylglycerols are mixed; they contain two or three different fatty acids. To name these compounds unambiguously, the name and position of each fatty acid must be specified.

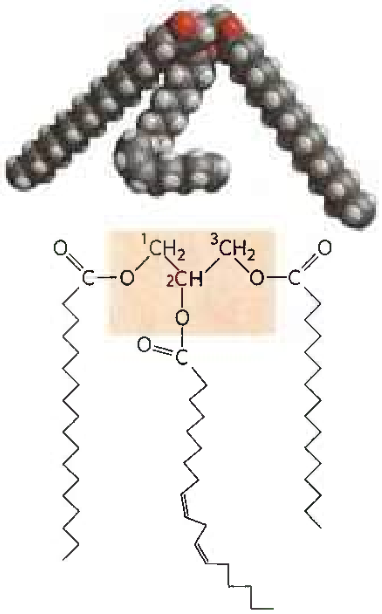
Because the polar hydroxyls of glycerol and the polar carboxylates of the fatty acids are bound in ester linkages, triacylglycerols are nonpolar, hydrophobic molecules, essentially insoluble in water. Lipids have lower specific gravities than water, which explains why mixtures of oil and water (oil-and-vinegar salad dressing, for example) have two phases: oil, with the lower specific gravity, floats on the aqueous phase.

Triacylglycerols Provide Stored Energy and Insulation

In most eukaryotic cells, triacylglycerols form a separate phase of microscopic, oily droplets in the aqueous cytosol, serving as depots of metabolic fuel. In vertebrates, specialized cells called adipocytes, or fat cells, store large amounts of triacylglycerols as fat droplets that nearly fill the cell (**Fig. 10-3a**). Triacylglycerols are also stored as oils in the seeds of many types of plants, providing energy and biosynthetic precursors during seed germination



Glycerol



1-Stearoyl, 2-linoleoyl, 3-palmitoyl glycerol, a mixed triacylglycerol

FIGURE 10-2 **Glycerol and a triacylglycerol.** The mixed triacylglycerol shown here has three different fatty acids attached to the glycerol backbone. When glycerol has different fatty acids at C-1 and C-3, C-2 is a chiral center.

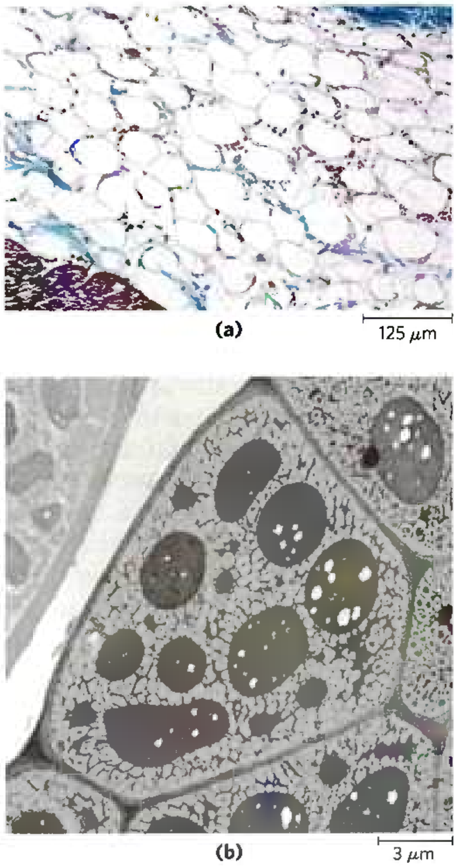


FIGURE 10-3 **Fat stores in cells.** (a) Cross section of human white adipose tissue. Each cell contains a fat droplet (white) so large that it squeezes the nucleus (stained red) against the plasma membrane. (b) Cross section of a cotyledon cell from a seed of the plant *Arabidopsis*. The large dark structures are protein bodies, which are surrounded by stored oils in the light-colored oil bodies. [(a) Biophoto Associates/Science Source. (b) Courtesy Howard Goodman, Department of Genetics, Harvard Medical School.]

(Fig. 10-3b). Adipocytes and germinating seeds contain **lipases**, enzymes that catalyze the hydrolysis of stored triacylglycerols, releasing fatty acids for export to sites where they are required as fuel.

P1 There are two significant advantages to using triacylglycerols as stored fuels, rather than polysaccharides such as glycogen and starch. First, the carbon atoms of fatty acids are more reduced than those of sugars, so oxidation of triacylglycerols yields more than twice as much energy, gram for gram, as the oxidation of carbohydrates. Second, because triacylglycerols are hydrophobic and therefore unhydrated, the organism that carries stored fuel in the form of fat does not have to carry the extra weight of water of hydration that is associated with stored polysaccharides (2 g per gram of polysaccharide). Humans have fat tissue (composed primarily of adipocytes) under the skin, in the abdominal cavity, and in the mammary glands. Moderately obese people with 15 to 20 kg of triacylglycerols deposited in their adipocytes could meet their energy needs for months by drawing on their fat stores. In contrast, the human body can store less than a day's energy supply in the form of glycogen. Carbohydrates such as glucose do offer certain advantages as quick sources of metabolic energy; one of those advantages is their ready solubility in water.

In some animals, triacylglycerols stored under the skin serve not only as energy stores but also as insulation against low temperatures. Seals, walruses, penguins, and other warm-blooded polar animals are amply padded with triacylglycerols. In hibernating animals (bears, for example), the huge fat reserves accumulated before hibernation serve the dual purposes of insulation and energy storage (see Box 17-1).

Partial Hydrogenation of Cooking Oils Improves Their Stability but Creates Fatty Acids with Harmful Health Effects

Most natural fats, such as those in vegetable oils, dairy products, and animal fat, are complex mixtures of simple and mixed triacylglycerols. These contain a variety of fatty acids differing in chain length and degree of saturation (**Fig. 10-4**). Vegetable oils such as corn (maize) oil and olive oil are composed largely of triacylglycerols with unsaturated fatty acids and thus are liquids at room temperature. Triacylglycerols containing only saturated fatty acids, such as tristearin, the major component of beef fat, are white, greasy solids at room temperature.

When lipid-rich foods are exposed too long to the oxygen in air, they may spoil and become rancid. The unpleasant taste and smell associated with rancidity result from the oxidative cleavage of double bonds in unsaturated fatty acids, which produces aldehydes and carboxylic acids of shorter chain length and therefore higher volatility; these compounds pass readily through the air to your nose. Throughout the twentieth century, partial hydrogenation of commercial vegetable oils was used to improve shelf life and to increase the stability of cooking oils at the high temperatures used in deep-frying. This process converts many of the cis double bonds in the fatty acids to single bonds and increases the melting temperature of the oils so that they are more nearly solid at room temperature (margarine is produced from vegetable oil in this way). Partial hydrogenation, however, has another, undesirable, effect: some cis double bonds are converted to trans double bonds. There is now strong evidence that dietary intake of trans fatty acids (often referred to simply as "trans fats") leads to a higher incidence of cardiovascular disease, and that avoiding these fats in the diet substantially reduces the risk of coronary heart disease. Dietary trans fatty acids raise the level of triacylglycerols and of LDL ("bad") cholesterol in the blood, and lower the level of HDL ("good") cholesterol, and these changes alone are enough to increase the risk of coronary heart disease. But trans fatty acids may have further adverse effects. They seem, for example, to increase the body's inflammatory response, which is another risk factor for heart disease. (See Chapter 21 for descriptions of LDL and HDL — low-density and high-density lipoprotein — cholesterol and their health effects.) Regulatory agencies around the world now limit or ban the use of trans fatty acids in prepared and packaged foods. ■

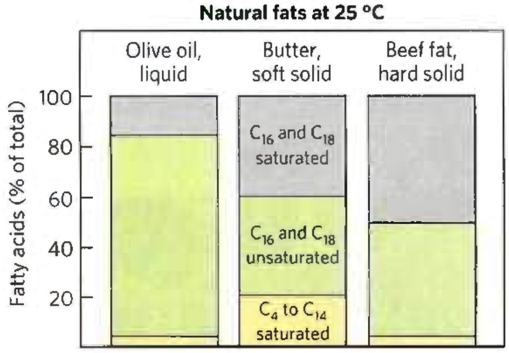
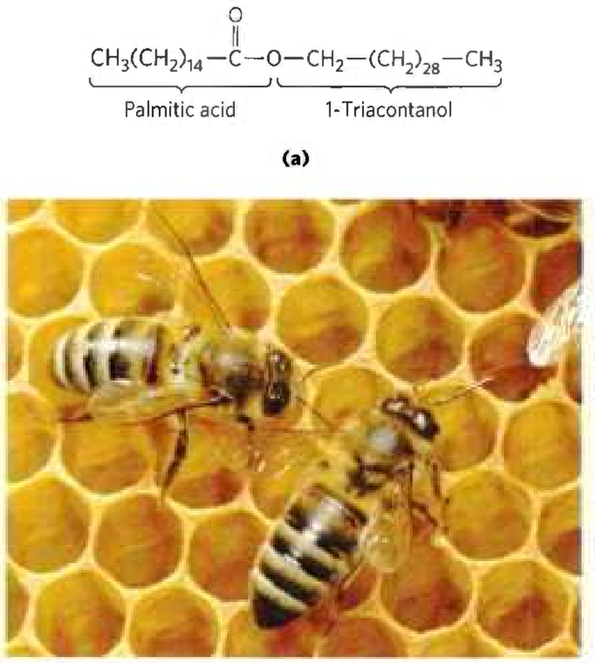


FIGURE 10-4 Fatty acid composition of three food fats. Olive oil, butter, and beef fat consist of mixtures of triacylglycerols differing in their fatty acid composition. The melting points of these fats — and hence their physical state at room temperature (25 °C) — are a direct function of their fatty acid composition. Olive oil has a high proportion of long-chain (C_{16} and C_{18}) unsaturated fatty acids, which accounts for its liquid state at 25 °C. The higher proportion of long-chain (C_{16} and C_{18}) saturated fatty acids in butter increases its melting point, so butter is a soft solid at room temperature. Beef fat, with an even higher proportion of long-chain saturated fatty acids, is a hard solid.

Waxes Serve as Energy Stores and Water Repellents

Biological waxes are esters of long-chain (C_{14} to C_{36}) saturated and unsaturated fatty acids with long-chain (C_{16} to C_{30}) alcohols (**Fig. 10-5**). Their melting points (60 to



$$\underbrace{CH_3(CH_2)_{14}-\overset{\overset{\displaystyle O}{\|}}{C}-}_{\text{Palmitic acid}}\underbrace{O-CH_2-(CH_2)_{28}-CH_3}_{\text{1-Triacontanol}}$$

FIGURE 10-5 Biological wax. (a) Triacontanoylpalmitate, the major component of beeswax, is an ester of palmitic acid with the alcohol triacontanol. (b) The beeswax of a honeycomb is firm at 25 °C and completely impervious to water. The term "wax" originates in the Old English *weax*, meaning "the material of the honeycomb." [(b) Irochka_T/Getty Images]

100 °C) are generally higher than those of triacylglycerols. In plankton, the free-floating microorganisms at the bottom of the food chain for marine animals, waxes are the chief storage form of metabolic fuel.

Waxes also serve a diversity of other functions related to their water-repellent properties and their firm consistency. Certain skin glands of vertebrates secrete waxes to protect hair and skin and keep it pliable, lubricated, and waterproof. Birds, particularly waterfowl, secrete waxes from preen glands to keep their feathers water-repellent. The shiny leaves of holly, rhododendrons, poison ivy, and many tropical plants are coated with a thick layer of waxes, which prevents excessive evaporation of water and protects against parasites.

Biological waxes find a variety of applications in the pharmaceutical, cosmetic, and other industries. Lanolin (from lamb's wool), beeswax (Fig. 10-5), carnauba wax (from a Brazilian palm tree), and wax extracted from the seeds of the jojoba bush are widely used in the manufacture of lotions, ointments, and polishes.

SUMMARY 10.1 *Storage Lipids*

- Lipids are water-insoluble cellular components of diverse structure that can be extracted from tissues by nonpolar solvents. Almost all fatty acids, the hydrocarbon components of many lipids, have an even number of carbon atoms (usually 12 to 24); they are either saturated or unsaturated, with double bonds almost always in the cis configuration.
- Triacylglycerols contain three fatty acid molecules esterified to the three hydroxyl groups of glycerol. Simple triacylglycerols contain only one type of fatty acid; mixed triacylglycerols contain two or three types.
- Triacylglycerols are present in many foods and are primarily used for storing energy and providing insulation in animals. Lipases release fatty acids from storage so they can be used as fuel.
- Since many natural fats can easily turn rancid, partial hydrogenation has been used to extend their shelf life. This process can produce trans fatty acids, which increase risk for coronary heart disease. As a result, their use in prepared and processed foods has become highly regulated.
- Waxes are esters of long-chain fatty acids and long-chain alcohols with diverse uses in biology and industry.

10.2 Structural Lipids in Membranes

The central architectural feature of biological membranes is a double layer of lipids, which acts as a barrier to the passage of polar molecules and ions. P2 Membrane lipids are amphipathic: one end of the molecule is hydrophobic, the other hydrophilic. The association of their hydrophobic regions with each other and their hydrophilic interactions with water direct their packing into sheets called membrane bilayers. In this section, we describe four general types of membrane lipids: **phospholipids**, which have hydrophobic regions composed of two fatty acids joined to glycerol or sphingosine; **glycolipids**, which contain a simple sugar or a complex oligosaccharide at the polar ends; **archaeal tetraether lipids**, which have two very long alkyl chains ether-linked to glycerol at both ends; and **sterols**, compounds characterized by a rigid system of four fused hydrocarbon rings (**Fig. 10-6**). Within these groups of membrane lipids, enormous diversity results from various combinations of fatty acid "tails" and polar "heads." The arrangement of these lipids in membranes, and their structural and functional roles therein, are considered in the next chapter.

Glycerophospholipids Are Derivatives of Phosphatidic Acid

P2 **Glycerophospholipids**, also called phosphoglycerides, are membrane lipids in which two fatty acids are attached in ester linkage to the first and second carbons of glycerol, and a highly polar or charged group is attached through a phosphodiester linkage to the third carbon. Glycerol is prochiral; it has no asymmetric carbons, but attachment of phosphate at one

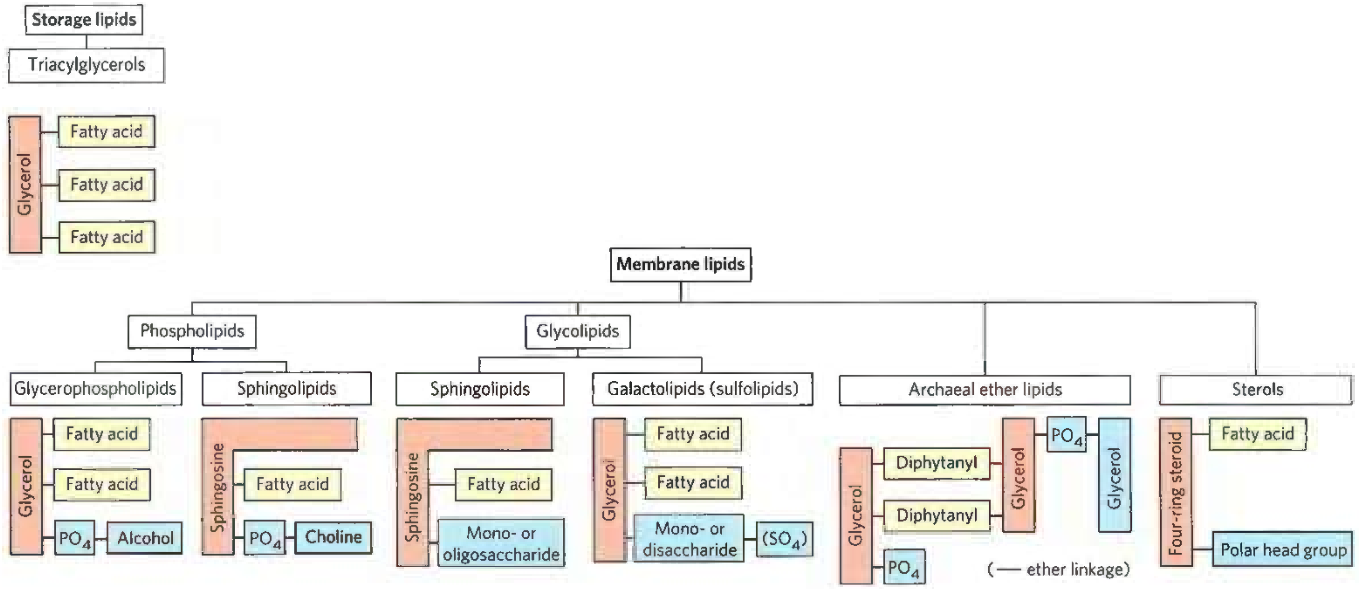
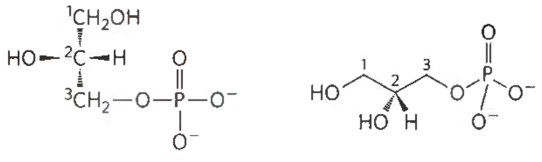

Storage lipids
Triacylglycerols
Glycerol
Fatty acid
Fatty acid
Fatty acid
Membrane lipids
Phospholipids
Glycolipids
Glycerophospholipids
Sphingolipids
Sphingolipids
Galactolipids (sulfolipids)
Archaeal ether lipids
Sterols
Glycerol
Fatty acid
Fatty acid
PO4
Alcohol
Sphingosine
Fatty acid
PO4
Choline
Sphingosine
Fatty acid
Mono- or oligosaccharide
Glycerol
Fatty acid
Fatty acid
Mono- or disaccharide
(SO4)
Glycerol
Diphytanyl
Diphytanyl
Glycerol
PO4
Glycerol
PO4
(— ether linkage)
Four-ring steroid
Fatty acid
Polar head group

FIGURE 10-6 Some common types of storage and membrane lipids. The triacylglycerol, phospholipid, glycolipid, and archaeal ether lipid types shown here have either glycerol or sphingosine as the backbone (light red screen), to which are attached one or more long-chain alkyl groups (yellow) and a polar head group (blue). Sterols have a core composed of four fused carbocylic rings called the steroid nucleus. In triacylglycerols, glycerophospholipids, galactolipids, and sulfolipids, the alkyl groups are fatty acids in ester linkage. Sphingolipids contain a single fatty acid, in amide linkage to the sphingosine backbone. The membrane lipids of archaea are variable; that shown here has two very long, branched alkyl chains, each end in ether linkage with a glycerol moiety. Sterols have many different types of alkyl chains that can be attached to the steroid D ring at C-17. In phospholipids, the polar head group is joined through a phosphodiester, whereas glycolipids have a direct glycosidic linkage between the head-group sugar and the backbone glycerol. Many sterols contain a polar head group such as a carbonyl or hydroxyl group attached at C-3 of the steroid A ring.

end converts it into a chiral compound, which can be correctly named either L-glycerol 3-phosphate, D-glycerol 1-phosphate, or *sn*-glycerol 3-phosphate (**Fig. 10-7**). Glycerophospholipids are named as derivatives of the parent compound, phosphatidic acid (**Fig. 10-8**), according to the polar alcohol in the head group. Phosphatidylcholine and phosphatidylethanolamine have choline and ethanolamine as their polar head groups, for example. Cardiolipin is a two-tailed glycerophospholipid in which two phosphatidic acid moieties share the same glycerol as their head group (Fig. 10-8). Cardiolipin is found in most bacterial membranes; in eukaryotic cells, cardiolipin is located almost exclusively in the inner mitochondrial membrane (where it is synthesized), a location consistent with the endosymbiosis hypothesis for the origin of these organelles (see Fig. 1-37).

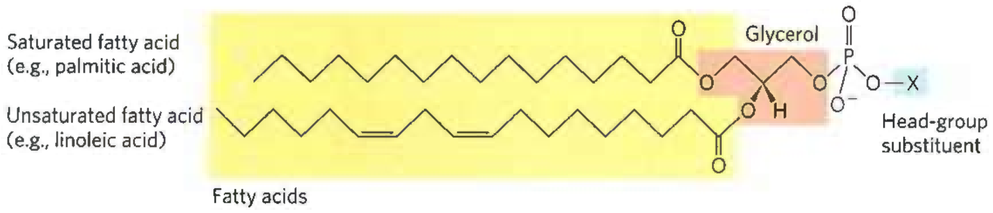
In all glycerophospholipids, the head group is joined to glycerol through a phosphodiester bond, in which the phosphate group bears a negative charge at neutral pH. The polar alcohol may be negatively charged (as in phosphatidylinositol 4,5-bisphosphate), neutral (phosphatidylserine), or positively charged (phosphatidylcholine, phosphatidylethanolamine). As we shall see in Chapter 11, these charges contribute greatly to the surface properties of membranes.



L-Glycerol 3-phosphate (*sn*-glycerol 3-phosphate)

FIGURE 10-7 L-Glycerol 3-phosphate, the backbone of phospholipids. Glycerol itself is not chiral, as it has a plane of symmetry through C-2. However, glycerol is prochiral — it can be converted to a chiral compound by adding a substituent such as phosphate to either of the —CH_2OH groups. One unambiguous nomenclature for glycerol phosphate is the D, L system (described on p. 73), in which the isomers are named according to their stereochemical relationships to glyceraldehyde isomers. By this system, the stereoisomer of glycerol phosphate found in most lipids is correctly named either L-glycerol 3-phosphate or D-glycerol 1-phosphate. Another way to specify stereoisomers is the *sn* (stereospecific numbering) system, in which C-1 is, by definition, the group of the prochiral compound that occupies the pro-S position. The common form of glycerol phosphate in phospholipids is, by this system, *sn*-glycerol 3-phosphate (in which C-2 has the R configuration). In archaea, the glycerol in lipids has the other configuration; it is D-glycerol 3-phosphate.

The fatty acids in glycerophospholipids can be any of a wide variety, so a given phospholipid (phosphatidylcholine, for example) may consist of several molecular species, each with its unique complement of fatty acids. The distribution of molecular species is specific to the organism, to the particular tissue within the organism, and to the particular glycerophospholipids in the same cell or tissue. In general, glycerophospholipids contain a C_{16} or C_{18} saturated fatty acid at C-1 and a C_{18} or C_{20} unsaturated fatty acid at C-2. With few exceptions, the



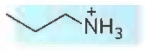
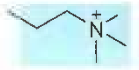
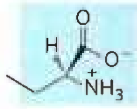
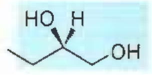
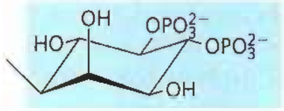
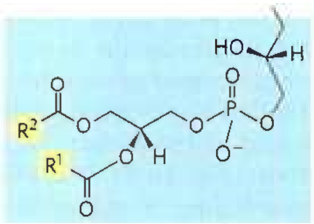
Name of glycerophospholipid	Head group	Formula of —X	Net charge (at pH 7)
Phosphatidic acid	—	—H	−2*
Phosphatidylethanolamine	Ethanolamine		0
Phosphatidylcholine	Choline		0
Phosphatidylserine	Serine		−1
Phosphatidylglycerol	Glycerol		−1
Phosphatidylinositol 4,5-bisphosphate	*myo*-Inositol 4,5-bisphosphate		−4*
Cardiolipin	Phosphatidyl-glycerol		−2

FIGURE 10-8 Glycerophospholipids. The common glycerophospholipids are diacylglycerols linked to head-group alcohols through a phosphodiester bond. Phosphatidic acid, a phosphomonoester, is the parent compound. Each derivative is named for the head-group alcohol, with the prefix "phosphatidyl-." In cardiolipin, two phosphatidic acids share a single glycerol (R^1 and R^2 are fatty acyl groups), resulting in a symmetric molecule. *Note that phosphate esters each have a charge of about −1.5; one of their —OH groups is only partially ionized at pH 7.

biological significance of the variation in fatty acids and head groups is not yet understood.

Some Glycerophospholipids Have Ether-Linked Fatty Acids

Some animal tissues and some unicellular organisms are rich in **ether lipids**, in which one of the two acyl chains is attached to glycerol in ether, rather than ester, linkage. The ether-linked chain may be saturated, as in the alkyl ether lipids, or it may contain a double bond between C-1 and C-2, as in **plasmalogens** (Fig. 10-9). Vertebrate heart tissue is uniquely enriched in ether lipids; about half of the heart phospholipids are plasmalogens. The membranes of halophilic bacteria, ciliated protists, and certain invertebrates also contain high proportions of ether lipids. The functional significance of ether lipids in these membranes is unknown; perhaps their resistance to the phospholipases that cleave ester-linked fatty acids from membrane lipids is important in some roles.

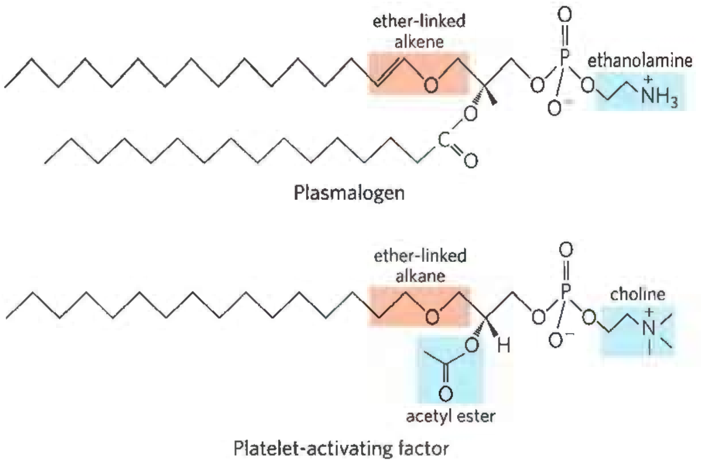


FIGURE 10-9 Ether lipids. Plasmalogens have an ether-linked alkenyl chain where most glycerophospholipids have an ester-linked fatty acid (compare to Fig. 10-8). Platelet-activating factor has a long ether-linked alkyl chain at C-1 of glycerol, but C-2 is ester-linked to acetic acid, which makes the compound much more water-soluble than most glycerophospholipids and plasmalogens. The head-group alcohol is ethanolamine in plasmalogens and choline in platelet-activating factor.

At least one ether lipid, **platelet-activating factor**, is a potent molecular signal. It is released from leukocytes called basophils and stimulates platelet aggregation and the release of serotonin (a vasoconstrictor) from platelets. It also exerts a variety of effects on liver, smooth muscle, heart, uterine, and lung tissues and plays an important role in inflammation and the allergic response.

Galactolipids of Plants and Ether-Linked Lipids of Archaea Are Environmental Adaptations

The second group of membrane lipids in Figure 10-6, the **glycolipids**, includes those that predominate in plant cells: the **galactolipids**, in which one or two galactose residues are connected by a glycosidic linkage to C-3 of a 1,2-diacylglycerol (**Fig. 10-10**). Galactolipids are localized in the thylakoid membranes (internal membranes) of chloroplasts; they make up 70% to 80% of the total membrane lipids of a vascular plant, and are therefore probably the most abundant membrane lipids in the biosphere.

Phosphate is often the limiting plant nutrient in soil, and perhaps the evolutionary pressure to conserve phosphate for more critical roles favored plants that made phosphate-free lipids. Plant membranes also contain sulfolipids, in which a sulfonated glucose residue is joined to a diacylglycerol in glycosidic linkage. The sulfonate group bears a negative charge like that of the phosphate group in phospholipids.

Some archaea that live in ecological niches with extreme conditions—for example, high temperatures (boiling water), low pH, or high ionic strength—have membrane lipids containing long-chain (32 carbons) branched hydrocarbons linked at each end to glycerol (Fig. 10-6). These linkages are through ether bonds, which are much more stable to hydrolysis at low pH and high temperature than are the ester bonds found in the lipids of bacteria and eukaryotes. In their fully extended form, these archaeal lipids are twice the length of phospholipids and sphingolipids, and can span the full width of the plasma membrane.

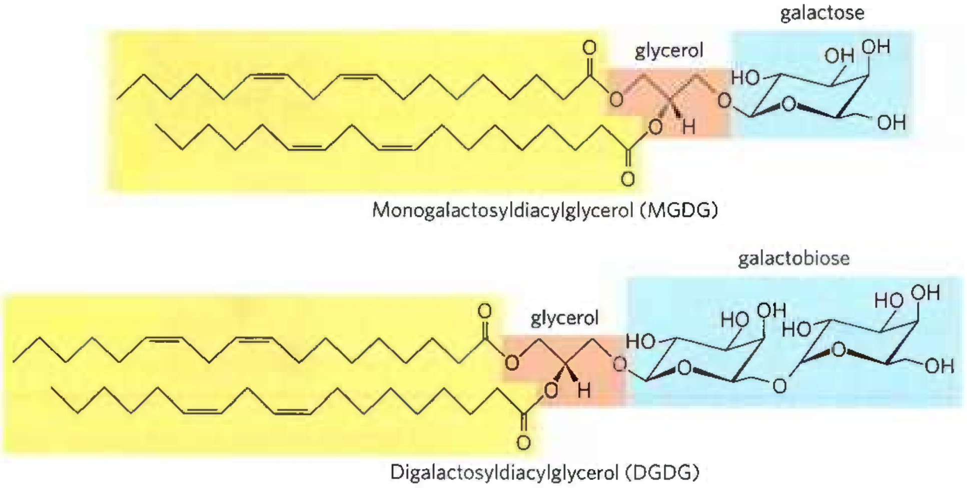

galactose
glycerol
Monogalactosyldiacylglycerol (MGDG)
galactobiose
glycerol
Digalactosyldiacylglycerol (DGDG)

FIGURE 10-10 Two galactolipids of chloroplast thylakoid membranes. In monogalactosyldiacylglycerols (MGDGs) and digalactosyldiacylglycerols (DGDGs), both acyl groups are polyunsaturated and the head groups are uncharged.

Sphingolipids Are Derivatives of Sphingosine

P2 **Sphingolipids**, a large class of membrane phospholipids and glycolipids, have a polar head group and two nonpolar tails, but unlike glycerophospholipids and galactolipids they contain no glycerol (Fig. 10-6). Sphingolipids are composed of one molecule of the long-chain amino alcohol sphingosine (also called 4-sphingenine) or one of its derivatives, one molecule of a long-chain fatty acid, and a polar head group that is joined by a glycosidic linkage in some cases and a phosphodiester in others (**Fig. 10-11**).

Carbons C-1, C-2, and C-3 of the sphingosine molecule are structurally analogous to the three carbons of glycerol in glycerophospholipids. When a fatty acid is attached in amide linkage to the —NH_2 on C-2, the resulting compound is a **ceramide**, which is structurally similar to a diacylglycerol. Ceramides are the structural parents of all sphingolipids.

There are three subclasses of sphingolipids, all derivatives of ceramide but differing in their head groups: sphingomyelins, neutral (uncharged) glycolipids, and gangliosides. **Sphingomyelins** contain phosphocholine or phosphoethanolamine as their polar head group and are therefore classified along with glycerophospholipids as phospholipids (Fig. 10-6). Indeed, sphingomyelins resemble phosphatidylcholines in their general properties and three-dimensional structure, and in having no net charge on their head groups (**Fig. 10-12**). Sphingomyelins are present in the plasma membranes of animal cells and are especially prominent in myelin, a membranous sheath that surrounds and insulates the axons of some neurons—thus the name "sphingomyelins."

Glycosphingolipids, which occur largely in the outer face of plasma membranes, have head groups with one or more sugars connected directly to the —OH at C-1 of the ceramide moiety; they do not contain phosphate. **Cerebrosides** have a single sugar linked to ceramide; those with galactose are characteristically found in the plasma membranes of cells in neural tissue, and those

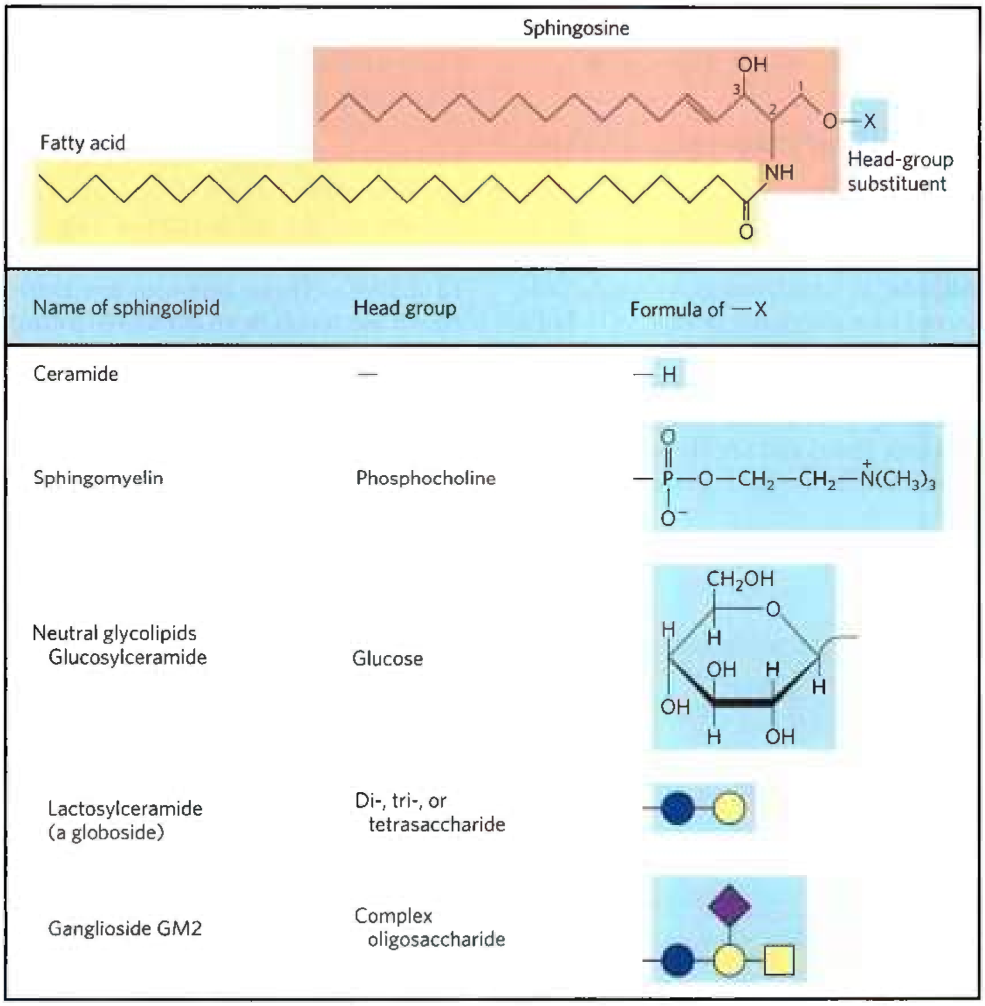

Sphingosine
OH
3
2
1
O—X
Fatty acid
NH
O
Head-group substituent
Name of sphingolipid
Head group
Formula of —X
Ceramide
—
—H
Sphingomyelin
Phosphocholine
$-\overset{O}{\overset{\|}{P}}-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3$ with O^- on P
Neutral glycolipids
Glucosylceramide
Glucose
CH_2OH
O
H
H
OH
H
H
OH
H
OH
Lactosylceramide
(a globoside)
Di-, tri-, or tetrasaccharide
Ganglioside GM2
Complex oligosaccharide

FIGURE 10-11 Sphingolipids. The first three carbons at the polar end of sphingosine are analogous to the three carbons of glycerol in glycerophospholipids. The amino group at C-2 bears a fatty acid in amide linkage. The fatty acid is usually saturated or monounsaturated, with 16, 18, 22, or 24 carbon atoms. Ceramide is the parent compound for this group. Other sphingolipids differ in the polar head group attached at C-1. Gangliosides have very complex oligosaccharide head groups. Standard symbols for sugars are used in this figure, as shown in Table 7-1.

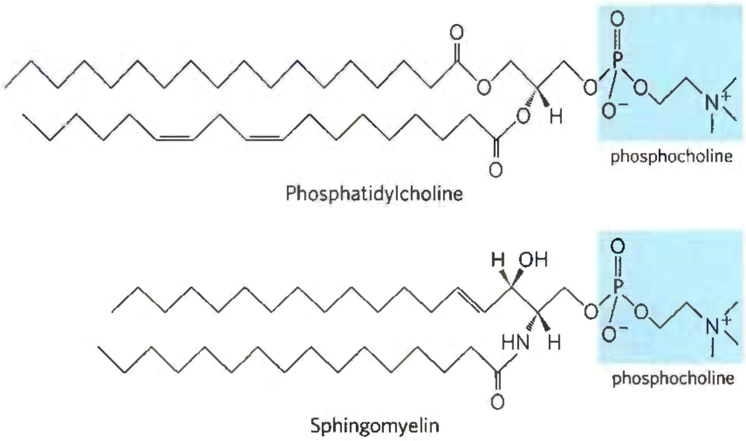
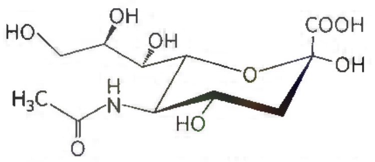


FIGURE 10-12 The similar molecular structures of two classes of membrane lipid. Phosphatidylcholine (a glycerophospholipid) and sphingomyelin (a sphingolipid) have similar dimensions and physical properties, but presumably play different roles in membranes.

with glucose are found in the plasma membranes of cells in nonneural tissues. **Globosides** are glycosphingolipids with two or more sugars, usually D-glucose, D-galactose, or *N*-acetyl-D-galactosamine. Cerebrosides and globosides are sometimes called **neutral glycolipids**, as they have no charge at pH 7.

Gangliosides, the most complex sphingolipids, have oligosaccharides as their polar head groups and one or more residues of *N*-acetylneuraminic acid (Neu5Ac), a sialic acid (often simply called "sialic acid"), at the termini. Deprotonated sialic acid gives gangliosides the negative charge at pH 7 that distinguishes them from globosides. Gangliosides with one sialic acid residue are in the GM (*M* for mono-) series, those with two sialic acid residues are in the GD (*D* for di-) series, and so on (GT, three sialic acid residues; GQ, four).



α-*N*-Acetylneuraminic acid (a sialic acid)
(Neu5Ac)

Sphingolipids at Cell Surfaces Are Sites of Biological Recognition

When sphingolipids were discovered more than a century ago by the physician-chemist Johann Thudichum, their biological role seemed as enigmatic as the Sphinx, for which he therefore named them. In humans, at least 60 different sphingolipids have been identified in cellular membranes. **P2** Many of these are especially prominent in the plasma membranes of neurons, and some are clearly recognition sites on the cell surface, but a specific function for only a few sphingolipids has been discovered thus far. The carbohydrate moieties of certain sphingolipids define the human blood groups and therefore determine the type of blood that individuals can safely receive in blood transfusions (**Fig. 10-13**).

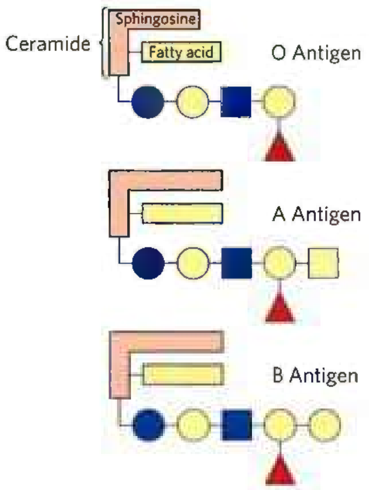


FIGURE 10-13 Glycosphingolipids as determinants of blood groups. The human blood groups (O, A, B) are determined in part by the oligosaccharide head groups of these glycosphingolipids. The same three oligosaccharides are also found attached to certain blood proteins of individuals of blood types O, A, and B, respectively. Standard symbols for sugars are used here (see Table 7-1).

Gangliosides are concentrated on the outer face of cell surface plasma membranes where they present points of recognition for extracellular molecules or the surfaces of neighboring cells. The kinds and amounts of gangliosides in the plasma membrane change dramatically during embryonic development. Tumor formation induces the synthesis of a new complement of gangliosides, and very low concentrations of a specific ganglioside have been found to induce differentiation of cultured neuronal tumor cells. Guillain-Barré syndrome is a serious autoimmune disorder in which the body makes antibodies against its own gangliosides, including those in neurons. The resulting inflammation damages the peripheral nervous system, leading to temporary (or sometimes permanent) paralysis. In cholera, cholera toxin produced by the intestinal bacterium *Vibrio cholerae* enters sensitive cells after attaching to specific gangliosides on the intestinal epithelial cell surface. Investigation of the biological roles of diverse gangliosides remains fertile ground for future research. ■

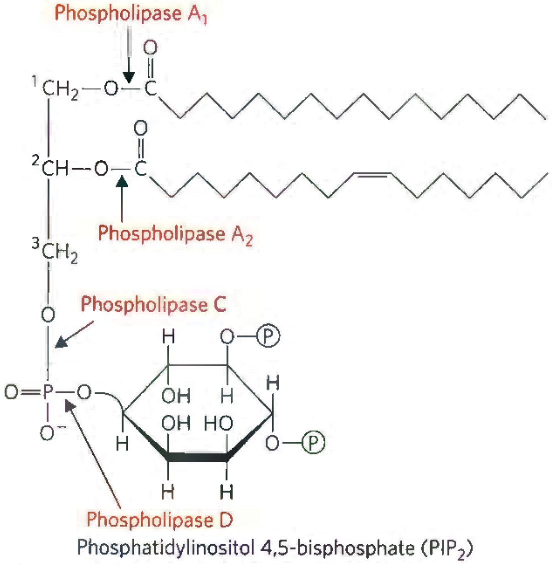


FIGURE 10-14 The specificities of phospholipases. Phospholipases A_1 and A_2 hydrolyze the ester bonds of intact glycerophospholipids at C-1 and C-2 of glycerol, respectively. When one of the fatty acids has been removed by a type A phospholipase, the second fatty acid is removed by a lysophospholipase (not shown). Phospholipases C and D each split one of the phosphodiester bonds in the head group. Some phospholipases act on only one type of glycerophospholipid, such as phosphatidylinositol 4,5-bisphosphate (PIP_2, shown here) or phosphatidylcholine; others are less specific.

Phospholipids and Sphingolipids Are Degraded in Lysosomes

Most cells continually degrade and replace their membrane lipids. For each hydrolyzable bond in a glycerophospholipid, there is a specific hydrolytic enzyme in the lysosome (**Fig. 10-14**). Phospholipases of the A type remove one of the two fatty acids, producing a lysophospholipid. (These esterases do not attack the ether link of plasmalogens.) Lysophospholipases remove the remaining fatty acid.

Gangliosides are degraded by a set of lysosomal enzymes that catalyze the stepwise removal of sugar units, finally yielding a ceramide. A genetic defect in any of these hydrolytic enzymes leads to the accumulation of gangliosides in the cell, with severe medical consequences (**Box 10-1**).

Sterols Have Four Fused Carbon Rings

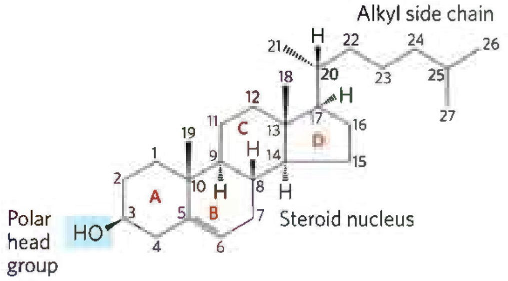
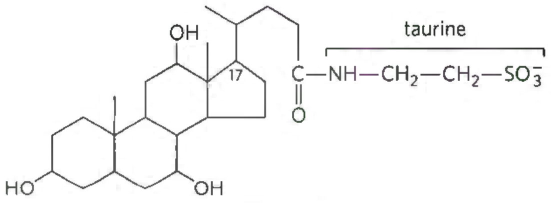


FIGURE 10-15 Cholesterol. In this chemical structure of cholesterol, the rings are labeled A through D to simplify reference to derivatives of the steroid nucleus. The C-3 hydroxyl group (shaded blue) is the polar head group. For storage and transport of the sterol, this hydroxyl group condenses with a fatty acid to form a sterol ester.

P2 **Sterols** are structural lipids present in the membranes of most eukaryotic cells. The characteristic structure of this group of membrane lipids is the steroid nucleus, consisting of four fused rings, three with six carbons and one with five (**Fig. 10-15**). The steroid nucleus is almost planar and is relatively rigid; the fused rings do not allow rotation about C—C bonds. **Cholesterol**, the major sterol in animal tissues, is amphipathic, with a polar head group (the hydroxyl group at C-3) and a nonpolar hydrocarbon body (the steroid nucleus and the hydrocarbon side chain at C-17) about as long as a 16-carbon fatty acid in its extended form. Similar sterols are found in other eukaryotes: stigmasterol in plants and ergosterol in fungi, for example. Bacteria cannot synthesize sterols; a few bacterial species, however, can incorporate exogenous sterols into their membranes. The sterols of all eukaryotes are synthesized from simple five-carbon isoprene subunits, as are the fat-soluble vitamins, quinones, and dolichols described in Section 10.3.

P3 In addition to their roles as membrane constituents, the sterols serve as precursors for a variety of products with specific biological activities. Steroid hormones, for example, are potent biological signals that regulate gene expression. **Bile acids** are polar derivatives of cholesterol that act as detergents in the intestine, emulsifying dietary fats to make them more readily accessible to digestive lipases.



Taurocholic acid
(a bile acid)

We return to cholesterol and other sterols in later chapters, to consider the structural role of cholesterol in biological membranes (Chapter 11), signaling by steroid hormones (Chapter 12), and the remarkable biosynthetic pathway to cholesterol and transport of cholesterol by lipoprotein carriers (Chapter 21).

SUMMARY 10.2 *Structural Lipids in Membranes*

- Lipids with polar heads and nonpolar tails are major components of membranes. The most abundant are the glycerophospholipids, which contain fatty acids esterified to two of the hydroxyl groups of glycerol. The third hydroxyl of glycerol is esterified with the polar head group via a phosphodiester bond. Common glycerophospholipids such as phosphatidylethanolamine and phosphatidylcholine differ in the structure of their head group.

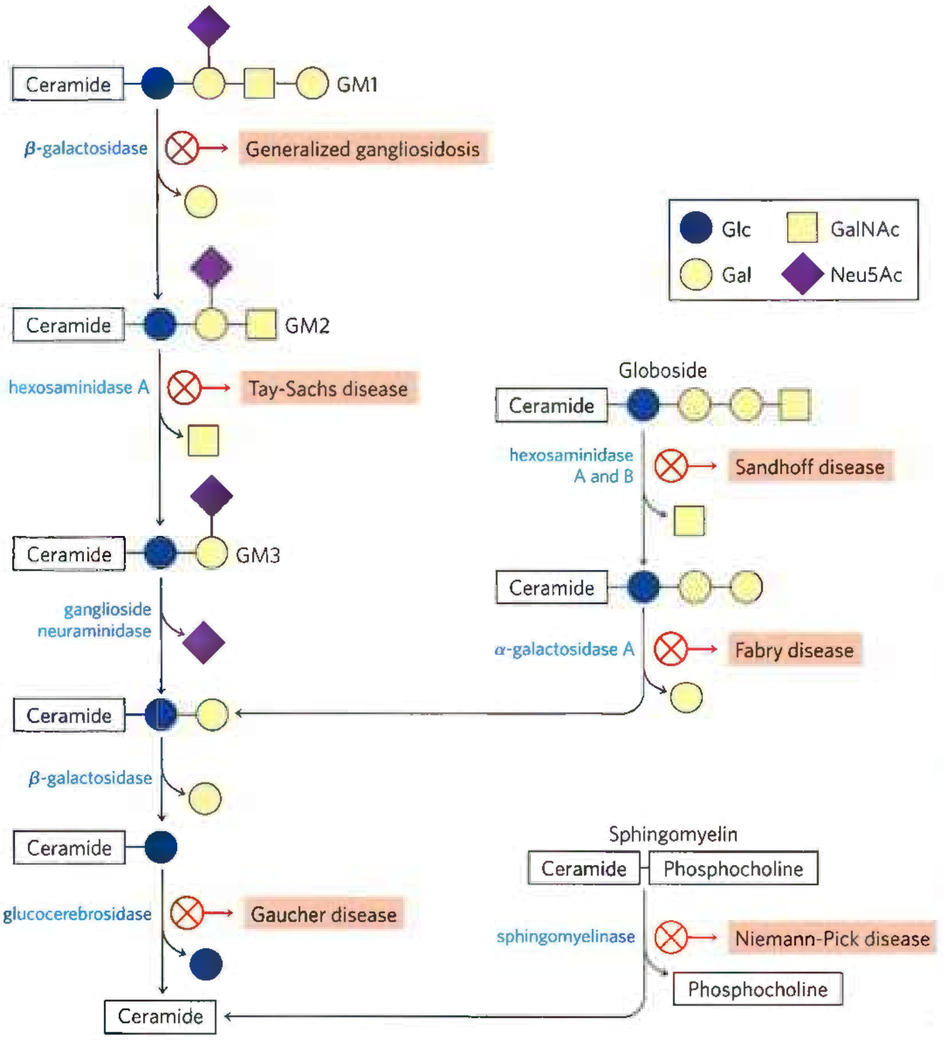
BOX 10-1 **MEDICINE**

Abnormal Accumulations of Membrane Lipids: Some Inherited Human Diseases

The polar lipids of membranes undergo constant metabolic turnover, the rate of their synthesis normally counterbalanced by the rate of breakdown. The breakdown of lipids is promoted by hydrolytic enzymes in lysosomes, with each enzyme capable of hydrolyzing a specific bond. When sphingolipid degradation is impaired by a defect in one of these enzymes (Fig. 1), partial breakdown products accumulate in the tissues, causing serious disease. More than 50 distinct lysosomal storage diseases have been discovered, each the result of a single mutation in one of the genes for a lysosomal protein.

For example, Niemann-Pick disease is caused by a rare genetic defect in the enzyme sphingomyelinase, the enzyme that cleaves phosphocholine from sphingomyelin. Sphingomyelin accumulates in the brain, spleen, and liver. The disease becomes evident in infants and causes intellectual disability and early death. More common is Tay-Sachs disease, in which ganglioside GM2 accumulates in the brain and spleen (Fig. 2) owing to lack of the enzyme hexosaminidase A. The symptoms of Tay-Sachs disease are progressive developmental delay and disability, paralysis, blindness, and death by the age of 3 or 4 years.

Genetic counseling can predict and avert many inheritable diseases. Tests on prospective parents can detect abnormal enzymes; then DNA testing can determine the exact nature of the defect and the risk it poses for offspring. Once a pregnancy occurs, fetal cells obtained by sampling a part of the placenta (chorionic villus sampling) or the fluid surrounding the fetus (amniocentesis) can be tested in the same way.



Ceramide
GM1
β-galactosidase
Generalized gangliosidosis
Ceramide
GM2
hexosaminidase A
Tay-Sachs disease
Ceramide
GM3
ganglioside neuraminidase
Ceramide
β-galactosidase
Ceramide
glucocerebrosidase
Gaucher disease
Ceramide
Glc
GalNAc
Gal
Neu5Ac
Globoside
Ceramide
hexosaminidase A and B
Sandhoff disease
Ceramide
α-galactosidase A
Fabry disease
Sphingomyelin
Ceramide
Phosphocholine
sphingomyelinase
Niemann-Pick disease
Phosphocholine

FIGURE 1 Pathways for the breakdown of GM1, globoside, and sphingomyelin to ceramide. A defect in the enzyme hydrolyzing a particular step is indicated by ⊗; the disease that results from accumulation of the partial breakdown product is noted.

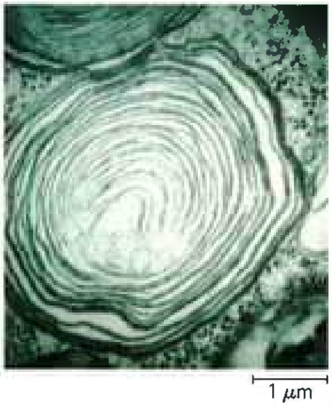


FIGURE 2 Electron micrograph of a portion of a brain cell from an infant with Tay-Sachs disease, obtained postmortem, showing abnormal ganglioside deposits in the lysosomes. [Otis Imboden/National Geographic Image Collection]

- Heart tissue plasmalogens and platelet-activating factor are examples of glycerophospholipids containing ether-linked acyl chains.
- Chloroplast membranes are rich in galactolipids, composed of a diacylglycerol with one or two linked galactose residues, and sulfolipids, diacylglycerols with a linked sulfonated sugar residue. Some archaea have unique membrane lipids, with long-chain alkyl groups ether-linked to glycerol at each end. These lipids are stable under the harsh conditions in which these archaea live.
- The sphingolipids contain sphingosine instead of glycerol. The three subclasses of sphingolipids are all ceramide derivatives: sphingomyelins, neutral glycolipids, and gangliosides. Sphingomyelins have choline head groups; the other classes have sugar components.
- Sphingolipids are abundant in the plasma membranes of neurons, and they define human blood groups.
- Phospholipases degrade glycerophospholipids and catalyze hydrolysis at specific positions within the structure.
- Sterols have four fused rings and a hydroxyl group. Cholesterol, the major sterol in animals, is both a structural component of membranes and precursor to a wide variety of steroids.

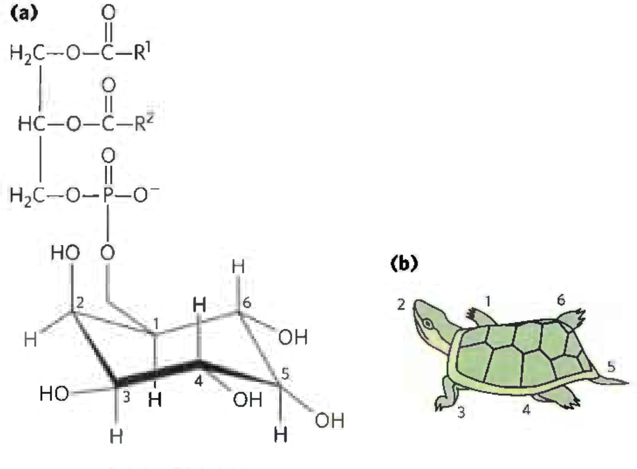
10.3 Lipids as Signals, Cofactors, and Pigments

The two functional classes of lipids considered thus far (storage lipids and structural lipids) are major cellular components; membrane lipids make up 5% to 10% of the dry mass of most cells, and storage lipids make up more than 80% of the mass of an adipocyte. With some important exceptions, these lipids play a *passive* role in the cell; lipid fuels are stored until oxidized by enzymes, and membrane lipids form impermeable barriers around cells and cellular compartments. P3 Another group of lipids, present in much smaller amounts, includes those with *active* roles in metabolic traffic as metabolites and messengers. Some serve as potent signals—as hormones, carried in the blood from one tissue to another, or as intracellular messengers generated in response to an extracellular signal (hormone or growth factor). Others function as enzyme cofactors in electron-transfer reactions in chloroplasts and mitochondria, or in the transfer of sugar moieties in a variety of glycosylation reactions. A third group consists of lipids with a system of conjugated double bonds: pigment molecules that absorb visible light. Some of these act as light-capturing pigments in vision and photosynthesis; others produce natural colorations, such as the orange of pumpkins and carrots and the yellow of canary feathers. Finally, a very large group of volatile lipids produced in plants consists of signaling molecules that pass through the air, allowing plants to communicate with each other and to invite animal friends and deter foes. In this section, we describe a few representatives of these biologically active lipids. In later chapters, we consider their synthesis and biological roles in more detail.

Phosphatidylinositols and Sphingosine Derivatives Act as Intracellular Signals

P3 Phosphatidylinositol (PI) and its phosphorylated derivatives **(Fig. 10-16)** act at several levels to regulate cell structure and metabolism. Phosphatidylinositol 4,5-bisphosphate (PIP_2; Fig. 10-14) in the cytoplasmic (inner) face of plasma membranes serves as a reservoir of messenger molecules that are released inside the cell in response to extracellular signals interacting with specific surface receptors. Extracellular signals such as the hormone vasopressin activate a specific phospholipase C in the membrane, which hydrolyzes PIP_2 to release two products that act as intracellular messengers: inositol 1,4,5-trisphosphate (IP_3), which is water soluble, and diacylglycerol, which remains associated with the plasma membrane. IP_3 triggers release of Ca^{2+} from the endoplasmic reticulum, and the combination of diacylglycerol and elevated cytosolic Ca^{2+} activates the enzyme protein kinase C. By phosphorylating specific proteins, this enzyme brings about the cell's response to the extracellular signal. This signaling mechanism is described more fully in Chapter 12 (see Fig. 12-15).

P3 Inositol phospholipids also serve as points of nucleation for supramolecular complexes involved in signaling or in exocytosis. Certain signaling proteins bind specifically to phosphatidylinositol 3,4,5-trisphosphate



Phosphatidylinositol

FIGURE 10-16 Phosphatidylinositol and its derivatives. (a) In phosphatidylinositol, the glycerol phospholipid is attached at C-1 of inositol. Phosphorylation of the remaining inositol hydroxyl groups forms signaling molecules such as phosphatidylinositol 4,5-bisphosphate (PIP_2; see Fig. 10-14), inositol 1,4,5-bisphosphate (IP_3), and phosphatidylinositol 3,4,5-trisphosphate (PIP_3). (b) A useful trick for remembering the inositol carbon-numbering scheme is to compare the chair conformation of inositol to a turtle. Begin numbering with C-1 as the right front flipper and move counterclockwise around the structure, with C-2 the head and C-5 the tail.

(PIP_3) in the plasma membrane, initiating the formation of multienzyme complexes at the membrane's cytosolic surface. Thus, formation of PIP_3 in response to extracellular signals brings the proteins together in signaling complexes at the surface of the plasma membrane (see Figs. 12-11 and 12-23).

Membrane sphingolipids also can serve as sources of intracellular messengers. Both ceramide and sphingomyelin (Fig. 10-11) are potent regulators of protein kinases, and ceramide or its derivatives are involved in the regulation of cell division, differentiation, migration, and programmed cell death (also called apoptosis; see Chapter 12).

Eicosanoids Carry Messages to Nearby Cells

P3 **Eicosanoids** are paracrine hormones, substances that act only on cells near the point of hormone synthesis instead of being transported in the blood to act on cells in other tissues or organs. These fatty acid derivatives have a variety of dramatic effects on vertebrate tissues. They are involved in reproductive function; in the inflammation, fever, and pain associated with injury or disease; in the formation of blood clots and the regulation of blood pressure; in gastric acid secretion; and in various other processes important in human health or disease.

Eicosanoids are derived from arachidonate (arachidonic acid; $20{:}4(\Delta^{5,8,11,14})$) and eicosapentaenoic acid (EPA; $20{:}5(\Delta^{5,8,11,14,17})$), from which they take their general name (Greek *eikosi*, "twenty"). There are four major classes of eicosanoids: prostaglandins, thromboxanes, leukotrienes, and lipoxins (**Fig. 10-17**). Eicosanoid names include letter designations for the functional groups on the ring and numbers indicating the number of double bonds in the hydrocarbon chain.

Prostaglandins (PG) contain a five-carbon ring, and their name derives from the prostate gland from which they were first isolated. PGE_2 and other series 2 prostaglandins are synthesized from arachidonate; series 3 prostaglandins are derived from EPA (see Fig. 21-12). Prostaglandins have an array of functions. Some stimulate contraction of the smooth muscle of the uterus during menstruation and labor. Others affect blood flow to specific organs, the wake-sleep cycle, and the responsiveness of certain tissues to hormones such as epinephrine and glucagon. Prostaglandins in a third group elevate body temperature (producing fever) and cause inflammation and pain.

The **thromboxanes (TX)** have a six-membered ring containing an ether. They are produced by platelets (also called thrombocytes) and act in the formation of blood clots and reduction of blood flow to the site of a clot. Nonsteroidal anti-inflammatory drugs (NSAIDs)—aspirin, ibuprofen, and meclofenamate, for example—inhibit the enzyme cyclooxygenase or COX (also called prostaglandin H_2 synthase), which catalyzes an early step in the pathway from arachidonate to series 2 prostaglandins and thromboxanes (Fig. 10-17) and from EPA to series 3 prostaglandins and thromboxanes (see Fig. 21-12).

Leukotrienes (LT), first found in leukocytes, contain three conjugated double bonds. They are powerful biological signals. For example, leukotriene D_4, derived from leukotriene A_4, induces contraction of the smooth muscle lining the airways to the lung. Overproduction of leukotrienes causes asthmatic attacks, and leukotriene synthesis is one target of antiasthmatic drugs such as prednisone. The strong contraction of the smooth muscle of the lungs that occurs during anaphylactic shock is part of the potentially fatal allergic reaction in individuals hypersensitive to bee stings, penicillin, or other agents.

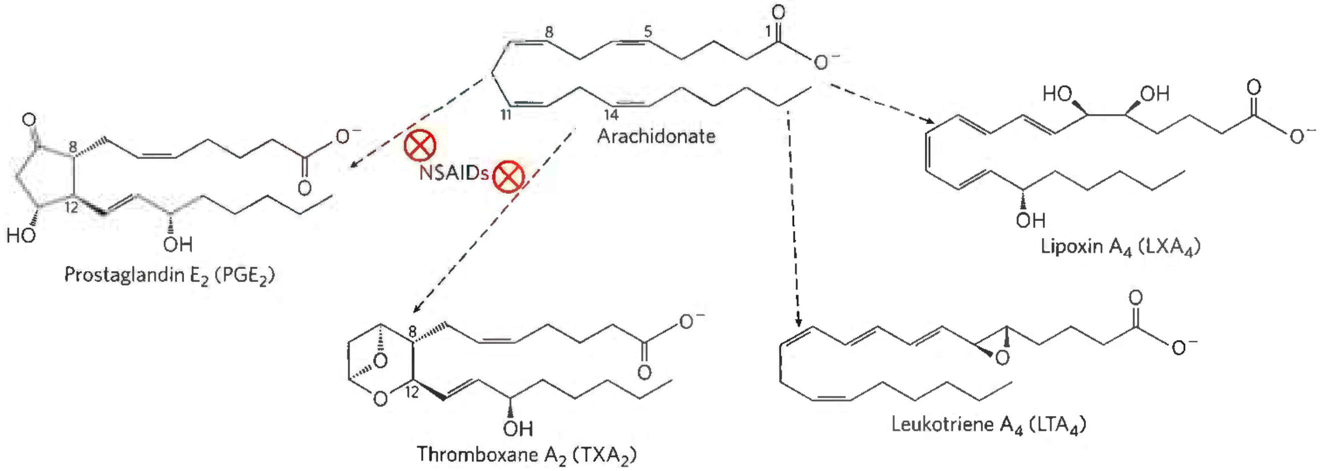


FIGURE 10-17 Arachidonic acid and some eicosanoid derivatives. Arachidonic acid (arachidonate at pH 7) is the precursor of eicosanoids, including the prostaglandins, thromboxanes, leukotrienes, and lipoxins. In prostaglandin E_2, C-8 and C-12 of arachidonate are joined to form the characteristic five-membered ring. In thromboxane A_2, the C-8 and C-12 are joined and an oxygen atom is added to form the six-membered ring. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen block the formation of prostaglandins and thromboxanes from arachidonate by inhibiting the enzyme cyclooxygenase (prostaglandin H_2 synthase). Leukotriene A_4 has a series of three conjugated double bonds, and no cyclic moiety. Lipoxins are also noncyclic derivatives of arachidonate, with several hydroxyl groups.

Lipoxins (LX), like leukotrienes, are linear eicosanoids. Their distinguishing feature is the presence of several hydroxyl groups along the chain (Fig. 10-17). These compounds are potent anti-inflammatory agents. Because their synthesis is stimulated by low doses (81 mg) of aspirin taken daily, this low dose is commonly prescribed for individuals with cardiovascular disease. ■

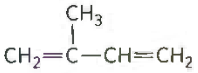
Steroid Hormones Carry Messages between Tissues

Steroids are oxidized derivatives of sterols; they have the sterol nucleus but lack the alkyl chain attached to ring D of cholesterol, and they are more polar than cholesterol. **P3** Steroid hormones move through the bloodstream (on protein carriers) from their site of production to target tissues, where they enter cells, bind to highly specific receptor proteins in the nucleus, and trigger changes in gene expression and thus metabolism. Because hormones have very high affinity for their receptors, very low concentrations of hormones (nanomolar or less) are sufficient to produce responses in target tissues. The major groups of steroid hormones are the male and female sex hormones and the hormones produced by the adrenal cortex, cortisol and aldosterone (**Fig. 10-18**). Prednisone is a steroid drug with strong anti-inflammatory activity, mediated in part by the inhibition of arachidonate release by phospholipase A_2 and consequent inhibition of the synthesis of prostaglandins, thromboxanes, leukotrienes, and lipoxins. Prednisone and similar drugs have a variety of medical applications, including the treatment of asthma and rheumatoid arthritis. ■

Vascular plants contain the steroidlike brassinolide (Fig. 10-18), a potent growth regulator that increases the rate of stem elongation and affects the orientation of cellulose microfibrils in the cell wall during growth.

Vascular Plants Produce Thousands of Volatile Signals

P3 Plants produce thousands of different lipophilic compounds, volatile substances that are used to attract pollinators, repel herbivores, attract organisms that defend the plant against herbivores, and communicate with other plants. Jasmonate, for example, derived from the fatty acid 18:3($\Delta^{9,12,15}$) in membrane lipids, triggers the plant's defenses in response to insect-inflicted damage. The methyl ester of jasmonate gives the characteristic fragrance of jasmine oil, which is widely used in the perfume industry. Many plant volatiles, including geraniol (the characteristic scent of geraniums), β-pinene (pine trees), limonene (limes), and menthol, are derived from fatty acids or from compounds made by the condensation of five-carbon isoprene units.

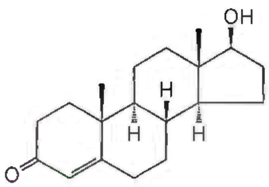


$$CH_2{=}\overset{\displaystyle CH_3}{\overset{|}{C}}{-}CH{=}CH_2$$

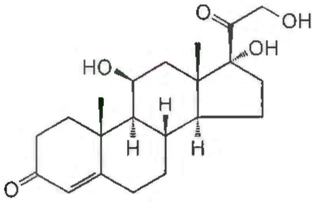
Isoprene

Vitamins A and D Are Hormone Precursors

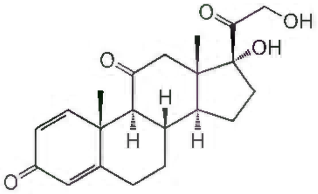
During early decades of the twentieth century, a major focus of research in physiological chemistry was the identification of **vitamins**, compounds that are essential to the health of humans and other vertebrates



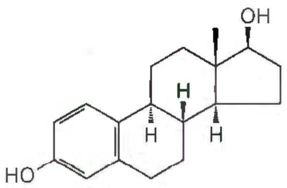
Testosterone
Male sex hormone
produced in the testes



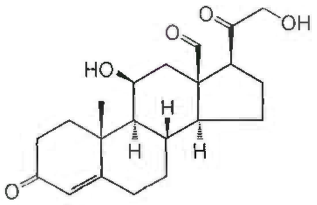
Cortisol
Hormone produced in the
adrenal cortex; regulates
glucose metabolism



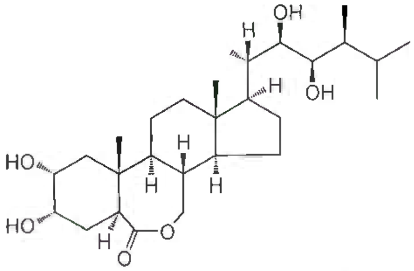
Prednisone
Synthetic steroid used as
an anti-inflammatory agent



β-Estradiol
Female sex hormone produced
in the ovaries and placenta

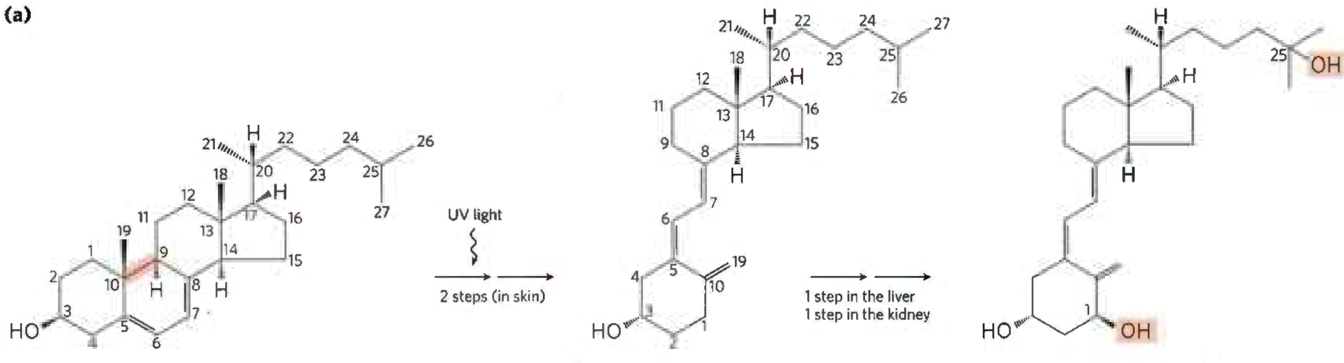


Aldosterone
Hormone produced in the
adrenal cortex; regulates
salt excretion



Brassinolide (a brassinosteroid)
Growth regulator found
in vascular plants

FIGURE 10-18 **Steroids derived from cholesterol.**



7-Dehydrocholesterol

Cholecalciferol (vitamin D_3)

1α,25-Dihydroxyvitamin D_3 (calcitriol)

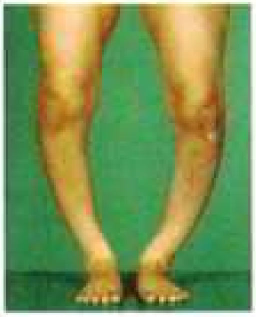


FIGURE 10-19 Vitamin D_3 production and metabolism. (a) Cholecalciferol (vitamin D_3) is produced in the skin by UV irradiation of 7-dehydrocholesterol, which breaks the bond shaded light red. In the liver, a hydroxyl group is added at C-25; in the kidney, a second hydroxylation at C-1 produces the active hormone, 1α,25-dihydroxyvitamin D_3. This hormone regulates the metabolism of Ca^{2+} in kidney, intestine, and bone. (b) Dietary vitamin D prevents rickets, a disease once common in cold climates where heavy clothing blocks the UV component of sunlight necessary for the production of vitamin D_3 in skin. Rickets results in weak or soft bones in children; it can often be identified by bowed legs and other bone deformities. [Biophoto Associates/Science Source]

but cannot be synthesized by these animals and must therefore be obtained in the diet. Early nutritional studies identified two general classes of such compounds: those soluble in nonpolar organic solvents (fat-soluble vitamins) and those that could be extracted from foods with aqueous solvents (water-soluble vitamins). P3 Eventually, the fat-soluble group was resolved into the four vitamin groups A, D, E, and K, all of which are isoprenoid compounds synthesized by the condensation of multiple isoprene units. Two of these (D and A) serve as hormone precursors.

Vitamin D_3, also called **cholecalciferol**, is normally formed in the skin from 7-dehydrocholesterol in a photochemical reaction driven by the UV component of sunlight (**Fig. 10-19a**). Vitamin D_3 is not itself biologically active, but it is converted by enzymes in the liver and kidney to 1α,25-dihydroxyvitamin D_3 (calcitriol), a hormone that regulates calcium uptake in the intestine and calcium levels in kidney and bone. Deficiency of vitamin D leads to defective bone formation and the disease rickets (Fig. 10-19b), for which administration of vitamin D produces a dramatic cure. Vitamin D_2 (ergocalciferol) is a commercial product formed by UV irradiation of the ergosterol of yeast. Vitamin D_2 is structurally similar to D_3, with slight modification to the side chain attached to the sterol D ring. Both have the same biological effects, and D_2 is commonly added to milk and butter as a dietary supplement. The product of vitamin D metabolism, 1α,25-dihydroxyvitamin D_3, regulates gene expression by interacting with specific nuclear receptor proteins. We discuss such regulation of gene expression in more detail in Chapter 28.

Vitamin A_1 (all-*trans*-retinol) and its oxidized metabolites retinoic acid and retinal act in the processes of development, cell growth and differentiation, and vision (**Fig. 10-20**). Vitamin A_1 or β-carotene in the diet can be converted enzymatically to all-*trans*-retinoic acid, a retinoid hormone that acts through a family of nuclear receptor proteins (RAR, RXR, PPAR) to regulate gene expression central to embryonic development, stem cell differentiation, and cell proliferation. All-*trans*-retinoic acid is used to treat certain types of leukemia, and it is the active ingredient in the drug tretinoin (Retin-A), used to treat severe acne and wrinkled skin. In the vertebrate eye, retinal bound to the protein opsin forms the photoreceptor pigment rhodopsin. The photochemical conversion of 11-*cis*-retinal to all-*trans*-retinal is the fundamental event in vision (see Fig. 12-19).

Unlike most vitamins, vitamin A can be stored for some time in the body (primarily as its ester with palmitic acid, in the liver). Vitamin A was first isolated from fish liver oils; eggs, whole milk, and butter are also good dietary sources. Another source is β-carotene (Fig. 10-20), the pigment that gives carrots, sweet potatoes, and other yellow vegetables their characteristic color. Carotene is one of a very large number (>700) of **carotenoids**, natural products with a characteristic extensive system of conjugated double bonds, which makes possible their strong absorption of visible light (450–470 nm).

Vitamin A deficiency in a pregnant woman can lead to congenital malformations and growth retardation in the infant. In adults, vitamin A is also essential to vision, immunity, and reproduction. Deficiency of vitamin A leads to a variety of symptoms, including dryness of the

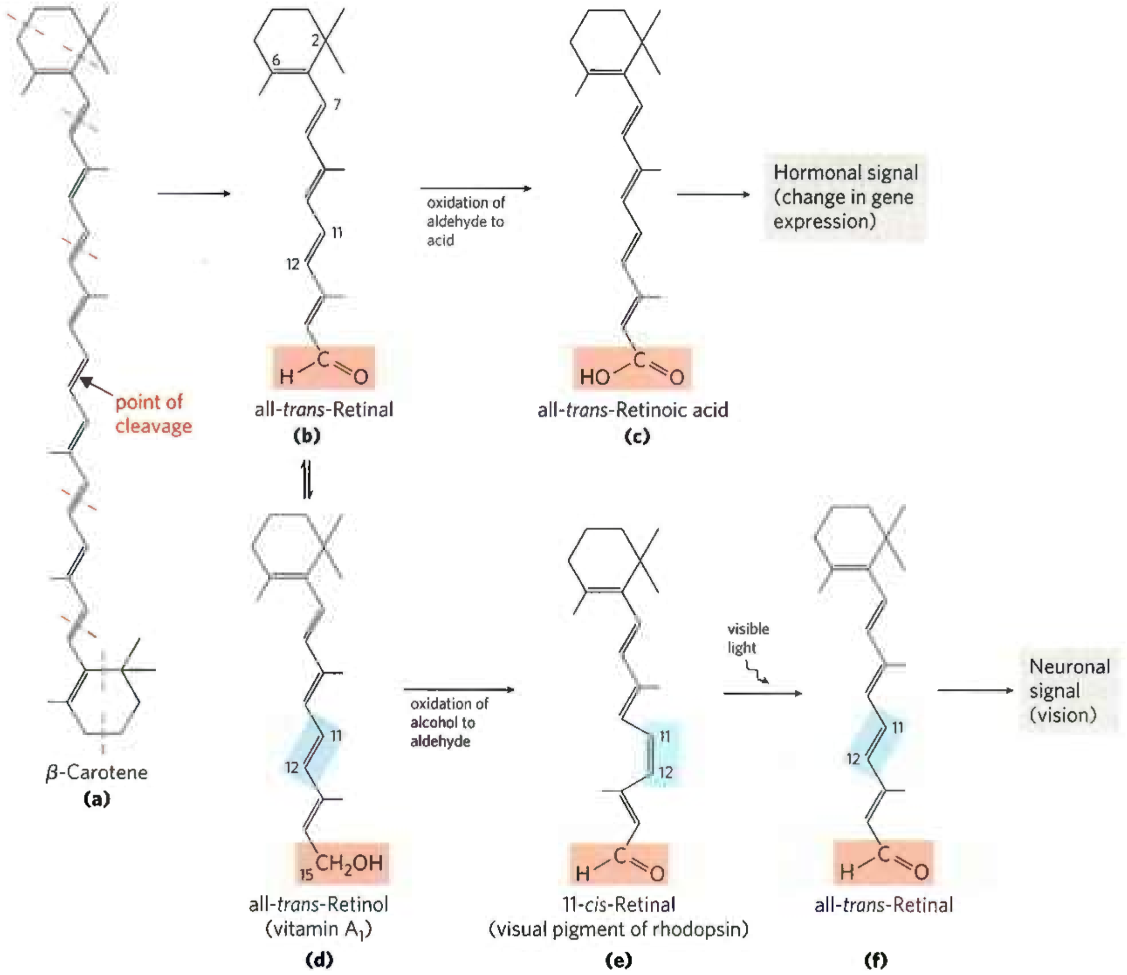

point of cleavage
β-Carotene
(a)
6
2
7
11
12
all-trans-Retinal
(b)
oxidation of aldehyde to acid
all-trans-Retinoic acid
(c)
Hormonal signal (change in gene expression)
15
all-trans-Retinol (vitamin A1)
(d)
oxidation of alcohol to aldehyde
11-cis-Retinal (visual pigment of rhodopsin)
(e)
visible light
all-trans-Retinal
(f)
Neuronal signal (vision)

FIGURE 10-20 Dietary β-carotene and vitamin A_1 as precursors of the retinoids. (a) β-Carotene is shown with its isoprene structural units set off by dashed red lines. Symmetric cleavage of β-carotene yields two molecules of all-*trans*-retinal (b), which can be either further oxidized to all-*trans*-retinoic acid, a retinoid hormone (c), or reduced to all-*trans*-retinol, vitamin A_1 (d). In the visual pathway, all-*trans*-retinol from this reaction, or obtained directly through the diet, can be converted to the aldehyde 11-*cis*-retinal (e). This product combines with the protein opsin to form rhodopsin (not shown), a visual pigment widespread in nature. In the dark, the retinal of rhodopsin is in the 11-*cis* form. When a rhodopsin molecule is excited by visible light, the 11-*cis*-retinal undergoes a series of photochemical reactions that convert it to all-*trans*-retinal (f), forcing a change in the shape of the entire rhodopsin molecule. This transformation in the rod cell of the vertebrate retina sends an electrical signal to the brain that is the basis of visual transduction.

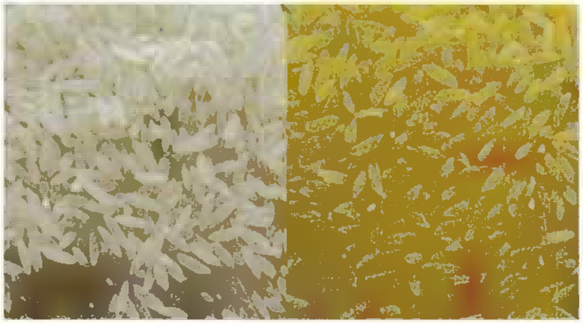


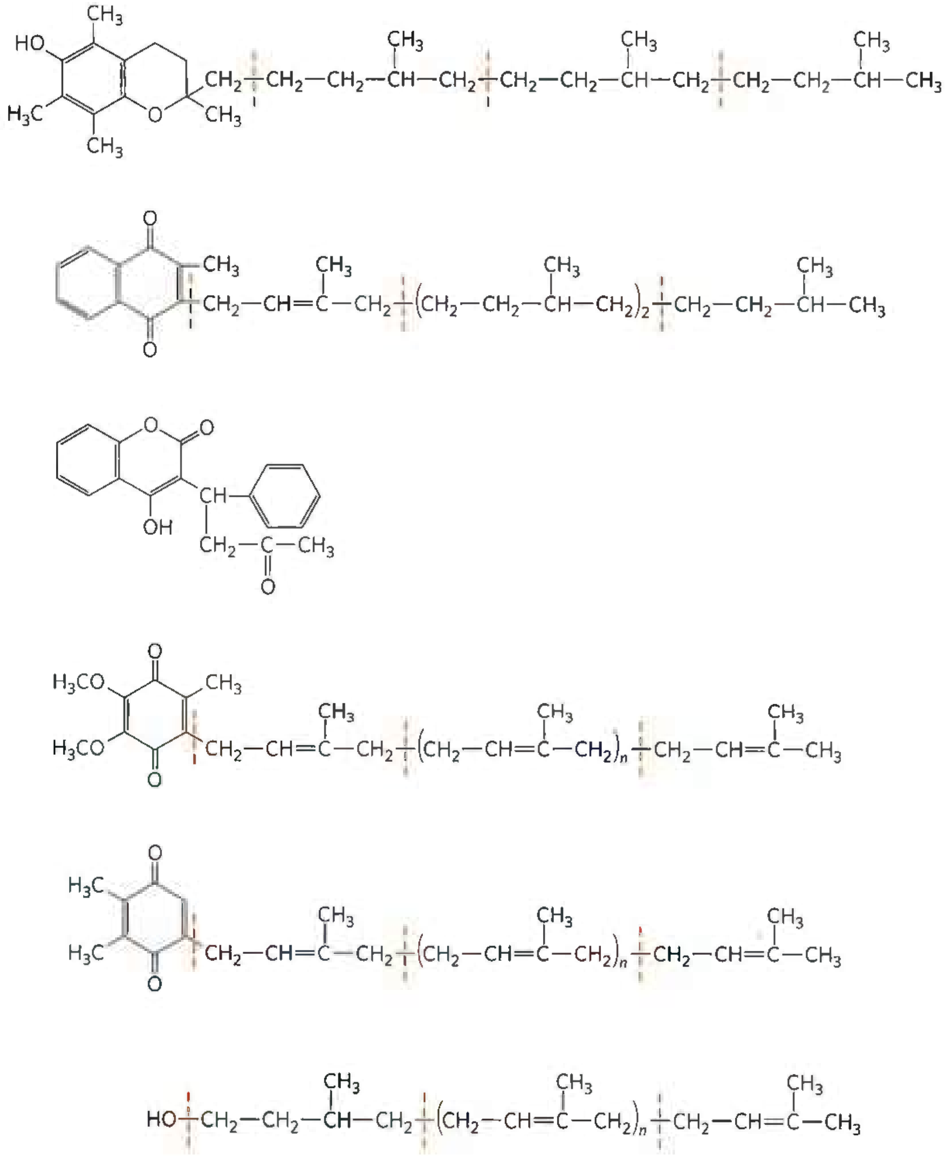
FIGURE 10-21 Carotene-enriched rice. Worldwide, more than 250 million children and pregnant women suffer from vitamin A deficiency, which causes at least 250,000 cases of irreversible blindness each year in children. Half of these children end up dying within a year of losing their sight. This deficiency is particularly prevalent where rice is a staple food. An international humanitarian effort — the Golden Rice Project — has made great strides in addressing this health crisis. Wild-type rice grains (left) do not produce β-carotene, the metabolic precursor of vitamin A. Rice plants have been genetically engineered to produce β-carotene in the grain, which takes on the yellow color of the carotene (right). A diet supplemented with Golden Rice provides enough β-carotene to prevent vitamin A deficiency and its tragic health consequences. [© Golden Rice Humanitarian Board (www.goldenrice.org)]

skin, eyes, and mucous membranes, and night blindness, an early symptom commonly used in diagnosing vitamin A deficiency. In the developing world, vitamin A deficiency causes an estimated 250,000 or more cases of blindness or death in children each year. One effective strategy for providing vitamin A is the metabolic engineering of rice strains to overproduce β-carotene. Rice has all the enzymatic machinery to produce β-carotene in its leaves, but these enzymes are less active in the grain. Introduction of two genes into the rice has resulted in "golden rice" with grains enriched in β-carotene (**Fig. 10-21**). ■

Vitamins E and K and the Lipid Quinones Are Oxidation-Reduction Cofactors

Vitamin E is the collective name for a group of closely related lipids called **tocopherols**, all of which contain a substituted aromatic ring and a long isoprenoid side chain (**Fig. 10-22a**). Because they are hydrophobic, tocopherols associate with cell membranes, lipid deposits, and lipoproteins in the blood. Tocopherols are biological antioxidants. The aromatic ring reacts with and destroys the most reactive forms of oxygen radicals and other free radicals, protecting unsaturated fatty acids from oxidation and preventing oxidative damage to membrane lipids, which can cause cell fragility. Tocopherols are found in eggs and vegetable oils and are especially abundant in wheat germ. Laboratory animals fed diets depleted of vitamin E develop scaly skin, muscular weakness and wasting, and sterility. Vitamin E deficiency in humans is very rare; the principal symptom is fragile erythrocytes.

The aromatic ring of **vitamin K** (Fig. 10-22b) undergoes a cycle of oxidation and reduction during the formation of active prothrombin, a blood plasma protein essential in blood clotting. Prothrombin is a proteolytic enzyme that splits peptide bonds in the blood protein fibrinogen to convert it to fibrin, the insoluble fibrous protein that holds blood clots together (see Fig. 6-43). Vitamin K deficiency, which slows blood clotting, is extremely uncommon in humans, aside from a small percentage of infants who suffer from hemorrhagic disease of the newborn, a potentially fatal disorder. In the United


(a) Vitamin E: an antioxidant

(b) Vitamin K_1: a blood-clotting cofactor (phylloquinone)

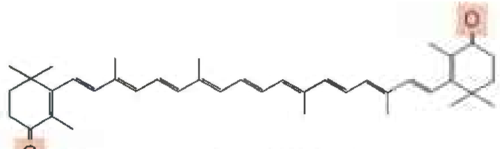
(c) Warfarin: a blood anticoagulant

(d) Ubiquinone: a mitochondrial electron carrier (coenzyme Q) (n = 4 to 8)

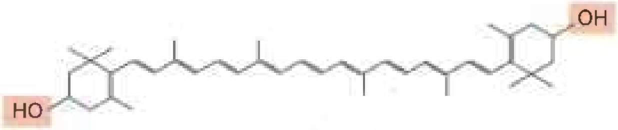
(e) Plastoquinone: a chloroplast electron carrier (n = 4 to 8)

(f) Dolichol: a sugar carrier (n = 9 to 22)

FIGURE 10-22 Some other biologically active isoprenoid compounds or derivatives. Units derived from isoprene are set off by dashed red lines. In most mammalian tissues, ubiquinone (also called coenzyme Q) has 10 isoprene units. Dolichols of animals have 17 to 21 isoprene units (85 to 105 carbon atoms), bacterial dolichols have 11, and dolichols of plants and fungi have 14 to 24.



Canthaxanthin
(bright red)



Zeaxanthin
(bright yellow)

FIGURE 10-23 Lipids as pigments in plants and bird feathers. Compounds with long conjugated systems absorb light in the visible region of the spectrum. Subtle differences in the chemistry of these compounds produce pigments of strikingly different colors. Birds acquire the pigments that color their feathers red or yellow by eating plant materials that contain carotenoid pigments, such as canthaxanthin and zeaxanthin. The differences in pigmentation between male and female birds are the result of differences in intestinal uptake and processing of carotenoids. [Gaertner/Alamy Stock Photo]

States, newborns are routinely given a 1 mg injection of vitamin K. Vitamin K$_1$ (phylloquinone) is found in green plant leaves; a related form, vitamin K$_2$ (menaquinone), is formed by bacteria living in the vertebrate intestine.

Warfarin (Fig. 10-22c) is a synthetic compound that inhibits the formation of active prothrombin. It is particularly poisonous to rats, causing death by internal bleeding. Ironically, this potent rodenticide is also an invaluable anticoagulant drug for treating humans at risk for excessive blood clotting, such as surgical patients and those with coronary thrombosis. ■

Ubiquinone (also called coenzyme Q) and plastoquinone (Fig. 10-22d, e) are isoprenoids that function as lipophilic electron carriers in the oxidation-reduction reactions that drive ATP synthesis in mitochondria and chloroplasts, respectively. Both ubiquinone and plastoquinone can accept either one or two electrons and either one or two protons (see Fig. 19-3).

Dolichols Activate Sugar Precursors for Biosynthesis

During assembly of the complex carbohydrates of bacterial cell walls, and during the addition of polysaccharide units to certain proteins (glycoproteins) and lipids (glycolipids) in eukaryotes, the sugar units to be added are chemically activated by attachment to isoprenoid alcohols called **dolichols** (Fig. 10-22f). These compounds have strong hydrophobic interactions with membrane lipids, anchoring the attached sugars to the membrane, where they participate in sugar-transfer reactions.

Many Natural Pigments Are Lipidic Conjugated Dienes

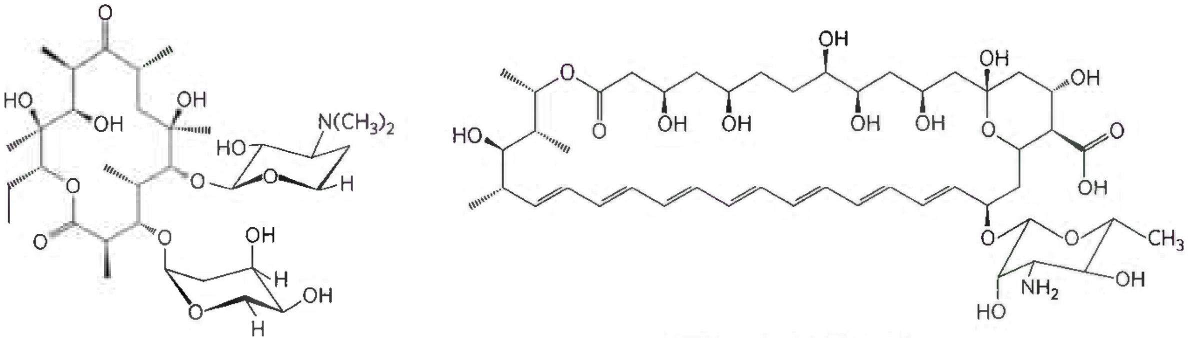
Conjugated dienes have carbon chains with alternating single and double bonds. Because this structural arrangement allows the delocalization of electrons, the compounds can be excited by low-energy electromagnetic radiation (visible light), giving them colors visible to humans and other animals. Carotene (Fig. 10-20) is yellow-orange; similar compounds give bird feathers their striking reds, oranges, and yellows (**Fig. 10-23**). Like sterols, steroids, dolichols, vitamins A, E, D, and K, ubiquinone, and plastoquinone, these pigments are synthesized from five-carbon isoprene derivatives; the biosynthetic pathway is described in detail in Chapter 21.

Polyketides Are Natural Products with Potent Biological Activities

Polyketides are a diverse group of lipids with biosynthetic pathways (Claisen condensations) similar to those for fatty acids. They are **secondary metabolites**, compounds that are not central to an organism's metabolism but serve some subsidiary function that gives the organism an advantage in some ecological niche. Many polyketides find use in medicine as antibiotics (erythromycin), antifungals (amphotericin B), or inhibitors of cholesterol synthesis (lovastatin) (**Fig. 10-24**). ■

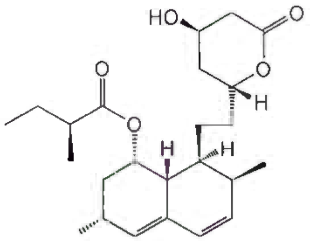
SUMMARY 10.3 *Lipids as Signals, Cofactors, and Pigments*

- Some types of lipids, although present in relatively small quantities, play critical roles as cofactors or signals.
- Membrane sphingolipids and derivatives of phosphatidylinositol such as PIP$_2$ and PIP$_3$ are used as signaling molecules or for nucleating formation of multiprotein complexes.
- Prostaglandins, thromboxanes, leukotrienes, and lipoxins, all of which are eicosanoids derived from arachidonate, are extremely potent hormones involved in reproduction, inflammation, regulating blood pressure, and other bodily processes. NSAIDs inhibit formation of some prostaglandins and thromboxanes.
- Steroid hormones, such as the sex hormones, are derived from sterols. They serve as powerful biological



Erythromycin (antibiotic)

Amphotericin B (antifungal)



Lovastatin (statin)

FIGURE 10-24 **Three polyketide natural products used in human medicine.**

signals and move through the bloodstream to alter gene expression in target cells.

- Plants produce volatile lipids to attract or repel other organisms and for communication. Many of these lipids are used as fragrances in perfumes.
- Vitamins D, A, E, and K are fat-soluble compounds made up of isoprene units. All play essential roles in the metabolism or physiology of animals. Vitamin D is precursor to a hormone that regulates calcium metabolism. Vitamin A furnishes the visual pigment of the vertebrate eye and is a regulator of gene expression during epithelial cell growth.
- Vitamins E and K and quinones can be oxidized or reduced by cells. Vitamin E functions in the protection of membrane lipids from oxidative damage, and vitamin K is essential for blood clotting. Quinones are essential for carrying electrons during the reactions that drive ATP synthesis in mitochondria and chloroplasts.
- Dolichols activate and anchor sugars to cellular membranes; the sugar groups are then used in the synthesis of complex carbohydrates, glycolipids, and glycoproteins.
- Lipidic conjugated dienes serve as pigments in flowers and fruits and give bird feathers their striking colors.
- Polyketides are natural products widely used in medicine.

10.4 Working with Lipids

Because lipids are insoluble in water, their extraction and subsequent fractionation require the use of organic solvents and some techniques not commonly used in the purification of water-soluble molecules such as proteins and carbohydrates. **P4** In general, complex mixtures of lipids are separated by differences in polarity or solubility in nonpolar solvents. Lipids that contain ester- or amide-linked fatty acids can be hydrolyzed by treatment with acid or alkali or with specific hydrolytic enzymes (phospholipases, glycosidases) to yield their components for analysis. Some methods commonly used in lipid analysis are shown in **Figure 10-25** and discussed below.

Lipid Extraction Requires Organic Solvents

Neutral lipids (triacylglycerols, waxes, pigments, and so forth) are readily extracted from tissues with ethyl ether, chloroform, or benzene, solvents that do not permit lipid clustering driven by the hydrophobic effect. Membrane lipids are more effectively extracted by more polar organic solvents, such as ethanol or methanol, which reduce the hydrophobic interactions among lipid molecules while also weakening the hydrogen bonds and electrostatic interactions that bind membrane lipids to membrane proteins. A commonly used extractant is a mixture of chloroform, methanol, and water, initially in volume proportions (1:2:0.8) that are miscible, producing a single phase. After tissue is homogenized in this solvent to extract all lipids, more water is added to the resulting extract, and the mixture separates into two phases: methanol/water (top phase) and chloroform (bottom phase). The lipids remain in the chloroform layer, and the more polar molecules such as proteins and sugars partition into the methanol/water layer (Fig. 10-25a).

Adsorption Chromatography Separates Lipids of Different Polarity

P4 Complex mixtures of tissue lipids can be fractionated by chromatographic procedures based on the different polarities of each class of lipid (Fig. 10-25b).

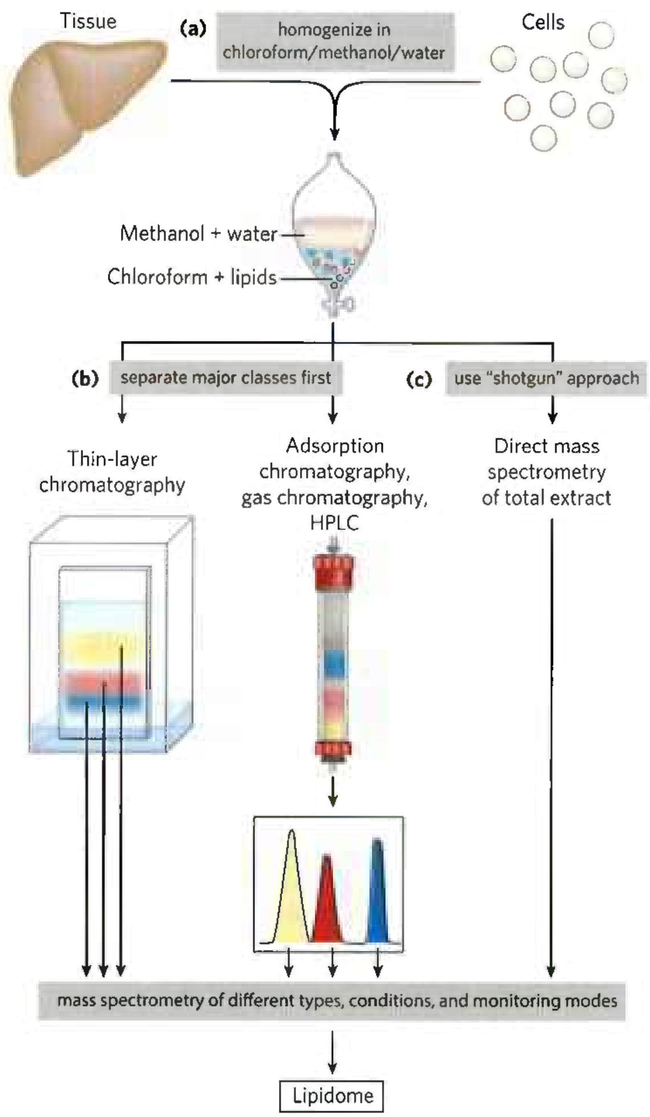

Tissue
(a)
homogenize in chloroform/methanol/water
Cells
Methanol + water
Chloroform + lipids
(b)
separate major classes first
(c)
use "shotgun" approach
Thin-layer chromatography
Adsorption chromatography, gas chromatography, HPLC
Direct mass spectrometry of total extract
mass spectrometry of different types, conditions, and monitoring modes
Lipidome

FIGURE 10-25 Common procedures in the extraction, separation, and identification of cellular lipids. (a) Tissue is homogenized in a chloroform/methanol/water mixture, which upon addition of water and removal of unextractable sediment by centrifugation yields two phases. (b) Major classes of extracted lipids in the chloroform phase may first be separated by thin-layer chromatography (TLC), in which lipids are carried up a silica gel–coated plate by a rising solvent front, with less-polar lipids traveling farther than more-polar or charged lipids, or by adsorption chromatography on a column of silica gel, through which solvents of increasing polarity are passed. For example, column chromatography with appropriate solvents can be used to separate closely related lipid species such as phosphatidylserine, phosphatidylglycerol, and phosphatidylinositol. Once separated, each lipid's complement of fatty acids can be determined by mass spectrometry. (c) Alternatively, in the "shotgun" approach, an unfractionated extract of lipids can be directly subjected to high-resolution mass spectrometry of different types and under different conditions to determine the total composition of all the lipids — that is, the lipidome.

In adsorption chromatography, an insoluble, polar material such as silica gel (a form of silicic acid, $Si(OH)_4$) is packed into a glass column, and the lipid mixture (in chloroform solution) is applied to the top of the column. (In high-performance liquid chromatography, or HPLC, the column is of smaller diameter and solvents are forced through the column under high pressure.) The polar lipids bind tightly to the polar silicic acid, but the neutral lipids pass directly through the column and emerge in the first chloroform wash. The polar lipids are then eluted, in order of increasing polarity, by washing the column with solvents of progressively higher polarity. Uncharged but polar lipids (cerebrosides, for example) are eluted with acetone, and very polar or charged lipids (such as glycerophospholipids) are eluted with methanol.

Thin-layer chromatography (TLC) on silicic acid employs the same principle (Fig. 10-25b). A thin layer of silica gel is spread onto a glass plate, to which it adheres. A small sample of lipids dissolved in chloroform is applied near one edge of the plate, which is dipped in a shallow container of an organic solvent or solvent mixture; the entire setup is enclosed in a chamber saturated with the solvent vapor. As the solvent rises on the plate by capillary action, it carries lipids with it. The less polar lipids move farthest, as they have less tendency to bind to the silicic acid. The separated lipids can be detected by spraying the plate with a dye (rhodamine) that fluoresces when associated with lipids, or by exposing the plate to iodine fumes. Iodine reacts reversibly with the double bonds in fatty acids, such that lipids containing unsaturated fatty acids develop a yellow or brown color. Several other spray reagents are also useful in detecting specific lipids. For subsequent analysis, regions containing separated lipids can be scraped from the plate and the lipids recovered by extraction with an organic solvent.

Gas Chromatography Resolves Mixtures of Volatile Lipid Derivatives

P4 Gas chromatography (GC) separates volatile components of a mixture according to their relative tendencies to dissolve in the inert material packed in the chromatography column or to volatilize and move through the column, carried by a current of an inert gas such as helium. Some lipids are naturally volatile, but most must first be derivatized to increase their volatility (that is, lower their boiling point). For an analysis of the fatty acids in a sample of phospholipids, the lipids are first transesterified: heated in a methanol/HCl or methanol/NaOH mixture to convert fatty acids esterified to glycerol into their methyl esters. These fatty acyl methyl esters are then loaded onto the GC column, and the column is heated to volatilize the compounds. Those fatty acyl esters that are most soluble in the column material will partition into (dissolve in) that material; the less-soluble lipids are carried by the stream of inert gas and emerge first from the column. The order of elution depends on the nature of the solid adsorbent in the column and on the boiling point of the components of the lipid mixture. With these techniques, mixtures of fatty acids of various chain lengths and various degrees of unsaturation can be completely resolved.

Specific Hydrolysis Aids in Determination of Lipid Structure

P4 Certain classes of lipids are susceptible to degradation under specific conditions. For example, all ester-linked fatty acids in triacylglycerols, phospholipids, and sterol esters are released by mild acid or alkaline treatment, and somewhat harsher hydrolysis conditions release amide-bound fatty acids from sphingolipids. Enzymes that specifically hydrolyze certain lipids are also useful in the determination of lipid structure. Phospholipases A, C, and D (Fig. 10-14) each split particular bonds in phospholipids and yield products with characteristic solubilities and chromatographic behaviors. Phospholipase C, for example, releases a water-soluble phosphoryl alcohol (such as phosphocholine from phosphatidylcholine) and a chloroform-soluble diacylglycerol, each of which can be characterized separately to determine the structure of the intact phospholipid. The combination of specific hydrolysis with characterization of the products by TLC, GC, or HPLC often allows determination of a lipid structure.

Mass Spectrometry Reveals Complete Lipid Structure

P4 To establish unambiguously the length of a hydrocarbon chain or the position of double bonds, mass spectrometric analysis of lipids or their volatile derivatives is invaluable. The chemical properties of similar lipids (for example, two fatty acids of similar length unsaturated at different positions, or two isoprenoids with different numbers of isoprene units) are very much alike, and their order of elution from the various chromatographic procedures often does not distinguish between them. When the eluate from a chromatography column is sampled by mass spectrometry, however, the components of a lipid mixture can be simultaneously separated and identified by their unique pattern of fragmentation (**Fig. 10-26**). With the increased resolution of mass spectrometry, it is possible to identify individual lipids in very complex mixtures without first fractionating the lipids in a crude extract. This "shotgun" method (Fig. 10-25c) avoids losses during the preliminary separation of lipid subclasses, and it is faster.

Lipidomics Seeks to Catalog All Lipids and Their Functions

As lipid biochemists have become aware of the thousands of different naturally occurring lipids, they have created a database analogous to the Protein Data Bank. The LIPID MAPS Lipidomics Gateway (www.lipidmaps.org) has its own classification system that places each lipid species in one of eight chemical categories, each designated by two letters (**Table 10-2**). Within each category, finer distinctions are indicated by numbered classes and subclasses.

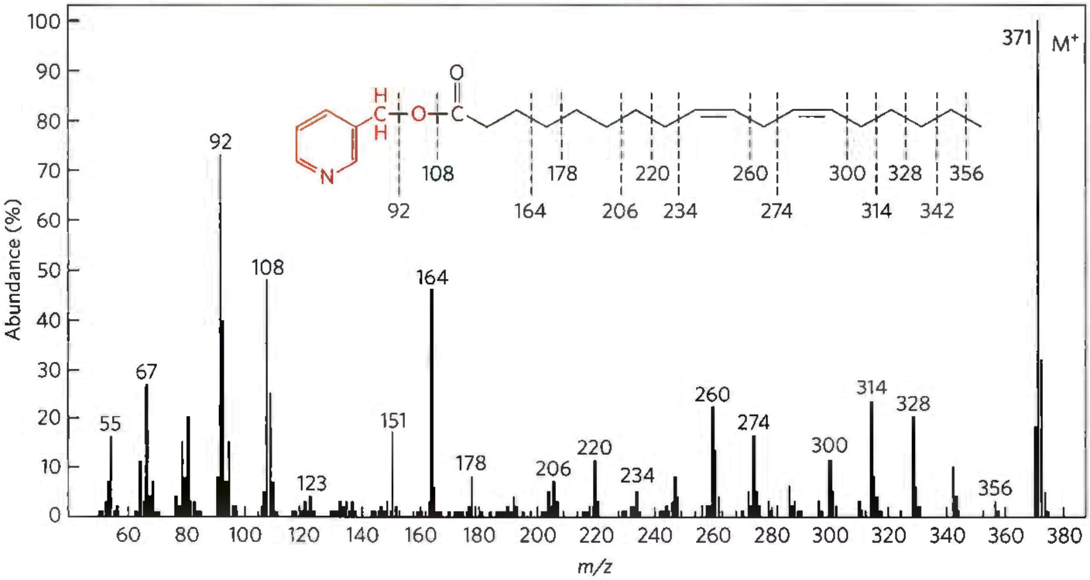


FIGURE 10-26 Determination of fatty acid structure by mass spectrometry. The fatty acid is first converted to a derivative that minimizes migration of the double bonds when the molecule is fragmented by electron bombardment. The derivative shown here is a picolinyl ester of linoleic acid—18:2($\Delta^{9,12}$) (M_r 371)—in which the alcohol is picolinol (red). When bombarded with a stream of electrons, this molecule is volatilized and converted to a parent ion (M^+; M_r 371), in which the N atom bears the positive charge, and a series of smaller fragments produced by breakage of C—C bonds in the fatty acid. The mass spectrometer separates these charged fragments according to their mass/charge ratio (m/z).

The prominent ions at $m/z = 92$, 108, 151, and 164 contain the pyridine ring of the picolinol and various fragments of the carboxyl group, showing that the compound is indeed a picolinyl ester. The molecular ion, M^+ ($m/z = 371$), confirms the presence of a C_{18} fatty acid with two double bonds. The uniform series of ions 14 atomic mass units (u) apart represents loss of each successive methyl and methylene group from the methyl end of the acyl chain (beginning at C-18; the right end of the molecule as shown here), until the ion at $m/z = 300$ is reached. This is followed by a gap of 26 u for the carbons of the terminal double bond, at $m/z = 274$; a further gap of 14 u for the C-11 methylene group, at $m/z = 260$; and so forth. By this means, the entire structure is determined, although these data alone do not reveal the configuration (cis or trans) of the double bonds. [Information from W. W. Christie, *Lipid Technol.* 8:64, 1996.]

TABLE 10-2 Eight Major Categories of Biological Lipids

Category	Category code	Examples
Fatty acids	FA	Oleate, stearoyl-CoA, palmitoylcarnitine
Glycerolipids	GL	Di- and triacylglycerols
Glycerophospholipids	GP	Phosphatidylcholine, phosphatidylserine, phosphatidyethanoloamine
Sphingolipids	SP	Sphingomyelin, ganglioside GM2
Sterol lipids	ST	Cholesterol, progesterone, bile acids
Prenol lipids	PR	Farnesol, geraniol, retinol, ubiquinone
Saccharolipids	SL	Lipopolysaccharide
Polyketides	PK	Tetracycline, erythromycin, aflatoxin B_1

For example, all glycerophosphocholines are GP01. The subgroup of glycerophosphocholines with two fatty acids in ester linkage is designated GP0101; the subgroup with one fatty acid ether-linked at position 1 and one ester-linked at position 2 is GP0102. The specific fatty acids are designated by numbers that give every lipid its own unique identifier, so that each individual lipid, including lipid types not yet discovered, can be unambiguously described in terms of a 12-character identifier, the LM_ID. One factor used in this classification system is the nature of the biosynthetic precursor. For example, prenol lipids (such as dolichols and vitamins E and K) are formed from isoprenyl precursors.

The eight chemical categories in Table 10-2 do not coincide perfectly with the less formal categorization according to biological function that we have used in this chapter. For example, the structural lipids of membranes include both glycerophospholipids and sphingolipids, which are separate categories in Table 10-2. Each method of classification has its advantages.

The application of mass spectrometric techniques with high throughput and high resolution can provide quantitative catalogs of all the lipids present in a specific cell type under particular conditions—the **lipidome**—and of the ways in which the lipidome changes with differentiation, disease such as cancer, or drug treatment. An animal cell contains more than a thousand different lipid species, each presumably having a specific function. These functions are known for a growing number of lipids, but the still largely unexplored lipidome offers a rich source of new problems for the next generation of biochemists and cell biologists to solve.

SUMMARY 10.4 *Working with Lipids*

- In the determination of lipid composition, lipids can first be extracted from tissues with organic solvents.
- Lipids in mixtures can be separated on the basis of their polarity and interactions with polar materials such as silica, using adsorption chromatography methods such as HPLC or TLC.
- GC volatilizes lipids so that they can be carried by a stream of inert gas and resolved based on their ability to partition into a soluble column material.
- Phospholipases specific for one of the bonds in a phospholipid can be used to generate simpler compounds for subsequent analysis.
- High-resolution mass spectrometry allows the analysis of crude mixtures of lipids without prefractionation—the "shotgun" approach.
- Lipidomics combines powerful analytical techniques to determine the full complement of lipids in a cell or a tissue (the lipidome) and to assemble annotated databases that allow comparisons between lipids of different cell types and under different conditions.

KEY TERMS

Terms in bold are defined in the glossary.

fatty acid	341
polyunsaturated fatty acid (PUFA)	343
omega-3 (ω-3) fatty acids	343
triacylglycerol	344
lipases	345
phospholipid	346
sterols	346
glycerophospholipid	346
ether lipid	348
plasmalogen	348
glycolipid	349
galactolipid	349
sphingolipid	350
ceramide	350
sphingomyelin	350
glycosphingolipid	350
cerebroside	350
globoside	351
ganglioside	351
sterol	352
cholesterol	352
bile acids	352
eicosanoid	355
prostaglandin (PG)	355
thromboxane (TX)	355
leukotriene (LT)	355
lipoxin (LX)	356
vitamin	356
vitamin D_3	357
cholecalciferol	357
vitamin A_1 (all-*trans*-retinol)	357
carotenoids	357
vitamin E	359
tocopherol	359
vitamin K	359
dolichol	360
polyketide	360
lipidome	364

PROBLEMS

1. **Operational Definition of Lipids** How is the definition of "lipid" different from the types of definitions used for other biomolecules such as amino acids, nucleic acids, and proteins?

2. **Structure of an Omega-3 Fatty Acid** The omega-3 fatty acid docosahexaenoic acid (DHA, $22{:}6(\Delta^{4,7,10,13,16,19})$) is the

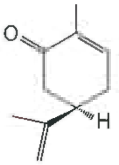
most abundant omega-3 fatty acid in the brain and an important component of breast milk. Draw the structure of this fatty acid.

3. Melting Points of Lipids The melting points of a series of 18-carbon fatty acids are stearic acid, 69.6 °C; oleic acid, 13.4 °C; linoleic acid, –5 °C; and linolenic acid, –11 °C.
(a) What structural aspect of these 18-carbon fatty acids can be correlated with the melting point?
(b) Draw all the possible triacylglycerols that can be constructed from glycerol, palmitic acid, and oleic acid. Rank them in order of increasing melting point.
(c) Branched-chain fatty acids are found in some bacterial membrane lipids. Would their presence increase or decrease the fluidity of the membrane (that is, give the lipids a lower or a higher melting point)? Why?

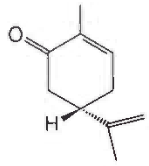
4. Catalytic Hydrogenation of Vegetable Oils Catalytic hydrogenation, used in the food industry, converts double bonds in the fatty acids of the oil triacylglycerols to $—CH_2—CH_2—$. How does this affect the physical properties of the oils?

5. Impermeability of Waxes What property of the waxy cuticles that cover plant leaves makes the cuticles impermeable to water?

6. Naming Lipid Stereoisomers Carvone, a member of the terpenoid family of chemicals, forms two enantiomers with quite different properties. One enantiomer, abundant in spearmint, smells sweet and minty. The other enantiomer, abundant in caraway seeds, smells spicy and of rye bread. Name the compounds abundant in spearmint and caraway seeds using the RS system.



Spearmint



Caraway

7. Chemical Reactivity of Lipids Soaps are salts of fatty acids and can be made by mixing triacylglycerols with a strong base such as NaOH. This saponification reaction produces glycerol and fatty acid salts. In a lab experiment, students saponify the triacylglycerol tristearin in the presence of ^{18}O-labeled water. What saponification reaction products will contain the ^{18}O label?

8. Hydrophobic and Hydrophilic Components of Membrane Lipids A common structural feature of membrane lipids is their amphipathic nature. For example, in phosphatidylcholine, the two fatty acid chains are hydrophobic and the phosphocholine head group is hydrophilic. Name the components that serve as the hydrophobic and hydrophilic units for each membrane lipid: **(a)** phosphatidylethanolamine **(b)** sphingomyelin **(c)** galactosylcerebroside **(d)** ganglioside **(e)** cholesterol.

9. Deducing Lipid Structure from Composition A biochemist completely digests a glycerophospholipid with a mixture of phospholipases A and D. HPLC and MS analysis reveals the presence of an amino acid of 105.09 Da, a saturated fatty acid of 256.43 Da, and an omega-3 monounsaturated fatty acid of 282.45 Da. Which amino acid does the glycerophospholipid contain? Draw the most likely structure of this glycerophospholipid.

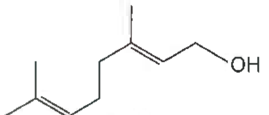
10. Deducing Lipid Structure from Molar Ratio of Components Complete hydrolysis of a glycerophospholipid yields glycerol, two fatty acids ($16{:}1(\Delta^9)$ and $16{:}0$), phosphoric acid, and serine in the molar ratio 1:1:1:1:1. Name this lipid and draw its structure.

11. Lipids in Blood Group Determination We note in Figure 10-13 that the structure of glycosphingolipids determines the blood groups A, B, and O in humans. It is also true that glycoproteins determine blood groups. How can both statements be true?

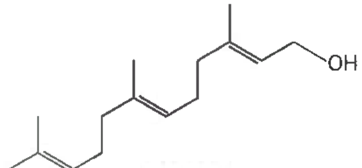
12. The Action of Phospholipases The venom of the Eastern diamondback rattler and the Indian cobra contains phospholipase A_2, which catalyzes the hydrolysis of fatty acids at the C-2 position of glycerophospholipids. The phospholipid breakdown product of this reaction is lysolecithin, which is derived from phosphatidylcholine. At high concentrations, this and other lysophospholipids act as detergents, dissolving the membranes of erythrocytes and lysing the cells. Extensive hemolysis may be life-threatening.
(a) All detergents are amphipathic. What are the hydrophilic and hydrophobic portions of lysolecithin?
(b) The pain and inflammation caused by a snake bite can be treated with certain steroids. What is the basis of this treatment?
(c) Though the high levels of phospholipase A_2 in venom can be deadly, this enzyme is necessary for a variety of normal metabolic processes. What are these processes?

13. Intracellular Messengers from Phosphatidylinositols The hormone vasopressin is an extracellular signal that activates a specific phospholipase C in the membrane. Cleavage of PIP_2 by phospholipase C generates two products. What are they? Compare their properties and their solubilities in water, and predict whether either would diffuse readily through the cytosol.

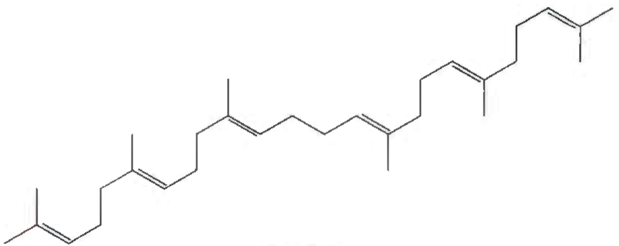
14. Isoprene Units in Isoprenoids Geraniol, farnesol, and squalene are called isoprenoids because they are synthesized from five-carbon isoprene units. In each compound, circle the five-carbon units representing isoprene units (see Fig. 10-22).



Geraniol



Farnesol



Squalene

15. Hydrolysis of Lipids Name the products of mild hydrolysis with dilute NaOH of **(a)** 1-stearoyl-2,3-dipalmitoylglycerol **(b)** 1-palmitoyl-2-oleoylphosphatidylcholine.

16. Effect of Polarity on Solubility Rank a triacylglycerol, a diacylglycerol, and a monoacylglycerol in order of decreasing solubility in water. Assume that each acylglycerol contains only palmitic acid.

17. Chromatographic Separation of Lipids Suppose that you apply a mixture of lipids to a silica gel column and then wash the column with increasingly polar solvents. The mixture consists of phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, cholesteryl palmitate (a sterol ester), sphingomyelin, palmitate, *n*-tetradecanol, triacylglycerol, and cholesterol. In what order will the lipids elute from the column? Explain your reasoning.

18. Identification of Unknown Lipids Johann Thudichum, who practiced medicine in London about 100 years ago, also dabbled in lipid chemistry in his spare time. He isolated a variety of lipids from neural tissue and characterized and named many of them. His carefully sealed and labeled vials of isolated lipids were rediscovered many years later.
(a) How would you confirm, using techniques not available to Thudichum, that the vials labeled "sphingomyelin" and "cerebroside" actually contain these compounds?
(b) How would you distinguish sphingomyelin from phosphatidylcholine by chemical, physical, or enzymatic tests?

BIOCHEMISTRY ONLINE

19. Using the LIPID MAPS Database to Find Solubility Information Lipidomics has identified thousands of cellular lipids. LIPID MAPS is an online database containing over 40,000 unique lipid structures, as well as information on the chemical and physical properties of each lipid (www.lipidmaps.org). One important parameter when working with lipids is log *P*, where *P* is the octanol:water partition coefficient, an indicator of lipophilicity.
(a) Look up cholesterol, sphingosine, linoleic acid, and stearic acid in LIPID MAPS and use the reported log *P* values to place them in order of increasing solubility in octanol.
(b) Pharmacologists often study log *P* values when developing new drugs. Why would knowing a drug's log *P* value be informative?

20. Characteristics of Lipid Transport Proteins Often when lipids are transported between different tissues, they are carried by proteins. In this exercise, you will explore the interactions between a lipid and a protein using the PDB (www.rcsb.org). Use the PDB identifier 2YG2 and study the structure of the complex between HDL-associated apolipoprotein M and sphingosine-1-phosphate. Navigate to 3D View: Structure to answer the following questions.
(a) What protein motif is adopted by apolipoprotein M?
(b) Which amino acid residues do you find lining the sphingosine binding pocket? What do they have in common?
(c) The phosphoryl group of sphingosine-1-phosphate is exposed on the surface of the protein. Why do you suppose it is important that the transport protein binds the hydrocarbon tail of sphingosine-1-phosphate but not necessarily the polar head group?

DATA ANALYSIS PROBLEM

21. Determining the Structure of the Abnormal Lipid in Tay-Sachs Disease Box 10-1, Figure 1, shows the pathway of breakdown of gangliosides in healthy (normal) individuals and in individuals with certain genetic diseases. Some of the data on which the figure is based were presented in a paper by Lars Svennerholm (1962). Note that the sugar Neu5Ac, *N*-acetylneuraminic acid, represented in the Box 10-1 figure as ◆, is a sialic acid.

Svennerholm reported that "about 90% of the monosialogangliosides isolated from normal human brain" consisted of a compound with ceramide, hexose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid in the molar ratio 1:3:1:1.
(a) Which of the gangliosides (GM1 through GM3 and globoside) in Box 10-1, Figure 1, fits this description? Explain your reasoning.
(b) Svennerholm reported that 90% of the gangliosides from a patient with Tay-Sachs disease had a molar ratio (of the same four components given above) of 1:2:1:1. Is this consistent with the Box 10-1 figure? Explain your reasoning.

To determine the structure in more detail, Svennerholm treated the gangliosides with neuraminidase to remove the *N*-acetylneuraminic acid. This resulted in an asialoganglioside that was much easier to analyze. He hydrolyzed it with acid, collected the ceramide-containing products, and determined the molar ratio of the sugars in each product. He did this for both the normal gangliosides and the Tay-Sachs gangliosides. His results are shown below.

Ganglioside	Ceramide	Glucose	Galactose	Galactosamine
Normal				
Fragment 1	1	1	0	0
Fragment 2	1	1	1	0
Fragment 3	1	1	1	1
Fragment 4	1	1	2	1
Tay-Sachs				
Fragment 1	1	1	0	0
Fragment 2	1	1	1	0
Fragment 3	1	1	1	1

(c) Based on these data, what can you conclude about the structure of the normal ganglioside? Is this consistent with the structure in Box 10-1? Explain your reasoning.
(d) What can you conclude about the structure of the Tay-Sachs ganglioside? Is this consistent with the structure in Box 10-1? Explain your reasoning.

Svennerholm also reported the work of other researchers who "permethylated" the normal asialoganglioside. Permethylation is the same as exhaustive methylation: a methyl group is added to every free hydroxyl group on a sugar. They found the following permethylated sugars: 2,3,6-trimethylglycopyranose; 2,3,4,6-tetramethylgalactopyranose; 2,4,6-trimethylgalactopyranose; and 4,6-dimethyl-2-deoxy-2-aminogalactopyranose.
(e) To which sugar of GM1 does each of the permethylated sugars correspond? Explain your reasoning.
(f) Based on all the data presented so far, what pieces of information about normal ganglioside structure are missing?

Reference

Svennerholm, L. 1962. The chemical structure of normal human brain and Tay-Sachs gangliosides. *Biochem. Biophys. Res. Comm.* 9:436–441.

Chapter 11

BIOLOGICAL MEMBRANES AND TRANSPORT

11.1 The Composition and Architecture of Membranes *367*

11.2 Membrane Dynamics *377*

11.3 Solute Transport across Membranes *385*

The first cell probably came into being when a membrane formed, enclosing a small volume of aqueous solution and separating it from the rest of the universe. Membranes define the external boundaries of cells and control the molecular traffic across that boundary; in eukaryotic cells, they also divide the internal space into discrete compartments to segregate processes and components. Proteins embedded in and associated with membranes organize complex reaction sequences and are central to both biological energy conservation and cell-to-cell communication.

In this chapter we focus on these principles that underlie the structure and function of biological membranes:

P1 **The biological membrane is a lipid bilayer with proteins of various functions (enzymes, transporters) embedded in or associated with the bilayer.** The hydrophobic effect stabilizes structures (lipid bilayers and vesicles) in which lipids with some polar and some nonpolar regions can protect their nonpolar regions from interaction with the very polar solvent, water. Membrane proteins are associated with the lipid bilayer more-or-less tightly, and proteins and lipids are both allowed limited lateral motion in the plane of the bilayer.

P2 **All of the internal membranes of cells are part of an interconnected, functionally specialized, and dynamic endomembrane system.** Proteins and lipids synthesized in the endoplasmic reticulum move through the Golgi apparatus, and they are targeted to the membranes of organelles or to the plasma membrane, where they provide the essential properties of these structures. During this membrane trafficking, some proteins are covalently altered, and some lipids are segregated into different organelles or are concentrated in one of the bilayer leaflets. Rafts are functionally specialized regions with unique lipid and protein compositions.

P3 **Although the lipid bilayer is impermeable to charged or polar solutes, cells of all kinds have many membrane transporters and ion channels that catalyze transmembrane movement of specific solutes.** Some transporters merely speed the movement of solutes in the direction that simple diffusion takes them, whereas others use an energy source to move solutes against a concentration gradient.

11.1 The Composition and Architecture of Membranes

We begin our discussion of biological membranes by looking at the stable bilayer structures formed spontaneously by phospholipids in water. In biological membranes we see this basic lipid bilayer combined with a wide array of membrane proteins specialized for one of the many roles that membranes play in cells. Each intracellular compartment has membranes with specific protein and lipid content, uniquely disposed across the two leaflets of the bilayer. The intracellular membranes together make up a dynamic endomembrane system that synthesizes and distributes membrane components to create functionally specialized cell compartments. Depending on its function, a protein may form one or several transmembrane segments, or it may adhere to the membrane bilayer through lipids attached to the protein, or it may alternate between membrane-associated and free forms.

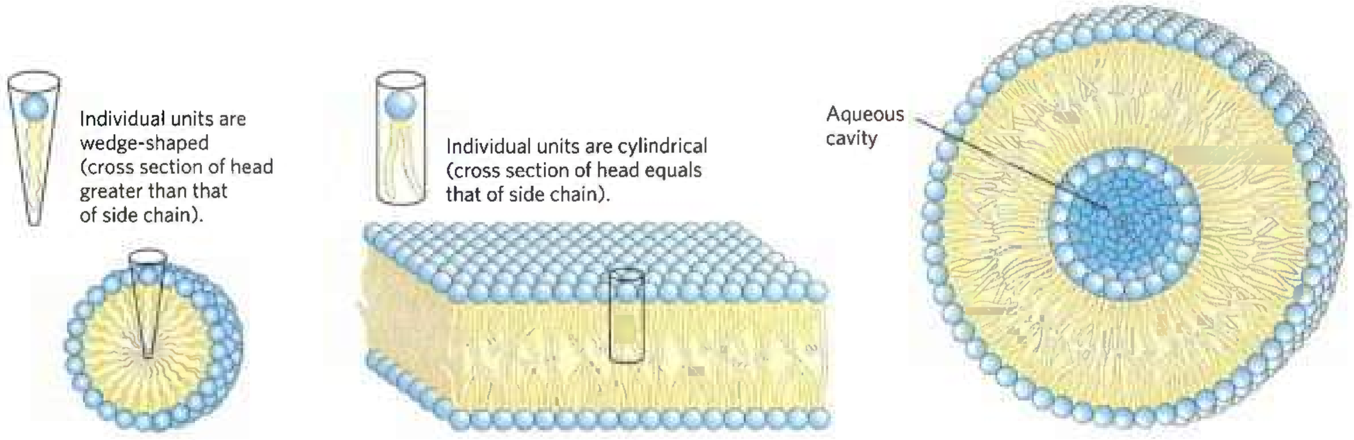
The Lipid Bilayer Is Stable in Water

Glycerophospholipids, sphingolipids, and sterols are virtually insoluble in water. Recall that glycerophospholipids contain two long-chain fatty acids (which are hydrophobic) and a head group consisting of glycerol and one of several polar or charged substituents such as phosphocholine or phosphoethanolamine (see Fig. 10-8). Sphingolipids are constructed from a long-chain alkyl amine (sphingosine), a saturated long-chain fatty acid, and a polar head group that may be as simple as phosphocholine or may be a complex oligosaccharide (see Fig. 10-11). Sterols (cholesterol in animal membranes) have a very nonpolar steroid nucleus of four fused rings, and a polar hydroxyl group at one end of the ring system (see Fig. 10-15). Each type of membrane lipid has its particular effect on membrane structure and function. When mixed with water, these lipids spontaneously form microscopic lipid aggregates, clustering together, with their hydrophobic moieties in contact with each other and their hydrophilic groups in contact with the surrounding water. The clustering reduces the amount of hydrophobic surface exposed to water. This minimizes the number of molecules in the shell of ordered water at the lipid-water interface (see Fig. 2-7), resulting in an increase in entropy. This hydrophobic effect provides the thermodynamic driving force for the formation and maintenance of these clusters of lipid molecules. The term **hydrophobic interactions** is sometimes used to describe the clustering of hydrophobic molecular surfaces in an aqueous environment, but the molecules are not interacting chemically; they are simply finding the lowest-energy environment by reducing the hydrophobic, or nonpolar, surface area exposed to water.

Depending on the precise conditions and the nature of the lipids, several types of lipid aggregate can form when amphipathic lipids are mixed with water (**Fig. 11-1**). **Micelles** are spherical structures that contain anywhere from a few dozen to a few thousand amphipathic molecules. These molecules are arranged with their hydrophobic regions aggregated in the interior, where water is excluded, and their hydrophilic head groups at the outer surface, in contact with water. Micelle formation is favored when the cross-sectional area of the head group is greater than that of the acyl side chain(s), as in free fatty acids, lysophospholipids (phospholipids lacking one fatty acid), and many detergents, such as sodium dodecyl sulfate (SDS; p. 88).

A second type of lipid aggregate in water is the **bilayer**, in which two lipid monolayers (leaflets) form a two-dimensional sheet. Bilayer formation is favored if the cross-sectional areas of the head group and acyl side chain(s) are similar, as in glycerophospholipids and sphingolipids. The hydrophobic portions in each monolayer, excluded from water, make contact with each other. The hydrophilic head groups interact with water at one or the other surface of the bilayer. Because the hydrophobic regions at its edges (Fig. 11-1b) are in contact with water, a bilayer sheet is relatively unstable and spontaneously folds back on itself to form a hollow sphere, called a **vesicle** or liposome (Fig. 11-1c). The continuous surface of vesicles eliminates exposed hydrophobic regions, allowing bilayers to achieve maximal stability in their aqueous environment. Vesicle formation also creates a separate internal aqueous compartment (the vesicle lumen). It is likely that the antecedents to the first living cells resembled lipid vesicles, their aqueous contents segregated from their surroundings by a lipid bilayer.

Studies of lipid bilayers in vitro (**Fig. 11-2**) show that the hydrocarbon core of the bilayer, made up of the $—CH_2—$ and $—CH_3$ of the fatty acyl groups, is about as nonpolar as decane, and about 3 nm (30 Å) thick, roughly the width of two extended fatty acyl chains. Biological membranes, to which we turn next, are 50–80 Å thick, when proteins protruding on either side are included.



Individual units are wedge-shaped (cross section of head greater than that of side chain).

Individual units are cylindrical (cross section of head equals that of side chain).

(a) Micelle

(b) Bilayer

(c) Vesicle

FIGURE 11-1 Amphipathic lipid aggregates that form in water. (a) In micelles, the hydrophobic chains of the fatty acids are sequestered at the core of the sphere. There is virtually no water in the hydrophobic interior. (b) In an open bilayer, all acyl side chains except those at the edges of the sheet are protected from interaction with water. (c) When a two-dimensional bilayer folds on itself, it forms a closed bilayer, a three-dimensional hollow vesicle (liposome) enclosing an aqueous cavity.

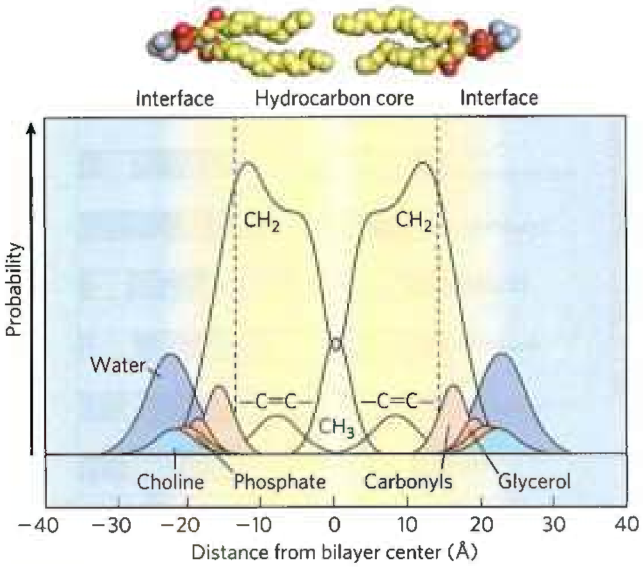


FIGURE 11-2 Distribution of membrane lipids across an artificial membrane. The membrane, composed of pure phosphatidylcholine in which both fatty acids are oleic acid ($18:1\Delta^9$), was studied with x-ray and neutron diffraction. The *y* axis shows the probability that one of the chemical moieties will be found at the position indicated on the *x* axis. The phosphatidylcholine molecules are shown at the same scale as the *x* axis. Fatty acyl chains in the hydrocarbon core are fluid and are about as nonpolar as decane. At either side, a polar region about 15 Å from the center of the bilayer contains the fatty acyl carbonyls. The glycerol, choline, and phosphate that make up the polar head group are at 18–20 Å, outside the hydrocarbon core and in contact with water. [Information from A. Rath and C. M. Deber, *Annu. Rev. Biophys.* 41:135, 2012, Fig. 5a, based on M. C. Wiener and S. H. White, *Biophys. J.* 61:434, 1992.]

Bilayer Architecture Underlies the Structure and Function of Biological Membranes

In the generalized membrane in **Figure 11-3**, phospholipids form a bilayer in which proteins are embedded, their hydrophobic domains in contact with the fatty acyl chains of membrane lipids. Some proteins protrude from only one side of the membrane; others have domains exposed on both sides. The orientation of both lipids and proteins in the bilayer is asymmetric, giving the membrane structural and functional "sidedness." **P1** The individual lipid and protein units in a membrane form a **fluid mosaic** with a pattern that, unlike a mosaic of ceramic tile and mortar, is able to change as lipids and proteins move in the plane of the membrane, while maintaining the permeability barrier to polar and charged solutes.

Membranes are not merely passive barriers. They are flexible, self-repairing, and selectively permeable. Their flexibility permits the shape changes that accompany cell growth and movement (such as amoeboid movement). With their ability to break and reseal, two membranes can fuse, as in exocytosis, or a single membrane-enclosed compartment can undergo fission to yield two sealed compartments, as in endocytosis or cell division, without creating gross leaks through cellular surfaces. Their selective permeability allows them to serve as molecular gatekeepers.

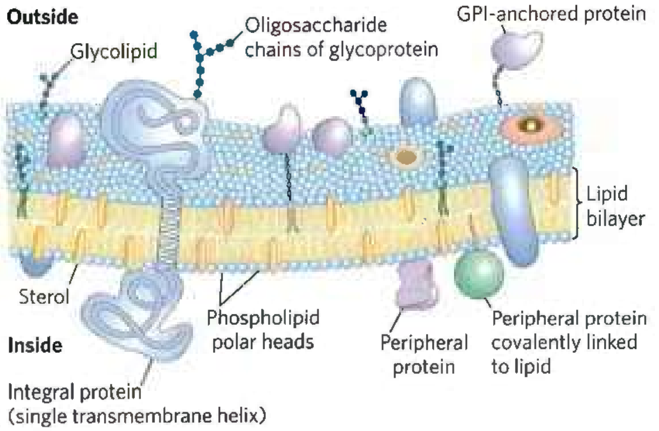

Outside
Glycolipid
Oligosaccharide chains of glycoprotein
GPI-anchored protein
Lipid bilayer
Sterol
Phospholipid polar heads
Peripheral protein
Peripheral protein covalently linked to lipid
Inside
Integral protein (single transmembrane helix)

FIGURE 11-3 Fluid mosaic model for plasma membrane structure. In this simplified and general model, the fatty acyl chains in the interior of the membrane form a fluid, hydrophobic region. Integral proteins float in this sea of lipid, with their nonpolar amino acid side chains protected from interaction with water.

These membrane functions are accomplished primarily by a broad array of proteins and enzymes in and on membranes. At the cell surface, transporters move specific organic solutes and inorganic ions across the membrane; receptors sense extracellular signals and trigger molecular changes in the cell; ion channels mediate electrical signaling between cells; and adhesion molecules hold neighboring cells together. The relative proportions of protein and lipid vary with the type of membrane, reflecting the diversity of biological roles. For example, certain neurons have a myelin sheath—an extended plasma membrane that wraps around the cell many times and acts as a passive electrical insulator. The myelin sheath consists primarily of lipids (good insulators). In contrast, the plasma membranes of bacteria and the membranes of mitochondria and chloroplasts, the sites of many enzyme-catalyzed processes, contain more protein than lipid (in mass per total mass).

Within the cell, membranes organize cellular processes such as the synthesis of lipids and certain proteins and the energy transductions that produce ATP in mitochondria and chloroplasts. Proteins associated with membranes are essentially confined to two-dimensional space, where intermolecular collisions of membrane proteins and lipids are far more probable than in the three-dimensional cytoplasm, so the efficiency of enzyme-catalyzed processes organized within membranes can be vastly increased.

The Endomembrane System Is Dynamic and Functionally Differentiated

P2 In eukaryotic cells, the endoplasmic reticulum (ER), Golgi apparatus, lysosomes, and various small vesicles that carry lipids and proteins throughout the cell are each surrounded and defined by a single membrane.

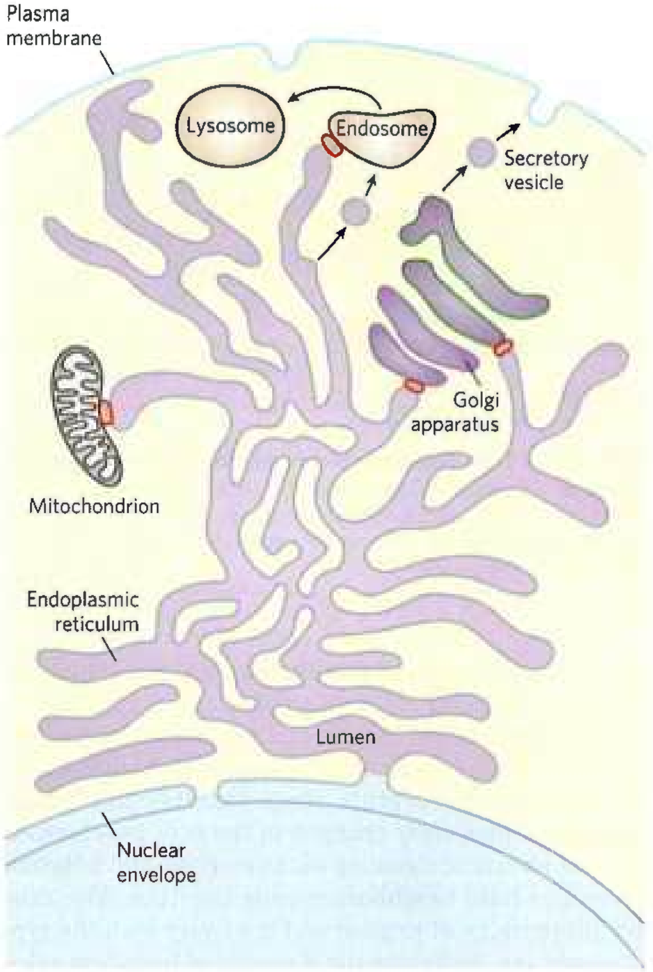


FIGURE 11-4 Trafficking in the endomembrane system of an animal cell. Lipids and proteins move from the site of their synthesis (ER) through the Golgi apparatus to the cell surface (or to organelles such as lysosomes). Mitochondria (and in plants, chloroplasts) are also part of the endomembrane system. Small vesicles bud off the ER, move to and fuse with the cis Golgi apparatus, exit the trans Golgi apparatus as secretory or transport vesicles, and fuse with the plasma membrane or with endosomes, giving rise to lysosomes. Individual lipid molecules can be carried throughout the cell by lipid transfer proteins (LTPs), which act at membrane contact points, shown here in red.

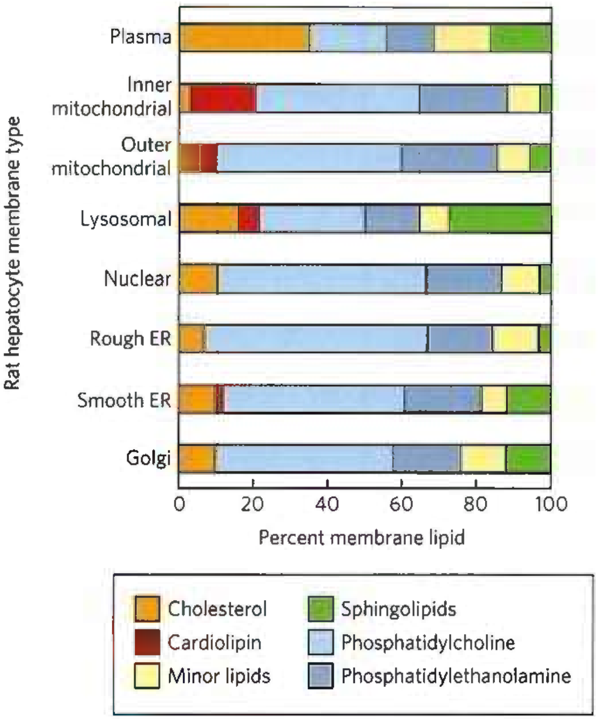


FIGURE 11-5 Lipid composition of the plasma and organelle membranes of a rat hepatocyte. The functional specialization of each membrane type is reflected in its unique lipid composition. Cholesterol is prominent in plasma membranes but barely detectable in mitochondrial membranes. Cardiolipin is a major component of the inner mitochondrial membrane but not of the plasma membrane. Phosphatidylserine, phosphatidylinositol, and phosphatidylglycerol are relatively minor components of most membranes but serve critical functions; phosphatidylinositol and its derivatives, for example, are important in signal transductions triggered by hormones. Sphingolipids, phosphatidylcholine, and phosphatidylethanolamine are present in most membranes but in varying proportions. Glycolipids, which are major components of the chloroplast membranes of plants, are virtually absent in animal cells.

This endomembrane system (**Fig. 11-4**) also includes three organelles with double membranes: the nucleus, mitochondrion, and (in plants) chloroplast. (Recall the likely origin of mitochondria and chloroplasts by an endosymbiont mechanism shown in Fig. 1-37.) The convoluted and tubular ER is dynamic and extends throughout the cell, touching the other compartments at specific membrane contact points. Although each compartment is closed, they actively exchange proteins and lipids in several ways.

Cells have mechanisms to target specific lipids to particular organelles. Each species, each tissue or cell type, and the organelles of each cell type have a characteristic set of membrane lipids. Plasma membranes, for example, are enriched in cholesterol and sphingolipids, but they contain no detectable cardiolipin (**Fig. 11-5**); mitochondrial membranes are very low in cholesterol and sphingolipids, but they contain most of the cell's phosphatidylglycerol and cardiolipin, which are synthesized within the mitochondria. Cardiolipin is specifically required for correct assembly of the respiratory complexes of mitochondria. In all but a few cases, the functional significance of these different combinations of lipids is not yet known.

Most membrane lipids and proteins are synthesized in the ER, and from there they move to their destination organelles or to the plasma membrane. In this process of membrane trafficking, small membrane vesicles bud from the ER, then move to and fuse with the cis Golgi apparatus. As lipids and proteins move across the Golgi apparatus to its trans side, they undergo covalent modifications required for their cellular function. In many cases, these covalent alterations act as molecular ZIP codes, dictating the eventual location of the mature protein.

P2 Membrane trafficking achieves striking changes in lipid composition and disposition across the bilayer (**Fig. 11-6**). Phosphatidylcholine is the principal phospholipid in the lumenal leaflet of the Golgi membrane,

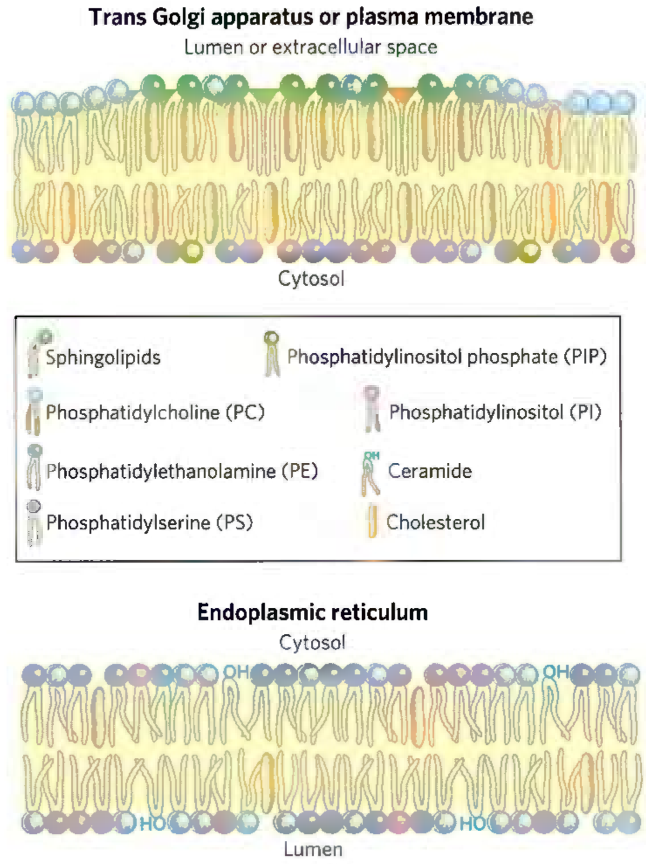

Trans Golgi apparatus or plasma membrane
Lumen or extracellular space
Cytosol
Sphingolipids
Phosphatidylinositol phosphate (PIP)
Phosphatidylcholine (PC)
Phosphatidylinositol (PI)
Phosphatidylethanolamine (PE)
Ceramide
Phosphatidylserine (PS)
Cholesterol
Endoplasmic reticulum
Cytosol
OH
OH
HO
HO
Lumen

FIGURE 11-6 Change in lipid composition of secretory vesicles with their passage through the Golgi apparatus. Both the lipid composition of the bilayer and the disposition of specific lipids between inner and outer leaflets change remarkably as a result of vesicle trafficking. In the trans Golgi apparatus and plasma membrane, sphingolipids are greatly enriched in the leaflet facing the lumen or extracellular space, and phosphatidylserine, phosphatidylethanolamine, and the inositol phospholipids are almost exclusively in the cytosolic leaflet. The plasma membrane is also enriched with cholesterol. [Information from G. Drin, *Annu. Rev. Biochem.* 83:51, 2014, Fig. 1.]

but in transport vesicles leaving the trans Golgi apparatus, phosphatidylcholine has largely been replaced by sphingolipids and cholesterol, which, following fusion of the transport vesicles with the plasma membrane, make up the majority of the lipids in the outer leaflet of the cell's plasma membrane. Plasma membrane lipids are asymmetrically distributed between the two leaflets of the bilayer. Choline-containing lipids (phosphatidylcholine and sphingomyelin) are typically found in the outer (extracellular, or exoplasmic) leaflet, whereas phosphatidylserine, phosphatidylethanolamine, and the phosphatidylinositols are almost exclusively in the inner (cytoplasmic) leaflet. Here the negatively charged serine and inositol phosphate head groups can interact electrostatically with positively charged regions of peripheral or amphitropic membrane proteins (described below).

A second route for redistributing lipids from their site of synthesis to their destination membrane is via **lipid transfer proteins** (**LTPs**; **Fig. 11-7**), which occur universally in various forms. LTPs are soluble in water, but they have a hydrophobic lipid-binding pocket in which they carry a lipid from one membrane to another through the cytosol. Some LTPs are bispecific: they carry, for example, a molecule of cholesterol to the plasma membrane, and they return with a phosphatidylinositol molecule. One class of lipid transfer protein forms a hydrophobic tunnel between two membranes at their contact points. In some cases, ATP is needed to drive the process, supplied by an ATP-binding cassette (ABC) transporter (described below). The rate of lipid movement from one membrane to another in vivo is significantly greater than the rate of vesicle budding and fusion.

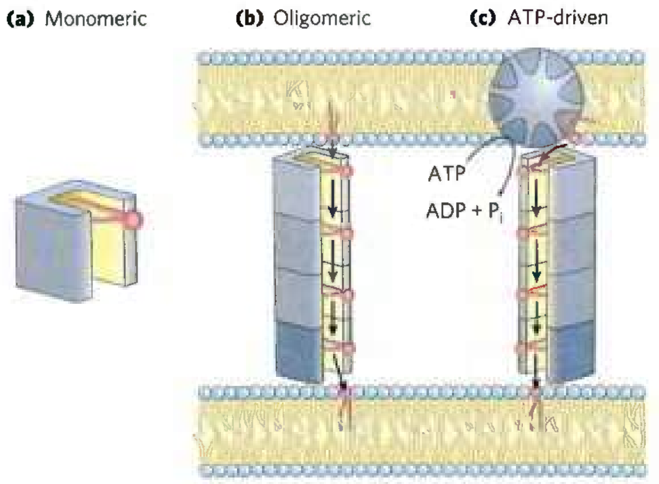


FIGURE 11-7 Lipid transfer protein (LTP) action. (a) Lipid transfer proteins move lipids and other nonpolar solutes through the very polar cytosol by providing a hydrophobic groove or pocket into which the hydrophobic solute can fit, with its nonpolar regions masked from the surrounding water. (b) Some LTPs form multisubunit structures to generate a hydrophobic groove long enough to connect two separate membranes at contact points (see Fig. 11-5). (c) To move lipid molecules against a concentration gradient (say, from a leaflet poor in phosphatidylserine to one relatively rich in it), the LTP is coupled to an ATP-dependent pump. [Information from L. H. Wong et al., *Nat. Rev. Mol. Cell Biol.* 20:85, 2019, Fig. 2.]

Finally, once phospholipids reach their destination membrane, they can undergo enzymatic remodeling of their fatty acid constituents, a subject discussed in Chapter 21.

Membrane Proteins Are Receptors, Transporters, and Enzymes

P2 The protein composition of membranes reflects each membrane's functional specialization. One large group of membrane proteins are the receptors for extracellular signals such as hormones and changes in membrane potential. Hundreds of membrane proteins are transporters that carry specific polar or charged compounds—sugars, amino acids, vitamins, a wide variety of metabolic intermediates, minerals (Mg^{2+}, Ca^{2+}, Na^+, K^+), and trace metal ions (Mn^{2+}, Ni^{2+}, Co^{2+})—across the plasma membrane or between organelles. All transporters and many receptors must span the membrane at least once to play their roles. Some membrane-associated enzymes also span the membrane, but many carry out their catalytic functions on one side or the other of their membrane setting, partially penetrating just one leaflet of the bilayer.

The synthesis and delivery to the site of function are more complicated for membrane proteins than for typical cytosolic proteins. Many of the proteins destined for the plasma membrane undergo extensive posttranslational modification as they pass through the ER and the Golgi apparatus. One covalent modification is the glycosylation (attachment of oligosaccharides) of proteins bound for the plasma membrane. In glycophorin, a glycoprotein of the erythrocyte plasma membrane, 60% of the mass consists of complex oligosaccharides covalently attached to specific amino acid residues (**Fig. 11-8**). For such glycoproteins, Ser, Thr, and Asn residues are the most common points of carbohydrate attachment (see Fig. 7-27), and the carbohydrates are invariably on the outer face of the plasma membrane. Another posttranslational modification of some proteins is the covalent attachment of one or more lipids, which serve as hydrophobic anchors to hold a protein to the membrane or as tags to target the protein to a specific location.

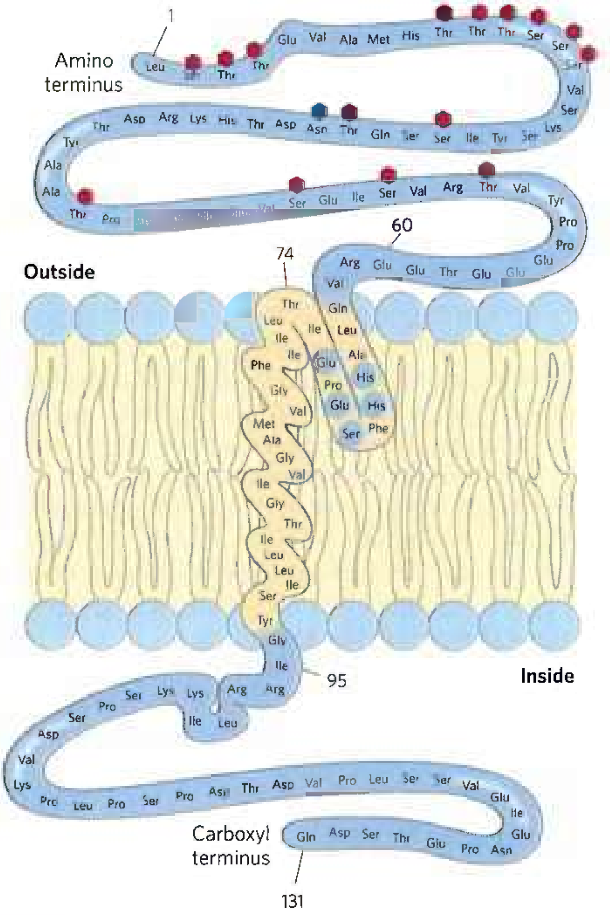


FIGURE 11-8 Transbilayer disposition of glycophorin in an erythrocyte. One hydrophilic domain, containing all the sugar residues, is on the outer surface, and another hydrophilic domain protrudes from the inner face of the membrane. Each red hexagon represents a tetrasaccharide (containing two Neu5Ac (sialic acid), Gal, and GalNAc) *O*-linked to a Ser or Thr residue; the blue hexagon represents an oligosaccharide *N*-linked to an Asn residue. The oligosaccharides are much larger than the hexagons used to represent them. A segment of 19 hydrophobic residues (residues 75 to 93) forms an α helix that traverses the membrane bilayer (see Fig. 11-13a). The segment from residues 64 to 74 has some hydrophobic residues and probably penetrates the outer face of the lipid bilayer, as shown. The functional unit of glycophorin is a homodimer; for clarity we show only one of the subunits here. [Information from V. T. Marchesi et al., *Annu. Rev. Biochem.* 45:667, 1976.]

Membrane Proteins Differ in the Nature of Their Association with the Membrane Bilayer

Integral membrane proteins are firmly embedded within the lipid bilayer and are removable only by agents such as detergents or organic solvents that overcome hydrophobic interactions (**Fig. 11-9**). **Peripheral membrane proteins** associate with the membrane through electrostatic interactions and hydrogen bonding with hydrophilic domains of integral proteins and with the charged head groups of membrane lipids. In the laboratory, they can be released from their membrane association by relatively mild treatments that interfere with electrostatic interactions or that break hydrogen bonds. **Amphitropic proteins** associate reversibly with membranes and are therefore found both in the membrane and in the cytosol. Their affinity for membranes results in some cases from the protein's noncovalent interaction with another membrane protein or lipid, and in other cases from the presence of one or more covalently attached lipids (see Fig. 11-16).

The firm attachment of integral proteins to membranes is the result of the hydrophobic interaction between the nonpolar core of the lipid bilayer and nonpolar side chains of the protein. **Monotopic** integral proteins have small hydrophobic domains that interact with only a single leaflet of the membrane (**Fig. 11-10**). These domains, which make up as little as a few percent of the protein's total mass, provide enough hydrophobic surface to hold the proteins firmly to the membrane. **Bitopic** proteins span the bilayer once, extending on either surface. They have a single hydrophobic sequence somewhere in the molecule. Glycophorin (Fig. 11-8) is bitopic. **Polytopic** proteins cross the membrane several times. They have multiple hydrophobic sequences, each of which, when in the α-helical conformation, is long enough (about 20 residues) to span the lipid bilayer. (Recall from Worked Example 4-1 that each residue in an α helix adds 1.5 Å to its length.)

One of the best-studied polytopic proteins, bacteriorhodopsin, has seven very hydrophobic internal sequences and spans the lipid bilayer seven times. Bacteriorhodopsin is a light-driven proton pump densely packed in regular arrays in the purple membrane of the bacterium *Halobacterium salinarum*. X-ray crystallography and cryo-electron microscopy reveal a structure in which each of the seven α-helical segments crosses the

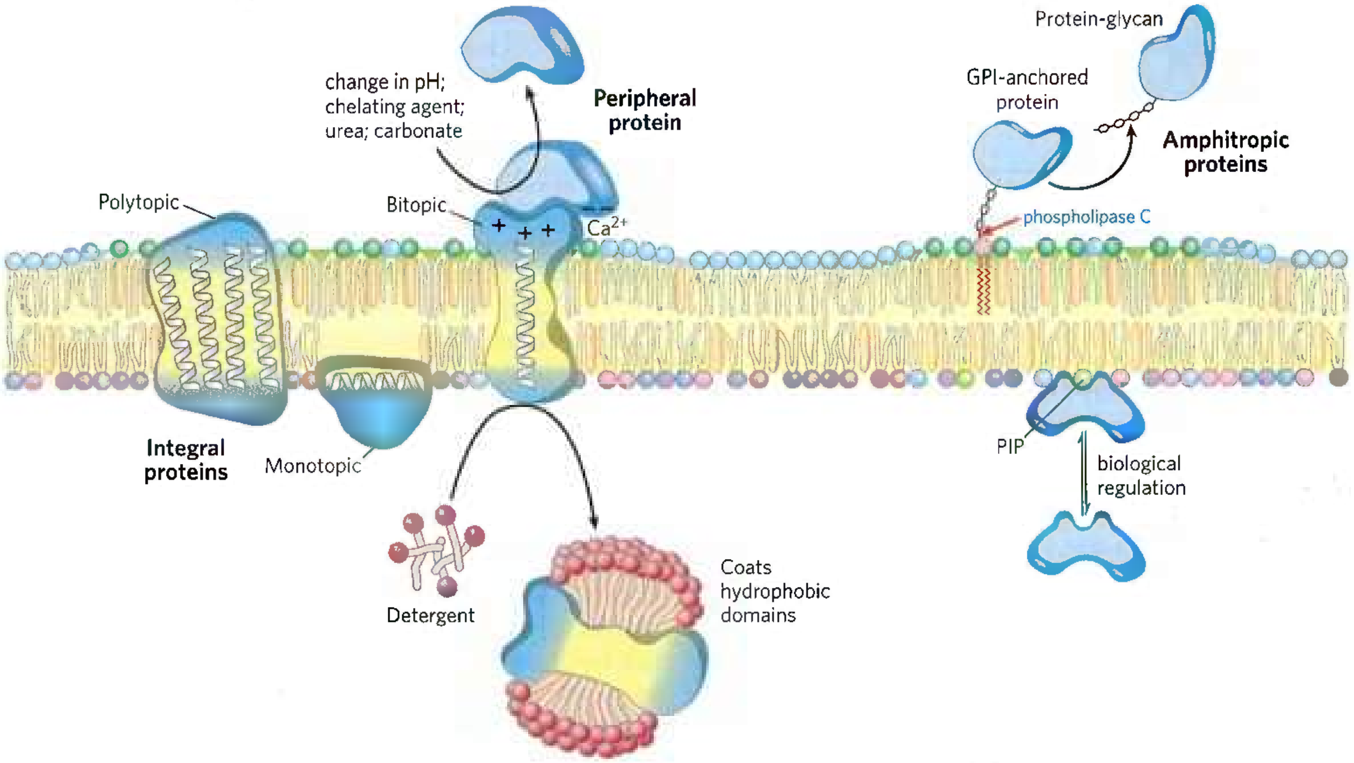

change in pH;
chelating agent;
urea; carbonate
Peripheral protein
Polytopic
Bitopic
+ + +
Ca^{2+}
Integral proteins
Monotopic
Detergent
Coats hydrophobic domains
Protein-glycan
GPI-anchored protein
Amphitropic proteins
phospholipase C
PIP
biological regulation

FIGURE 11-9 Integral, peripheral, and amphitropic proteins. Membrane proteins can be operationally distinguished by the conditions required to release them from the membrane. Integral proteins can only be removed from membranes with harsh treatment such as detergent. Peripheral proteins are more loosely associated. Amphitropic proteins have a reversible association with membranes that depends on a regulatory process, such as reversible palmitoylation or phosphorylation.

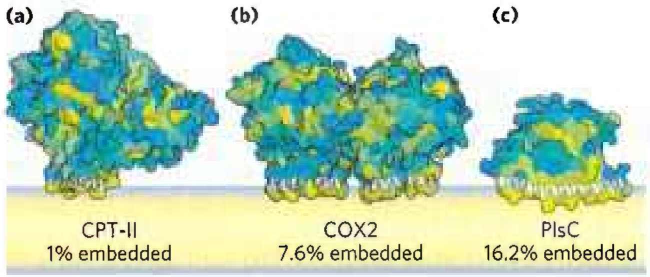

(a)
(b)
(c)
CPT-II
1% embedded
COX2
7.6% embedded
PlsC
16.2% embedded

FIGURE 11-10 Monotopic proteins penetrate only one leaflet. Three typical monotopic membrane proteins have relatively little of their structure (1% to 16%) associated with lipids in one leaflet of the bilayer. They are colored to show surface hydrophobicity, with yellow hydrophobic, blue hydrophilic, and hues of green in between. Each of these proteins catalyzes a reaction that involves a hydrophobic substrate. (a) CPT-II is a mammalian carnitine palmitoyltransferase II; (b) COX2 is a mammalian prostaglandin H2 synthase-1; (c) PlsC is a bacterial acyltransferase (1-acyl-*sn*-glycerol-6-phosphate acyltransferase). [Data from (a) PDB ID 2H4T, Y. S. Hsiao et al., *Biochem. Biophys. Res. Commun.* 346:974, 2006; (b) PDB ID 1CQE, D. Picot et al., *Nature* 367:243, 1994; (c) PDB ID 5KYM, R. M. Robertson et al., *Nat. Struct. Mol. Biol.* 24:666, 2017.]

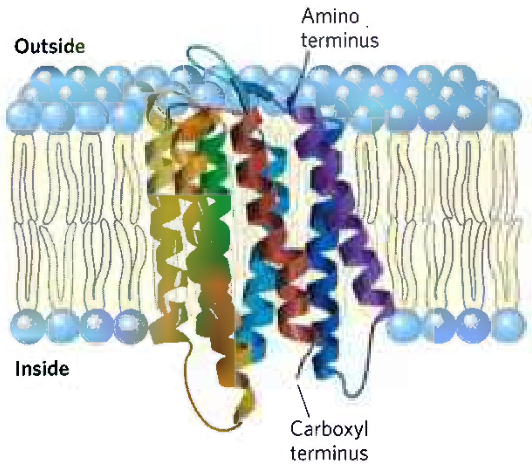

Outside
Amino terminus
Inside
Carboxyl terminus

FIGURE 11-11 Bacteriorhodopsin, a membrane-spanning protein. The single polypeptide chain folds into seven hydrophobic α helices, each of which traverses the lipid bilayer roughly perpendicular to the plane of the membrane. The seven transmembrane helices are clustered, and the space around and between them is filled with the acyl chains of membrane lipids. The light-absorbing pigment retinal (see Fig. 10-20) is buried deep within the membrane in contact with several of the helical segments (not shown). The helices are colored to correspond with the hydropathy plot in Figure 11-13. [Data from PDB ID 2AT9, K. Mitsuoka et al., *J. Mol. Biol.* 286:861, 1999.]

lipid bilayer, and all are connected by nonhelical loops at the inner and outer faces of the membrane (**Fig. 11-11**). In the amino acid sequence of bacteriorhodopsin, seven segments of about 20 hydrophobic residues can be identified, each forming an α helix that spans the bilayer. The seven helices are clustered together and oriented not quite perpendicular to the bilayer plane, a motif that (as we shall see in Chapter 12) is very common in membrane proteins involved in signal reception. The hydrophobic interaction keeps the nonpolar amino acid residues firmly anchored among the fatty acyl groups of the membrane lipids in the membrane's lipid core.

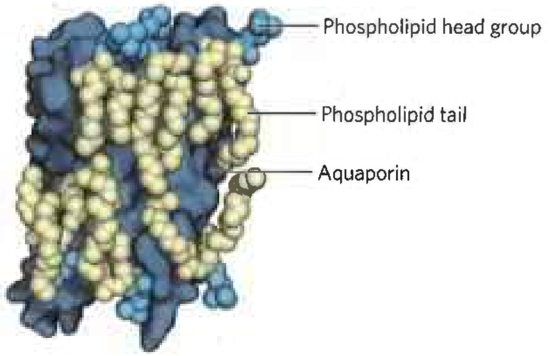


FIGURE 11-12 Lipid annuli associated with an integral protein. The crystal structure of sheep aquaporin, a transmembrane water channel, includes a shell of phospholipids positioned with their head groups (blue) at the expected positions on the inner and outer membrane surfaces and their hydrophobic acyl chains (gold) intimately associated with the surface of the protein exposed to the bilayer. The lipid forms a "grease seal" around the protein, which is depicted by a dark blue surface representation. [Data from PDB ID 2B6O, T. Gonen et al., *Nature* 438:633, 2005.]

Many membrane proteins co-crystalize with phospholipid molecules, which are presumed to be positioned in the native membranes as they are in the protein crystals. Many of these phospholipid molecules lie on the protein surface, their head groups interacting with polar amino acid residues at the inner and outer membrane–water interfaces and their side chains associated with nonpolar residues. Other phospholipids are found at the interfaces between monomers of multisubunit membrane proteins, where they form a "grease seal" (**Fig. 11-12**).

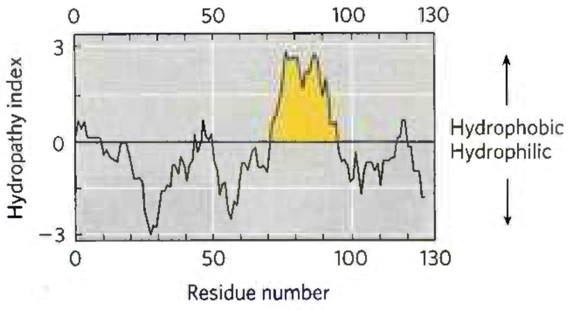
The Topology of an Integral Membrane Protein Can Often Be Predicted from Its Sequence

Determination of the location of a membrane protein relative to the bilayer—that is, its topology—is generally much more difficult than determining its amino acid sequence, either directly or by gene sequencing. The amino acid sequences of many thousands of membrane proteins are known, but fewer three-dimensional structures have been established by x-ray crystallography or cryo-electron microscopy. The presence of unbroken sequences of more than 20 hydrophobic residues in a membrane protein is commonly taken as evidence that these sequences traverse the lipid bilayer, acting as hydrophobic anchors or forming transmembrane channels. All known integral proteins have at least one such sequence. Application of this logic to entire genomic sequences leads to the conclusion that in many organisms, 20% to 30% of all proteins are integral proteins.

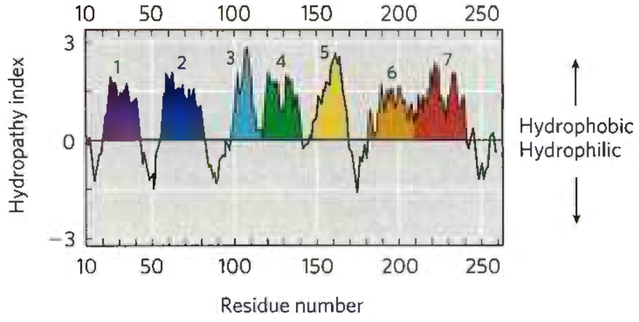
What can we predict about the secondary structure of the membrane-spanning portions of integral proteins? At 1.5 Å (0.15 nm) per amino acid residue, an α-helical sequence of 20 to 25 residues is just long enough to span the thickness (30 Å) of the lipid bilayer. A polypeptide chain surrounded by lipids, having no water molecules with which to hydrogen-bond, will tend to form α helices or β sheets in which intrachain hydrogen bonding is maximized. If the side chains of all amino acids in a helix are nonpolar, the helix is further stabilized in the surrounding lipids by the hydrophobic effect.

Several simple methods of analyzing amino acid sequences yield reasonably accurate predictions of secondary structure for transmembrane proteins. The relative polarity of each amino acid has been determined experimentally by measuring the free-energy change accompanying the movement of that amino acid side chain from a hydrophobic environment into water. This free energy of transfer, which can be expressed as a **hydropathy index** (see Table 3-1), ranges from highly exergonic for charged or polar residues to highly endergonic for amino acids with aromatic or aliphatic hydrocarbon side chains. The overall hydropathy index (hydrophobicity) of a sequence of amino acids is estimated by summing the free energies of transfer for the residues in the sequence. To scan a polypeptide sequence for potential membrane-spanning segments, an investigator calculates the hydropathy index for successive segments (called windows) of a given size, from 7 to 20 residues. For a window of seven residues, for example, the average indices for residues 1 to 7, 2 to 8, 3 to 9, and so on, are plotted as in **Figure 11-13** (plotted for the middle residue in each window—residue 4 for residues 1 to 7, for example). A region with more than 20 residues of high hydropathy index is presumed to be a transmembrane segment. When the sequences of membrane proteins of known three-dimensional structure are scanned using simple online bioinformatics tools, we find a reasonably good correspondence between predicted and known membrane-spanning segments. Hydropathy analysis predicts a single hydrophobic helix for glycophorin (Fig. 11-13a) and seven transmembrane segments for bacteriorhodopsin (Fig. 11-13b)—in agreement with structures known from x-ray crystallography.

Not all integral proteins are composed of transmembrane α helices. Another structural motif common in bacterial and mitochondrial membrane proteins is the **β barrel** (see Fig. 4-16b), in which 20 or more transmembrane segments form β sheets that line a cylinder (**Fig. 11-14**). The same factors that favor α helix formation in the hydrophobic interior of a lipid bilayer also stabilize β barrels: when no water molecules are available to hydrogen-bond with the carbonyl oxygen and nitrogen of the peptide bond, maximal intrachain hydrogen bonding gives the most stable conformation. Planar β sheets do not maximize these interactions and are generally not found in the membrane interior; β barrels allow all possible hydrogen bonds and are common among membrane proteins. **Porins**, proteins that allow certain polar solutes to cross the outer membrane of gram-negative bacteria such as *E. coli*, have many-stranded β barrels lining the polar transmembrane



(a) Glycophorin



(b) Bacteriorhodopsin

FIGURE 11-13 Hydropathy plots. Average hydropathy index (see Table 3-1) is plotted against residue number for two integral membrane proteins. The hydropathy index for each amino acid residue in a sequence of defined length, or "window," is used to calculate the average hydropathy for that window. The horizontal axis shows the residue number in the middle of the window. (a) Glycophorin from human erythrocytes has a single hydrophobic sequence between residues 75 and 93 (yellow); compare this with Figure 11-8. (b) Bacteriorhodopsin, known from independent physical studies to have seven transmembrane helices (see Fig. 11-11), has seven hydrophobic regions. Note, however, that the hydropathy plot is ambiguous in the region of segments 6 and 7. X-ray crystallography has confirmed that this region has two transmembrane segments.

passage. The outer membranes of mitochondria and chloroplasts also contain a variety of β barrels, as do the outer membranes of the modern bacteria descended from those believed to have evolved to mitochondria and chloroplasts.

A polypeptide is more extended in the β conformation than in an α helix; just seven to nine residues of β conformation are needed to span a membrane. Recall that in the β conformation, alternating side chains project above and below the sheet (see Fig. 4-5). In β strands of membrane proteins, every second residue in the membrane-spanning segment is hydrophobic and interacts with the lipid bilayer; aromatic side chains are commonly found at the lipid-protein interface. The other residues may or may not be hydrophilic.

A further remarkable feature of many transmembrane proteins of known structure is the presence of Tyr and Trp residues at the interface between lipid and water (**Fig. 11-15**). The side chains of these residues seem to serve as membrane interface anchors, able to interact simultaneously with the central lipid phase and the aqueous phases on either side of the membrane. Another generalization about amino acid location relative to the bilayer is described by the **positive-inside rule**: the positively charged Lys and Arg residues in the extramembrane loop of membrane proteins occur more commonly on the cytoplasmic face of plasma membranes.

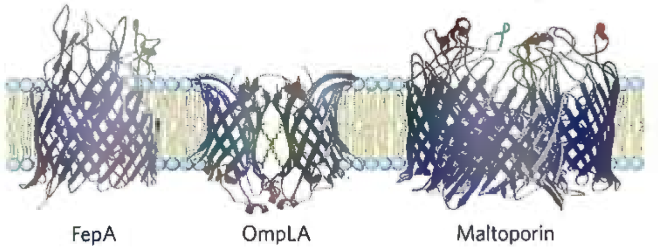


FIGURE 11-14 Polytopic integral proteins with β-barrel structure. Three proteins of the *E. coli* outer membrane are shown, viewed in the plane of the membrane. FepA, involved in iron uptake, has 22 membrane-spanning β strands. Outer membrane phospholipase A, or OmpLA, is a 12-stranded β barrel that exists as a dimer in the membrane. Maltoporin, a maltose transporter, is a trimer; each monomer consists of 16 β strands. [Data from FepA, PDB ID 1FEP, S. K. Buchanan et al., *Nat. Struct. Biol.* 6:56, 1999; OmpLA, PDB ID 1QD5, H. J. Snijder et al., *Nature* 401:717, 1999; maltoporin, PDB ID 1MAL, T. Schirmer et al., *Science* 267:512, 1995.]

Covalently Attached Lipids Anchor or Direct Some Membrane Proteins

Some membrane proteins are covalently linked to one or more lipids, which may be of several types: long-chain fatty acids, isoprenoids, sterols, or *g*lycosylated derivatives of *p*hosphatidyl*i*nositol (GPIs; **Fig. 11-16**). The attached lipid provides a hydrophobic anchor that inserts into the lipid bilayer and holds the protein at the membrane surface. The strength of the hydrophobic interaction between a bilayer and a single hydrocarbon chain linked to a protein is barely enough to anchor the protein securely, but many proteins have more than one attached lipid moiety. Furthermore, other interactions, such as ionic attractions between positively charged Lys residues in the protein and negatively charged lipid head groups, can add to the anchoring effect of a covalently bound lipid. For example, the plasma membrane protein MARCKS (*m*yristoylated *a*lanine-*r*ich *C*-*k*inase *s*ubstrate), which interacts with actin filaments in the process of cell motility, has a covalently attached myristoyl moiety, but it also contains the sequence

KKKKKRFSFKKSFKLSGFSFKKNKK
151 175

which adds to the protein's affinity for the membrane. Three clusters of positively charged Lys and Arg residues (screened blue) interact with the negatively charged head group of phosphatidylinositol 4,5-bisphosphate (PIP_2) on the cytoplasmic face of the plasma membrane; five aromatic residues (screened yellow) insert into the lipid bilayer. When the head-group phosphates of PIP_2 are enzymatically removed, MARCKS loses its hold on

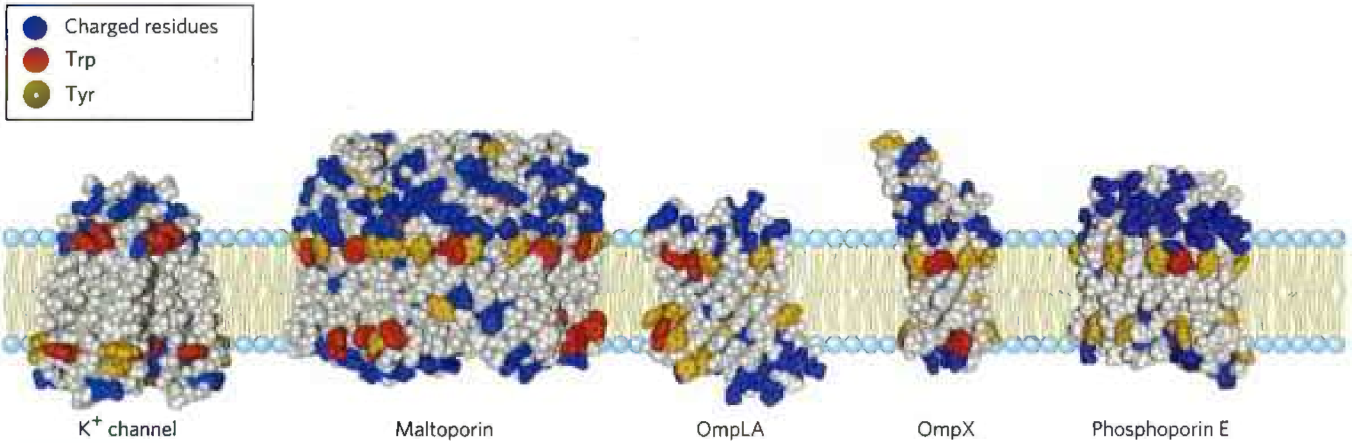
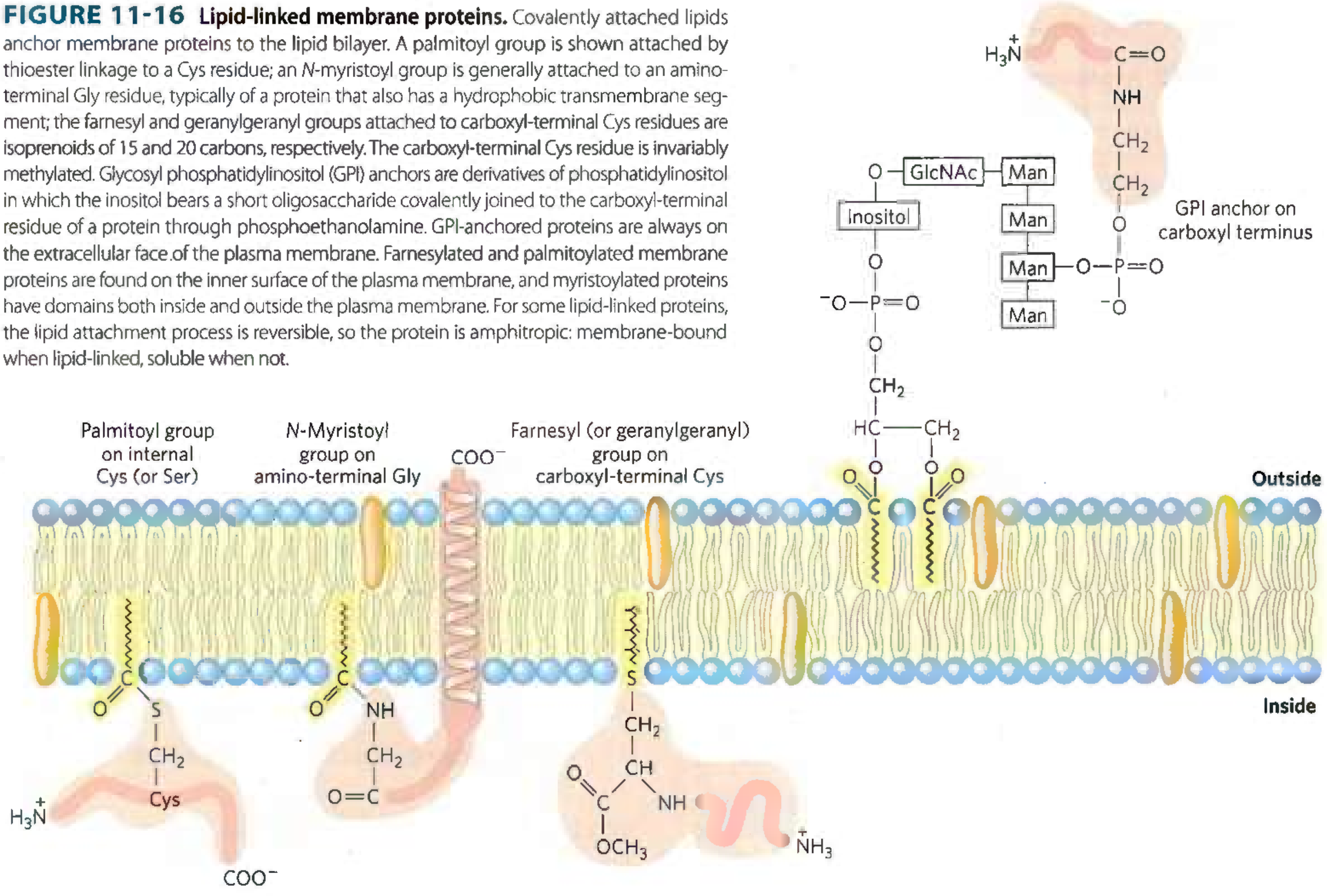

● Charged residues
● Trp
● Tyr
K^+ channel
Maltoporin
OmpLA
OmpX
Phosphoporin E

FIGURE 11-15 Tyr and Trp residues of integral proteins clustering at the water-lipid interface. The detailed structures of these five integral proteins are known from crystallographic studies. The K^+ channel is from the bacterium *Streptomyces lividans* (see Fig. 11-45); maltoporin, OmpLA, OmpX, and phosphoporin E are proteins of the outer membrane of *E. coli*. Residues of Tyr and Trp are found predominantly where the nonpolar region of acyl chains meets the polar head-group region. Charged residues (Lys, Arg, Glu, Asp) are found almost exclusively in the aqueous phases. [Data from K^+ channel, PDB ID 1BL8, D. A. Doyle et al., *Science* 280:69, 1998; maltoporin, PDB ID 1AF6, Y. F. Wang et al., *J. Mol. Biol.* 272:56, 1997; OmpLA, PDB ID 1QD5, H. J. Snijder et al., *Nature* 401:717, 1999; OmpX, PDB ID 1QJ9, J. Vogt and G. E. Schulz, *Structure* 7:1301, 1999; phosphoporin E, PDB ID 1PHO, S. W. Cowan et al., *Nature* 358:727, 1992.]

FIGURE 11-16 Lipid-linked membrane proteins. Covalently attached lipids anchor membrane proteins to the lipid bilayer. A palmitoyl group is shown attached by thioester linkage to a Cys residue; an *N*-myristoyl group is generally attached to an amino-terminal Gly residue, typically of a protein that also has a hydrophobic transmembrane segment; the farnesyl and geranylgeranyl groups attached to carboxyl-terminal Cys residues are isoprenoids of 15 and 20 carbons, respectively. The carboxyl-terminal Cys residue is invariably methylated. Glycosyl phosphatidylinositol (GPI) anchors are derivatives of phosphatidylinositol in which the inositol bears a short oligosaccharide covalently joined to the carboxyl-terminal residue of a protein through phosphoethanolamine. GPI-anchored proteins are always on the extracellular face of the plasma membrane. Farnesylated and palmitoylated membrane proteins are found on the inner surface of the plasma membrane, and myristoylated proteins have domains both inside and outside the plasma membrane. For some lipid-linked proteins, the lipid attachment process is reversible, so the protein is amphitropic: membrane-bound when lipid-linked, soluble when not.


$H_3\overset{+}{N}$
C=O
NH
CH_2
CH_2
O
GPI anchor on carboxyl terminus
O
GlcNAc
Man
Man
Man
Man
Inositol
O
$^-O-P=O$
O
$O-P=O$
^-O
CH_2
HC
CH_2
O
O
O
C
O
C
Palmitoyl group on internal Cys (or Ser)
N-Myristoyl group on amino-terminal Gly
COO^-
Farnesyl (or geranylgeranyl) group on carboxyl-terminal Cys
Outside
Inside
O
C
S
CH_2
Cys
$H_3\overset{+}{N}$
COO^-
O
C
NH
CH_2
O=C
S
CH_2
CH
O
C
NH
OCH_3
$\overset{+}{N}H_3$

the plasma membrane and dissociates. The reversible nature of the association of MARCKS with the membrane makes it an amphitropic protein.

Beyond merely anchoring a protein to the membrane, the attached lipid may have a more specific role. In the plasma membrane, **GPI-anchored proteins** are exclusively on the outer face and are clustered in certain regions, as discussed later in the chapter (p. 380), whereas other types of lipid-linked proteins (with farnesyl or geranylgeranyl groups attached; Fig. 11-16) are exclusively on the inner face. In polarized epithelial cells (such as intestinal epithelial cells), in which apical and basal surfaces have different roles, GPI-anchored proteins are directed specifically to the apical surface. Attachment of a specific lipid to a newly synthesized membrane protein therefore has a targeting function, directing the protein to its correct cellular location.

SUMMARY 11.1 *The Composition and Architecture of Membranes*

- Biological phospholipids and sterols spontaneously form a lipid bilayer to protect their hydrophobic hydrocarbon chains from energetically unfavorable interaction with water.
- In the fluid mosaic model, a lipid bilayer is the basic structural unit, and proteins associate with or span the bilayer. Biological membranes are flexible, self-repairing, and selectively permeable. They regulate the traffic of small molecules into and out of the cell and among the organelles, and they provide a scaffold on which proteins assemble into catalytic and structural aggregates that function in two-dimensional space.
- Proteins and lipids are trafficked through the dynamic endomembrane system of a eukaryote from their point of synthesis to their appropriate cellular locations. Small vesicles and soluble transporter proteins ensure that each membrane leaflet has its unique complement of lipids and proteins to achieve its specialized function.
- Cells have hundreds of membrane transporters that carry polar solutes and ions in and out of cells and throughout the endomembrane system. The plasma membrane contains protein receptors that sense signals from outside the cell and carry their message into the cell. Many enzymes are associated with membranes, where they interact with each other in essentially two-dimensional space.
- Integral proteins are embedded within membranes, their nonpolar amino acid side chains stabilized by contact with the lipid bilayer. Peripheral proteins associate with membranes through electrostatic interactions and hydrogen bonding with membrane phospholipids and integral proteins. Amphitropic proteins associate reversibly with the membrane.
- Many integral membrane proteins span the lipid bilayer several times, with hydrophobic sequences of about 20 amino acid residues forming transmembrane α helices. Multistranded β barrels are also common in integral proteins of bacterial and mitochondrial membranes. A hydropathy plot of the amino acid sequence identifies segments that are likely to cross the lipid bilayer as helices or barrels.
- Covalent attachment of a hydrophobic molecule such as a fatty acid serves to anchor some membrane proteins to the bilayer.

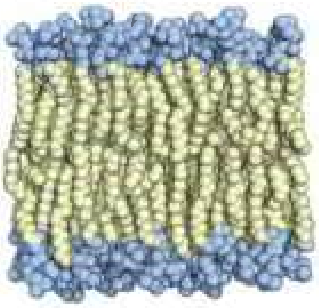
11.2 Membrane Dynamics

One remarkable feature of all biological membranes is their plasticity—their ability to change shape without losing their integrity and becoming leaky. The bases for this property are the noncovalent interactions among lipids in the bilayer and the mobility allowed to individual lipids because they are not covalently anchored to one another. We turn now to the dynamics of membranes: the motions that occur and the transient structures that are allowed by these motions.

Acyl Groups in the Bilayer Interior Are Ordered to Varying Degrees

Although the lipid bilayer structure is stable, its individual phospholipid molecules have much freedom of motion (**Fig. 11-17**), depending on the temperature and the lipid composition. Below normal physiological temperatures, P1 the lipids in a bilayer form a gel-like **liquid-ordered (L_o) state**, in which all types of motion of individual lipid molecules are strongly constrained (Fig. 11-17a). Above physiological temperatures, individual hydrocarbon chains of fatty acids are in constant motion produced by rotation about the carbon–carbon bonds of the long acyl side chains and by lateral diffusion of individual lipid molecules in

(a) Liquid-ordered state L_o



Heat produces thermal motion of side chains ($L_o \rightarrow L_d$ transition).

(b) Liquid-disordered state L_d

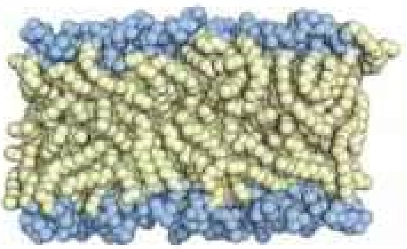


FIGURE 11-17 Two extreme states of bilayer lipids. (a) In the liquid-ordered (L_o) state, polar head groups are uniformly arrayed at the surface, and the acyl chains are nearly motionless and packed with regular geometry. (b) In the liquid-disordered (L_d) state, or fluid state, acyl chains undergo much thermal motion and have no regular organization. The state of membrane lipids in biological membranes is maintained somewhere between these extremes. [Information from H. Heller et al., *J. Phys. Chem.* 97:8343, 1993.]

the plane of the bilayer. This is the **liquid-disordered (L_d) state** (Fig. 11-17b). In the transition from the L_o state to the L_d state, the general shape and dimensions of the bilayer are maintained; what changes is the degree of motion (lateral and rotational) allowed to individual lipid molecules.

At temperatures in the physiological range for a mammal (about 20 to 40 °C), long-chain saturated fatty acids (such as 16:0 and 18:0) tend to pack into an L_o gel phase, but the kinks in unsaturated fatty acids interfere with packing, favoring the L_d state (see Fig. 10-1). Shorter-chain fatty acyl groups are more mobile than longer-chain fatty acyl groups and thus also favor the L_d state. The sterol content of a membrane, which varies greatly with organism and organelle, is another important determinant of lipid state. Sterols (such as cholesterol) have paradoxical effects on bilayer fluidity: they interact with phospholipids containing unsaturated fatty acyl chains, compacting them and constraining their motion in bilayers. In contrast, their association with sphingolipids and phospholipids having long, saturated fatty acyl chains tends to make a bilayer fluid that would otherwise, without cholesterol, adopt the L_o state. In biological membranes composed of a variety of phospholipids and sphingolipids, cholesterol tends to associate with sphingolipids and to form regions in the L_o state surrounded by cholesterol-poor regions in the L_d state (see the discussion of membrane rafts below).

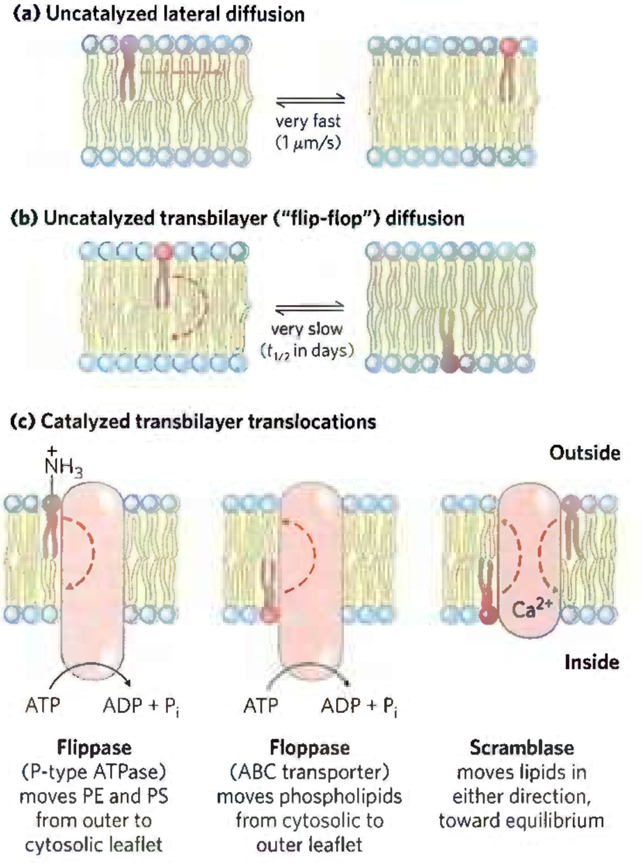


FIGURE 11-18 Motion of single phospholipids in a bilayer. (a) Lateral diffusion within a leaflet is very rapid, but (b) uncatalyzed movement from one leaflet to the other is very slow. (c) Three types of phospholipid translocators in the plasma membrane. PE is phosphatidylethanolamine; PS is phosphatidylserine.

Transbilayer Movement of Lipids Requires Catalysis

At physiological temperatures, lateral diffusion *in the plane* of the bilayer is very rapid, but the movement of a lipid molecule from one leaflet of the bilayer to the other occurs very slowly, if at all, in most membranes (**Fig. 11-18**). Transbilayer — or "flip-flop" — movement requires that a polar or charged head group leave its aqueous environment and move into the hydrophobic interior of the bilayer, a process with a large, positive free-energy change. There are, however, situations in which such movement is essential. For example, in the ER, membrane glycerophospholipids are synthesized on the cytosolic face, whereas sphingolipids are synthesized or modified on the lumenal surface. To get from their site of synthesis to their eventual point of deposition, these lipids must undergo flip-flop diffusion.

P2 Membrane proteins called flippases, floppases, and scramblases (Fig. 11-18c) facilitate the transbilayer movement (translocation) of individual lipid molecules. Like enzymes, these translocators act by providing a path that is energetically more favorable and therefore much faster than the uncatalyzed movement. The combination of asymmetric biosynthesis of membrane lipids, no uncatalyzed flip-flop diffusion, and the presence of selective, energy-dependent lipid translocators accounts for the transbilayer asymmetry in lipid composition discussed in Section 11.1.

Flippases catalyze translocation of the *amino*-phospholipids phosphatidylethanolamine and phosphatidylserine from the extracellular to the cytoplasmic leaflet of the plasma membrane, contributing to the asymmetric distribution of phospholipids: phosphatidylethanolamine and phosphatidylserine primarily in the cytoplasmic leaflet, and the sphingolipids and phosphatidylcholine in the outer leaflet. Keeping phosphatidylserine out of the extracellular leaflet is important: its exposure on the outer surface triggers apoptosis (programmed cell death; see Chapter 12) and engulfment by macrophages that carry phosphatidylserine receptors. Flippases also act in the ER, where they move newly synthesized phospholipids from their site of synthesis in the cytosolic leaflet to the lumenal leaflet. Flippases consume about one ATP per molecule of phospholipid translocated, and they are structurally and functionally related to the P-type ATPases (active transporters) described in Section 11.3.

There are three other types of lipid-translocating activities: floppases, scramblases, and phosphatidylinositol transfer proteins. **Floppases** move plasma membrane phospholipids and sterols from the cytoplasmic leaflet to the extracellular leaflet and, like flippases, are ATP-dependent. Floppases are members of the ABC transporter family, described on page 395; all ABC transporters actively transport hydrophobic substrates outward across the plasma membrane. Each floppase specializes in movement of specific lipids: cholesterol, phosphatidylcholine, sphingomyelin, and phosphatidylserine. **Scramblases** are proteins that move any membrane phospholipid across the bilayer down its concentration gradient (from the leaflet where it has a higher concentration to the leaflet where it has a lower concentration); their activity is not dependent on ATP, although some require Ca^{2+}. Scramblase activity leads to controlled randomization of the head-group composition on the two faces of the bilayer. The activity of some scramblases rises sharply with an increase in cytosolic Ca^{2+} concentration, which may result from cell activation, cell injury, or apoptosis. Finally, a group of proteins that act primarily to move phosphatidylinositol lipids across lipid bilayers, the **phosphatidylinositol transfer proteins**, are believed to have important roles in lipid signaling and membrane trafficking.

Lipids and Proteins Diffuse Laterally in the Bilayer

P2 Individual lipid molecules can move laterally in the plane of the membrane by changing places with neighboring lipid molecules; that is, they undergo Brownian movement within the bilayer (Fig. 11-18a). This rapid lateral diffusion in the plane of the bilayer tends to randomize the positions of individual molecules in a few seconds.

Lateral diffusion can be shown experimentally by attaching fluorescent probes to the head groups of lipids and using fluorescence microscopy to follow the probes over time (**Fig. 11-19**). In one technique, a small region (5 μm^2) of a cell surface with fluorescence-tagged lipids is bleached by intense laser radiation so that the irradiated patch no longer fluoresces when viewed with less-intense (nonbleaching) light in the fluorescence microscope. However, within milliseconds, the region recovers its fluorescence as unbleached lipid molecules diffuse into the bleached patch and bleached lipid molecules diffuse away from it. The rate of *f*luorescence *r*ecovery *a*fter *p*hotobleaching, or **FRAP**, is a measure of the rate of lateral diffusion of the lipids. Using the FRAP technique, researchers have shown that some membrane lipids diffuse laterally at rates of up to 1 $\mu m/s$. At this rate, a lipid molecule could move from one end of a eukaryotic cell to the other in a few seconds.

Another technique, single particle tracking, has allowed researchers to follow the movement of a *single* lipid molecule in the plasma membrane on a much shorter time scale. Results from these studies confirm that lipid molecules diffuse laterally with rapidity within small, discrete regions of the cell surface but that movement from one such region to a nearby region ("hop diffusion") is rarer; membrane lipids behave as though corralled by fences that they can occasionally cross by hop diffusion (**Fig. 11-20**).

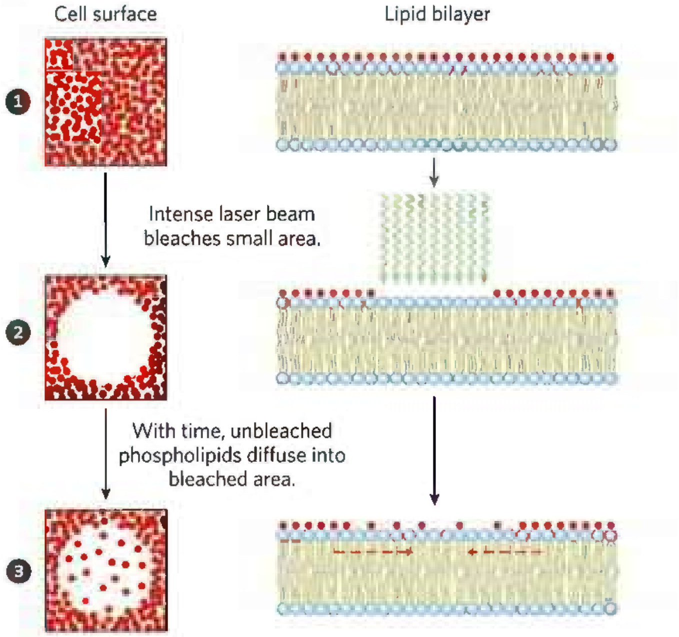

Cell surface
Lipid bilayer
1
Intense laser beam bleaches small area.
2
With time, unbleached phospholipids diffuse into bleached area.
3

FIGURE 11-19 Measurement of lateral diffusion rates of lipids by fluorescence recovery after photobleaching (FRAP). Lipids in the outer leaflet of the plasma membrane are labeled by reaction with a membrane-impermeant fluorescent probe (red) so that the surface is uniformly labeled when viewed with a fluorescence microscope. A small area is bleached with a laser, then recovers its fluorescence. With time, unbleached lipid molecules diffuse into the bleached region, and it again becomes fluorescent. The FRAP method can also be used to measure lateral diffusion of membrane proteins labeled with a fluorescent tag.

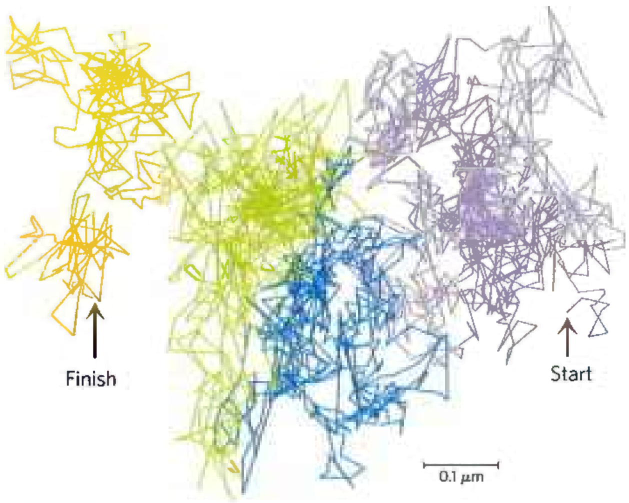


FIGURE 11-20 Hop diffusion of individual lipid molecules. The motion of a single fluorescently labeled lipid molecule in a cell surface is recorded on video by fluorescence microscopy, with a time resolution of 25 μs (equivalent to 40,000 frames/s). The track shown here represents a molecule followed for 56 ms (2,250 frames); the trace begins in the purple area and continues through blue, green, and orange. The pattern of movement indicates rapid diffusion within a confined region (about 250 nm in diameter, shown by a single color), with occasional hops into an adjoining region. This finding suggests that the lipids are corralled by molecular fences that they occasionally jump. [Data from Takahiro Fujiwara, Ken Ritchie, Hideji Murakoshi, Ken Jacobson, and Akihiro Kusumi.]

Like membrane lipids, many membrane proteins are free to diffuse laterally in the plane of the bilayer and are in constant motion, as shown by the FRAP technique with fluorescence-tagged plasma membrane proteins. Some membrane proteins associate to form large aggregates ("patches") on the surface of a cell or an organelle in which individual protein molecules do not move relative to one another; for example, acetylcholine receptors form dense, near-crystalline patches on neuronal plasma membranes at synapses. Other membrane proteins are anchored to internal structures that prevent their free diffusion. In the erythrocyte membrane, both glycophorin and the chloride-bicarbonate exchanger (p. 389) are tethered to spectrin, a filamentous cytoskeletal protein (**Fig. 11-21**). One possible explanation for the pattern of lateral diffusion of lipid molecules shown in Figure 11-20 is that membrane proteins immobilized by their association with spectrin form the "fences" that define the regions within which relatively unrestricted lipid motion can occur.

Sphingolipids and Cholesterol Cluster Together in Membrane Rafts

P2 We have seen that diffusion of membrane lipids from one bilayer leaflet to the other does not occur unless catalyzed and that the different lipid species of the plasma membrane are asymmetrically distributed in the two leaflets of the bilayer (Fig. 11-6). Even within a single leaflet, the lipid distribution is not uniform. Glycosphingolipids (cerebrosides and gangliosides), which typically contain long-chain saturated fatty acids, form transient clusters in the outer leaflet; these clusters largely exclude glycerophospholipids, which typically contain one unsaturated fatty acyl group and a shorter saturated acyl group. The long, saturated acyl groups of sphingolipids can form more compact, more stable associations with the long ring system of cholesterol than can the shorter, often unsaturated, chains of phospholipids. The cholesterol-sphingolipid **microdomains** of the plasma membrane make the bilayer slightly thicker and more ordered (less fluid) than neighboring regions, which are rich in phospholipids. These microdomains are more difficult to dissolve with nonionic detergents; they behave like liquid-ordered sphingolipid **rafts** adrift on an ocean of liquid-disordered phospholipids (**Fig. 11-22**). Proteins with relatively short hydrophobic helical sections (19 to 20 residues) cannot span the thicker bilayer in rafts, and thus they tend to be excluded. Proteins with longer hydrophobic helices (24 to 25 residues) segregate into the thicker bilayer regions of rafts, where the entire length of the helix is stabilized by the hydrophobic effect.

Lipid rafts are remarkably enriched in two classes of integral proteins, with two specific types of covalently attached lipids. The integral proteins of one class have two long-chain saturated fatty acids (two palmitoyl groups, or a palmitoyl and a myristoyl group) covalently attached through Cys residues. Caveolin is one such protein; there are many others. Those of the second class, the GPI-anchored proteins, have a glycosyl phosphatidylinositol on their carboxyl-terminal residue (Fig. 11-16). Presumably, these lipid anchors, like the long, saturated acyl chains of sphingolipids,

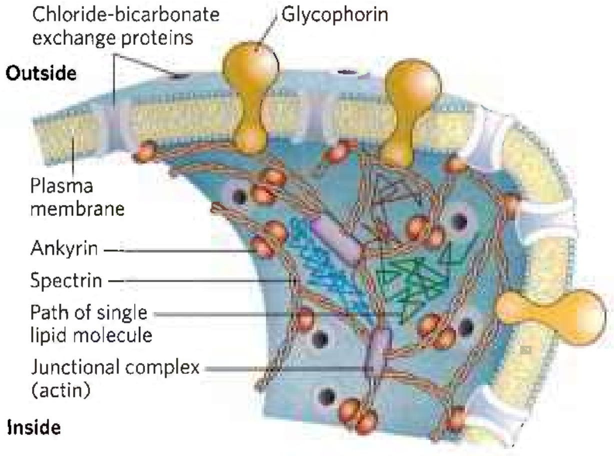

Chloride-bicarbonate exchange proteins
Glycophorin
Outside
Plasma membrane
Ankyrin
Spectrin
Path of single lipid molecule
Junctional complex (actin)
Inside

FIGURE 11-21 Restricted motion of the erythrocyte chloride-bicarbonate exchanger and glycophorin. The proteins span the membrane and are tethered to spectrin, a cytoskeletal protein, by another protein, ankyrin, limiting their lateral mobility. Ankyrin is anchored in the membrane by a covalently bound palmitoyl side chain (see Fig. 11-16). Spectrin, a long, filamentous protein, is cross-linked at junctional complexes containing actin. A network of cross-linked spectrin molecules attached to the cytoplasmic face of the plasma membrane stabilizes the membrane, making it resistant to deformation. This network of anchored membrane proteins may form the "corral" suggested by the experiment shown in Figure 11-20; the lipid tracks shown here are confined to different regions defined by the tethered membrane proteins. Occasionally a lipid molecule (green track) jumps from one corral to another (blue track), then another (red track).

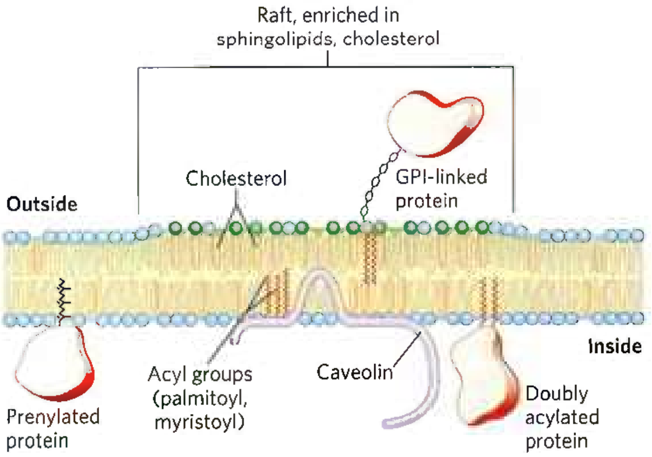


FIGURE 11-22 Membrane microdomains (rafts). Stable associations of sphingolipids and cholesterol in the outer leaflet produce a microdomain, slightly thicker than other membrane regions, that is enriched with specific types of membrane proteins. GPI-anchored proteins are prominent in the outer leaflet of these rafts, and proteins with one or several covalently attached long-chain acyl groups are common in the inner leaflet. Inwardly curved rafts called caveolae are especially enriched in proteins called caveolins (see Fig. 11-23).

form more stable associations with the cholesterol and long acyl groups in rafts than they form with the surrounding phospholipids. (It is notable that other lipid-linked proteins, those with covalently attached isoprenyl groups such as farnesyl, are *not* preferentially associated with the outer leaflet of sphingolipid rafts; see Fig. 11-22.) The "raft" and "sea" domains of the plasma membrane are not rigidly separated; membrane proteins can move into and out of lipid rafts in a fraction of a second. But in the shorter time scale (microseconds) more relevant to many membrane-mediated biochemical processes, many of these proteins reside primarily in a raft.

The approximate fraction of the cell surface occupied by rafts can be as high as 50% in some cases; the rafts cover half of the ocean. Indirect measurements in cultured fibroblasts suggest a diameter of roughly 50 nm for an individual raft, which corresponds to a patch containing a few thousand sphingolipids and perhaps 10 to 50 membrane proteins. Because most cells express more than 50 different kinds of plasma membrane proteins, it is likely that a single raft contains only a subset of membrane proteins and that this segregation of membrane proteins is functionally significant. Their presence in a single raft would hugely increase the likelihood of their collision. Certain membrane receptors and signaling proteins, for example, seem to be segregated together in membrane rafts. Experiments show that signaling through these proteins can be disrupted by manipulations that deplete the plasma membrane of cholesterol and destroy lipid rafts.

Plasma membranes of many cells have specialized rafts called **caveolae** ("little caves"), which can represent about half of the total area of the plasma membrane (**Fig. 11-23a**). Closely associated with caveolae is **caveolin**, an integral protein with two globular domains connected by a hairpin-shaped hydrophobic domain that binds the protein to the cytoplasmic leaflet of the plasma membrane (Fig. 11-23b). Three palmitoyl groups attached to the carboxyl-terminal globular domain further anchor the protein to the membrane. Caveolins form dimers and associate with cholesterol-rich regions in the membrane. The presence of caveolin dimers forces the associated lipid bilayer to curve inward, forming caveolae. Caveolae involve *both* leaflets of the bilayer — the cytoplasmic leaflet, from which the caveolin globular domains project, and the extracellular leaflet, a cholesterol and sphingolipid raft with associated GPI-anchored proteins. Caveolae are implicated in a variety of cellular functions, including membrane trafficking within cells and the transduction of external signals into cellular responses.

Caveolae may also provide a means of expanding the cell surface. The lipid bilayer itself is not elastic, but if existing caveolae lose their associated caveolin as the result of a regulatory signal, the caveolae flatten into

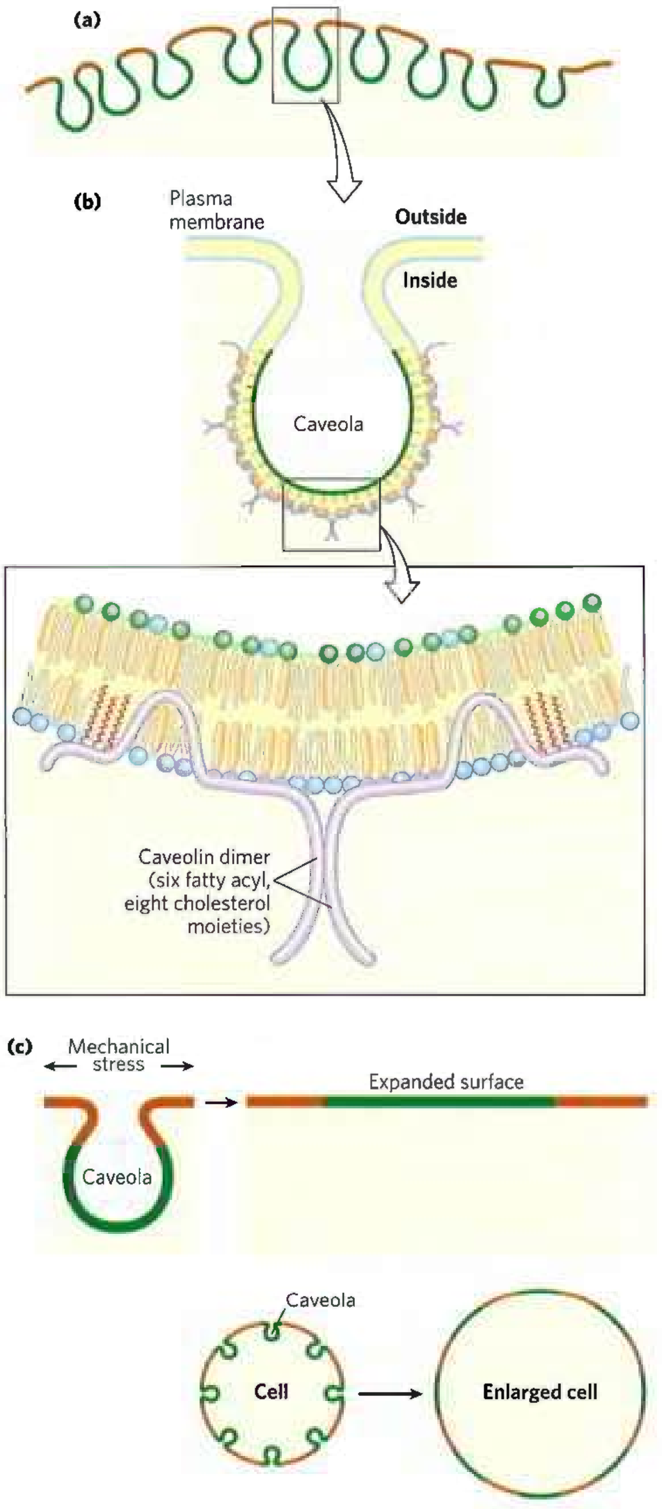


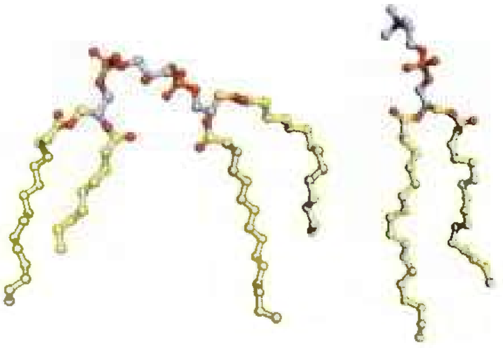
FIGURE 11-23 Caveolin forces inward curvature of a membrane. (a) Caveolae are small invaginations in the plasma membrane. (b) Cartoon showing the location and role of a caveolin dimer in causing inward membrane curvature. Each caveolin monomer has a central hydrophobic domain and three long-chain acyl groups (red), which hold the molecule to the inside of the plasma membrane. When several caveolin dimers are concentrated in a small region (a raft), they force a curvature in the lipid bilayer, forming a caveola. Cholesterol molecules in the bilayer are shown in orange. (c) Flattening of caveolae allows the plasma membrane to expand in response to various stresses. [Information from R. G. Parton, *Nat. Rev. Mol. Cell Biol.* 8:185–194, 2007.]

the plasma membrane (Fig. 11-23c). The effect is to add surface area, allowing the cell to expand without bursting in response to osmotic or other stress.

Membrane Curvature and Fusion Are Central to Many Biological Processes

Caveolins are not unique in their ability to induce curvature in membranes. **P2** Changes of curvature are central to one of the most remarkable features of biological membranes: their ability to undergo fusion with other membranes without losing their continuity. Within the eukaryotic endomembrane system, the membranous compartments constantly reorganize. Vesicles bud from the ER to carry newly synthesized lipids and proteins to other organelles and to the plasma membrane. Exocytosis, endocytosis, cell division, fusion of egg and sperm cells, and entry of a membrane-enveloped virus into a host cell all involve a membrane reorganization that requires the fusion of two membrane segments without loss of continuity (**Fig. 11-24**). Most of these processes begin with a local increase in membrane curvature.

Cardiolipin, present primarily in mitochondrial membranes of eukaryotes, but also a major component of the membrane lipids of bacteria, can create or recognize membrane curvature. It is cone-shaped—its head group is small relative to its four fatty acyl chains—so it can act as a wedge in the monolayer, contracting that monolayer relative to the other.



Membrane curvature is the result. In *E. coli,* cardiolipin is highly localized at the two poles of the rod-shaped cell, where its curvature is sharpest. It seems very likely that other membrane lipids will be found to influence local curvature of the bilayer.

A protein that is intrinsically curved may force a bilayer to curve by binding to it (**Fig. 11-25**); the binding energy provides the driving force for the increase in bilayer curvature. Alternatively, multiple subunits of a scaffold protein may assemble into curved supramolecular complexes and stabilize curves that spontaneously form in the bilayer. For example, a superfamily of proteins containing **BAR domains** (named for the first three members of the family that were identified: *B*IN1, *a*mphiphysin, and *R*VS167) can assemble into a crescent-shaped scaffold that binds to the membrane surface, forcing or favoring membrane curvature. BAR domains consist of coiled coils that form long, thin, curved dimers with a positively charged concave surface that tends to form ionic interactions with the negatively charged head groups of membrane lipids PIP_2 and PIP_3. The enzymatic formation of these inositol lipids can tag a plasma membrane area for creation of inward curvature by a BAR protein (Fig. 11-25). Some of these BAR proteins also have an amphipathic helix (having one polar face and one hydrophobic face; see Fig. 11-32) that inserts like a wedge into one leaflet of the bilayer, expanding its area relative to the other leaflet and thereby forcing curvature. Such a protein might also serve as a detector of preexisting membrane curvature due to differences in lipid composition of two leaflets of a bilayer.

Septins make up a family of GTP-binding proteins (14 genes in humans) that polymerize at curved regions of the plasma membrane and participate in cellular processes such as cell division, exocytosis, phagocytosis, and apoptosis. All septins have an amphipathic helix that can submerge its hydrophobic side in one leaflet of the bilayer, forcing one leaflet of the bilayer to expand laterally, either causing or sensing local curvature in the membranes. Studies of cells with mutations in this helix

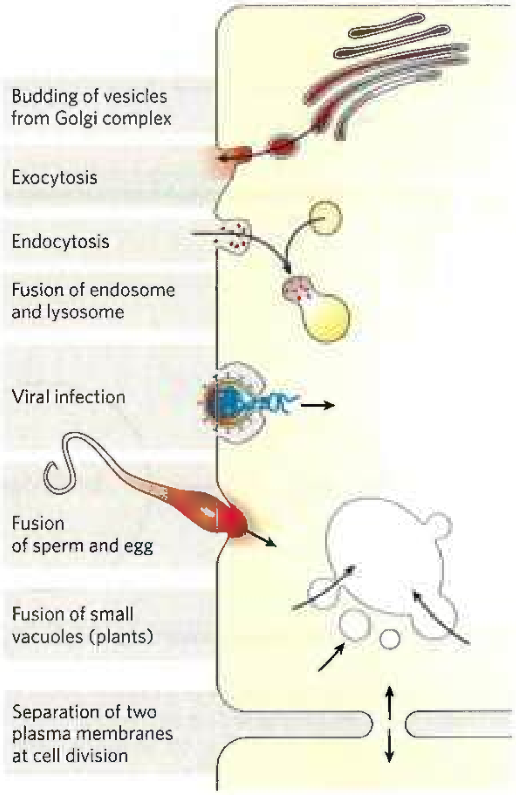
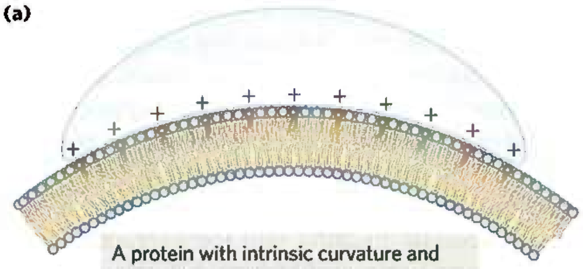
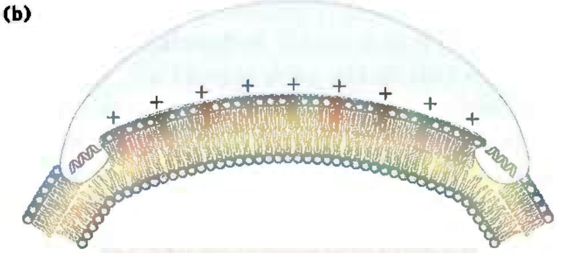

Budding of vesicles from Golgi complex
Exocytosis
Endocytosis
Fusion of endosome and lysosome
Viral infection
Fusion of sperm and egg
Fusion of small vacuoles (plants)
Separation of two plasma membranes at cell division

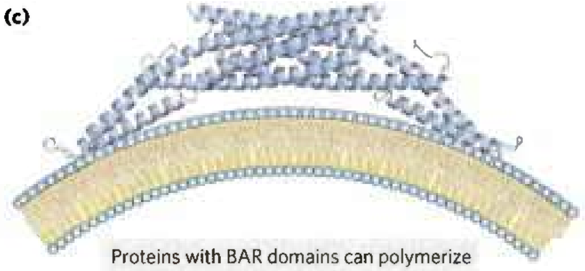
FIGURE 11-24 Membrane fusion. The fusion of two membranes is central to a variety of cellular processes involving organelles and the plasma membrane.



A protein with intrinsic curvature and with a high density of positive charge on its concave surface interacts with negatively charged phospholipid head groups, favoring curvature of the bilayer.



A protein with one or several amphipathic helices inserted into one leaflet of the bilayer crowds the lipids in that leaflet, forcing the membrane to bend.



Proteins with BAR domains can polymerize into a superstructure that favors and maintains the curvature.

FIGURE 11-25 Three models for protein-induced curvature of membranes. [Information from (a, b) B. Qualmann et al., *EMBO J.* 30:3501, 2011, Fig. 1; (c) B. J. Peter et al., *Science* 303:495, 2004, Fig. 1A.]

show its biological importance in vesicle trafficking and neurotransmitter release.

Specific fusion of two membranes requires mediation by **fusion proteins**. Fusion proteins ensure (1) that the two membranes recognize each other; (2) that their surfaces become closely apposed, which requires removal of the water molecules normally associated with the polar head groups of lipids; (3) that their bilayer structures become locally disrupted, resulting in fusion of the outer leaflets of the two membranes (hemifusion); and (4) that their bilayers fuse to form a single continuous bilayer. The fusion occurring in receptor-mediated endocytosis, or regulated secretion, also requires (5) that the process is triggered at the appropriate time or in response to a specific signal. Fusion proteins mediate these events, bringing about specific recognition and a transient local distortion of the bilayer structure that favors membrane fusion. (Note that these fusion proteins are unrelated to the products encoded by two fused genes, also called fusion proteins, discussed in Chapter 9.)

A well-studied example of membrane fusion occurs at synapses, when intracellular (neuronal) vesicles loaded with neurotransmitter fuse with the plasma membrane. Yeast cells provide another experimentally accessible system in which vesicles fuse with the plasma membrane, releasing their secretion products. Both processes involve a family of proteins called SNAREs (*snap receptors*; **Fig. 11-26**). SNAREs in the cytoplasmic face of the intracellular vesicle are called **v-SNAREs** (*v* for vesicle); those in the target membrane with which the vesicle fuses (the plasma membrane during exocytosis) are **t-SNAREs** (*t* for target). The protein NSF (*N*-ethylmaleimide *s*ensitive *f*actor) regulates the interactions among SNAREs. During fusion, a v-SNARE and a t-SNARE bind to each other and undergo a structural change that produces a bundle of long, thin rods made up of helices from both SNAREs and two helices from the protein SNAP25 (Fig. 11-26). The two SNAREs initially interact at their ends, then zip up into the bundle of helices. This structural change pulls the two membranes into contact and initiates the fusion of their lipid bilayers. An alternative designation of SNARE types is based on structural features of the proteins: R-SNAREs have an Arg residue critical to their function, and Q-SNAREs have a critical Gln residue. Typically, R-SNAREs act as v-SNAREs, and Q-SNAREs act as t-SNAREs.

The complex of SNAREs and SNAP25 is the target of several powerful neurotoxins. *Clostridium botulinum* toxin is a bacterial protease that cleaves specific bonds in SNARE proteins, preventing neurotransmission and causing paralysis and death. Because of its very high specificity for these proteins, purified botulinum toxin has served as a powerful tool for dissecting the mechanism of neurotransmitter release in vivo and in vitro. Used in small amounts, botulinum toxin (Botox) is used in medicine to treat disorders of eye and neck muscles, as well as cosmetically for the removal of skin wrinkles. Tetanus toxin, produced by the bacterium *Clostridium tetani*, also is a protease with high specificity for SNARE proteins. It causes painful muscle spasms and rigidity of voluntary muscles—hence the characteristic symptom "lockjaw." ■

Integral Proteins of the Plasma Membrane Are Involved in Surface Adhesion, Signaling, and Other Cellular Processes

Several families of integral proteins in the plasma membrane provide specific points of attachment between cells or between a cell and proteins of the extracellular matrix. **Integrins** are surface adhesion proteins that mediate a cell's interaction with the extracellular matrix and with other cells, including some pathogens.

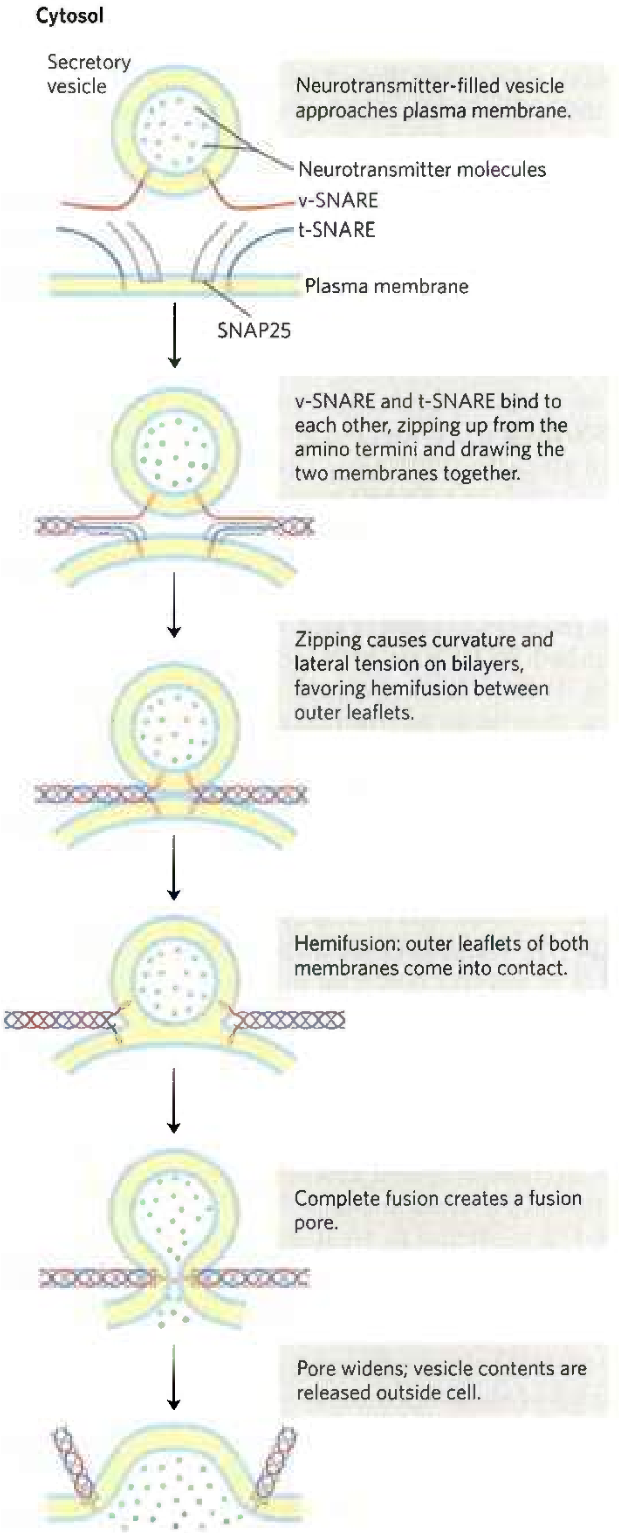

Cytosol
Secretory vesicle
Neurotransmitter-filled vesicle approaches plasma membrane.
Neurotransmitter molecules
v-SNARE
t-SNARE
Plasma membrane
SNAP25
v-SNARE and t-SNARE bind to each other, zipping up from the amino termini and drawing the two membranes together.
Zipping causes curvature and lateral tension on bilayers, favoring hemifusion between outer leaflets.
Hemifusion: outer leaflets of both membranes come into contact.
Complete fusion creates a fusion pore.
Pore widens; vesicle contents are released outside cell.

FIGURE 11-26 Membrane fusion during neurotransmitter release at a synapse. The secretory vesicle membrane contains the v-SNARE synaptobrevin (red). The target (plasma) membrane contains the t-SNAREs syntaxin (blue) and SNAP25 (violet). When a local increase in [Ca^{2+}] signals release of neurotransmitter, the v-SNARE, SNAP25, and t-SNARE interact, forming a coiled bundle of four α helices, pulling the two membranes together and disrupting the bilayer locally. This leads first to hemifusion, joining the outer leaflets of the two membranes, then to complete membrane fusion and neurotransmitter release. When fusion is complete, the SNARE complex is disassembled. [Information from Y. A. Chen and R. H. Scheller, *Nat. Rev. Mol. Cell Biol.* 2:98, 2001.]

Integrins also carry signals in both directions across the plasma membrane, integrating information about the extracellular and intracellular environments. All integrins are heterodimeric proteins composed of two unlike subunits, α and β, each anchored to the plasma membrane by a single transmembrane helix. The large extracellular domains of the α and β subunits combine to form a specific binding site for extracellular proteins such as collagen and fibronectin, which contain a common determinant of integrin binding, the sequence Arg–Gly–Asp (RGD).

Other plasma membrane proteins involved in surface adhesion are the **cadherins**, which undergo homophilic ("with the same kind") interactions with identical cadherins in an adjacent cell. **Selectins** have extracellular domains that, in the presence of Ca^{2+}, bind specific polysaccharides on the surface of an adjacent cell. Selectins are present primarily in the various types of blood cells and in the endothelial cells that line blood vessels (see Fig. 7-29). They are an essential part of the blood-clotting process.

SUMMARY 11.2 *Membrane Dynamics*

- Lipids in a biological membrane can exist in liquid-ordered or liquid-disordered states. Membrane fluidity is affected by temperature, fatty acid composition, and sterol content.
- Flip-flop diffusion of lipids between the inner and outer leaflets of a membrane occurs only when the diffusion is specifically catalyzed by flippases, floppases, scramblases, or PI transporters.
- Proteins and lipids can diffuse laterally within the plane of the membrane, but this mobility is limited by interactions of membrane proteins with internal cytoskeletal structures and interactions of lipids with lipid rafts.
- One class of lipid rafts is enriched for sphingolipids and cholesterol with a subset of membrane proteins that are GPI-linked or attached to several long-chain fatty acyl moieties. Caveolins are integral proteins that associate with the inner leaflet of the plasma membrane, forcing it to curve inward to form caveolae, which are involved in membrane transport, signaling, and the expansion of plasma membranes.
- Proteins containing BAR domains cause local membrane curvature and mediate the fusion of two membranes, which accompanies processes such as endocytosis, exocytosis, and viral invasion. Septins are proteins that sense or cause membrane curvature. Membrane fusion depends on SNARE proteins, which draw two membranes close together and favor fusion.
- Integrins, cadherins, and selectins are transmembrane proteins of the plasma membrane that act both to attach cells to each other and to carry messages between the extracellular matrix and the cytoplasm.

11.3 Solute Transport across Membranes

Every living cell must acquire from its surroundings the raw materials for biosynthesis and for energy production, and must release the byproducts of metabolism to its environment; both processes require that small compounds or inorganic ions cross the plasma membrane. Within the eukaryotic cell, different compartments have different concentrations of ions and of metabolic intermediates and products, and these, too, must move across intracellular membranes in tightly regulated processes. **P3** A few nonpolar compounds can dissolve in the lipid bilayer and cross a membrane unassisted, but for any polar compound or ion, a specific membrane protein carrier is essential. Approximately 2,000 genes in the human genome encode proteins that function in transporting solutes across membranes. In some cases, a membrane protein simply facilitates the diffusion of a solute down its concentration gradient; but transport can also occur against a gradient of concentration, electrical potential, or both, and in these cases, as we shall see, the transport process requires energy. Ions may also diffuse across membranes via ion channels formed by proteins, or they may be carried across by ionophores, small molecules that mask the charge of ions and allow them to diffuse through the lipid bilayer. **Figure 11-27** summarizes the various types of transport mechanisms discussed in this section.

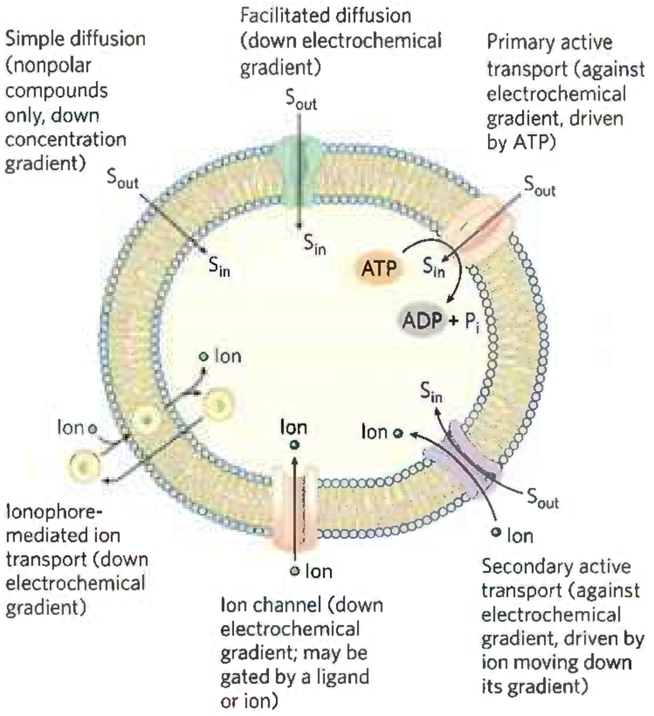

Simple diffusion (nonpolar compounds only, down concentration gradient)
Facilitated diffusion (down electrochemical gradient)
Primary active transport (against electrochemical gradient, driven by ATP)
S_{out}
S_{out}
S_{out}
S_{in}
S_{in}
ATP
S_{in}
ADP + P_i
Ion
Ion
Ion
Ion
S_{in}
S_{out}
Ion
Ion
Ionophore-mediated ion transport (down electrochemical gradient)
Ion channel (down electrochemical gradient; may be gated by a ligand or ion)
Secondary active transport (against electrochemical gradient, driven by ion moving down its gradient)

FIGURE 11-27 Summary of transporter types. Some types (ionophores, ion channels, and passive transporters) simply speed transmembrane movement of solutes (S) down their electrochemical gradients, whereas others (active transporters) can pump solutes against a gradient, using ATP or a gradient of a second solute to provide the energy.

Transport May Be Passive or Active

When two aqueous compartments containing unequal concentrations of a soluble compound or ion are separated by a permeable divider (membrane), the solute moves by **simple diffusion** from the region of higher concentration, through the membrane, to the region of lower concentration, until the two compartments have equal solute concentrations (**Fig. 11-28a**). When ions of opposite charge are separated by a permeable membrane, there is a transmembrane electrical gradient, a **membrane potential,** V_m (expressed in millivolts). This membrane potential produces a force opposing ion movements that increase V_m and driving ion movements that reduce V_m (Fig. 11-28b). **P3** Thus, the direction in which a charged solute tends to move spontaneously across a membrane depends on both the chemical gradient (the difference in solute concentration) and the electrical gradient (V_m) across the membrane. Together these two factors are referred to as the **electrochemical gradient** or **electrochemical potential.** This behavior of solutes is in accord with the second law of thermodynamics: molecules tend to spontaneously assume the distribution of greatest randomness and lowest energy.

Membrane proteins that act by increasing the rate of solute movement across membranes are called transporters or carriers. **P3** Transporters are of two general types: passive and active. **Passive transporters** simply facilitate movement down a concentration gradient, increasing the transport rate. This process is called **passive transport** or

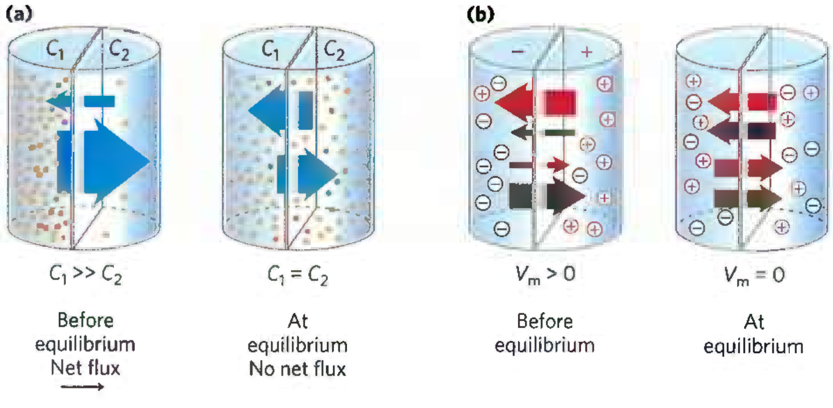

(a)
C_1
C_2
C_1
C_2
$C_1 >> C_2$
Before equilibrium Net flux
$C_1 = C_2$
At equilibrium No net flux
(b)
−
+
$V_m > 0$
Before equilibrium
$V_m = 0$
At equilibrium

FIGURE 11-28 Movement of solutes across a permeable membrane. (a) Net movement of an electrically neutral solute is toward the side of lower solute concentration until equilibrium is achieved. The solute concentrations on the left and right sides of the membrane, as shown here, are designated C_1 and C_2. The rate of transmembrane solute movement (indicated by the arrows) is proportional to the concentration ratio. (b) Net movement of an electrically charged solute is dictated by a combination of the electrical potential (V_m) and the ratio of chemical concentrations (C_2/C_1) across the membrane; net ion movement continues until this electrochemical potential reaches zero.

facilitated diffusion. **Active transporters** (sometimes called pumps) can move substrates across a membrane against a concentration gradient or an electrical potential, a process called **active transport**. **Primary active transporters** use energy provided directly by a chemical reaction; **secondary active transporters** couple uphill transport of one substrate with downhill transport of another.

Transporters and Ion Channels Share Some Structural Properties but Have Different Mechanisms

To pass through a lipid bilayer, a polar or charged solute must first give up its interactions with the water molecules in its hydration shell, then diffuse about 3 nm (30 Å) through a substance (lipid) in which it is poorly soluble (**Fig. 11-29a**). The energy used to strip away the hydration shell and to move the polar compound from water into lipid, then through the lipid bilayer, is regained as the compound leaves the membrane on the other side and is rehydrated. However, the intermediate stage of transmembrane passage is a high-energy state comparable to the transition state in an enzyme-catalyzed chemical reaction. In both cases, an activation barrier must be overcome to reach the intermediate stage (Fig. 11-29; compare with Fig. 6-3). The energy of activation ($\Delta G^\ddagger$) for translocation of a polar solute across the bilayer is so large that pure lipid bilayers are virtually impermeable to polar and charged species on time scales relevant to cell growth and division.

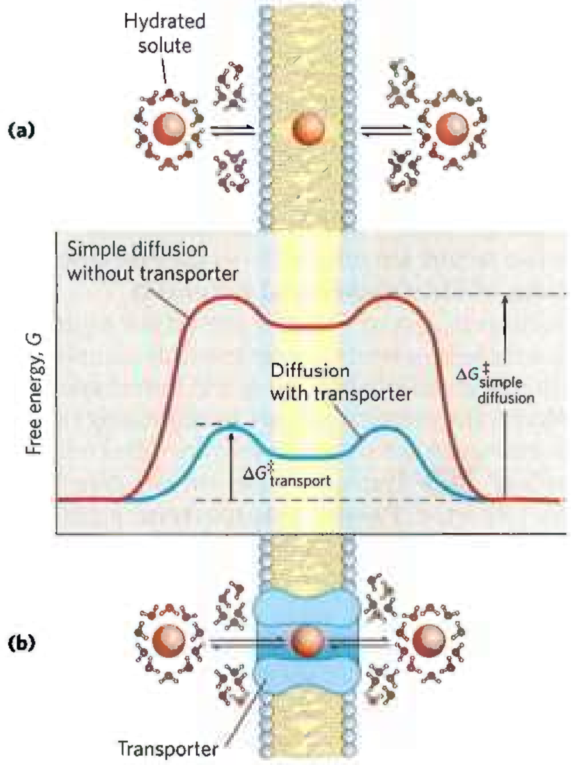
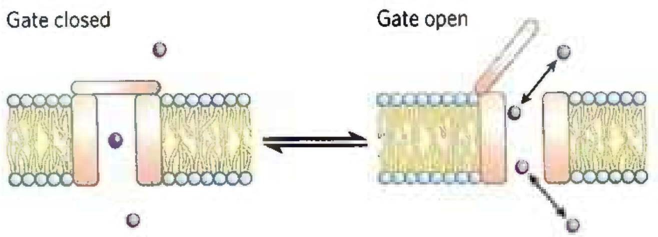

Hydrated solute
(a)
Simple diffusion without transporter
Diffusion with transporter
Free energy, G
$\Delta G^\ddagger_{\text{simple diffusion}}$
$\Delta G^\ddagger_{\text{transport}}$
(b)
Transporter

FIGURE 11-29 Energy changes accompanying passage of a hydrophilic solute through the lipid bilayer of a biological membrane. (a) In simple diffusion, removal of the hydration shell is highly endergonic, and the energy of activation ($\Delta G^\ddagger$) for diffusion through the bilayer is very high. (b) A transporter protein reduces the $\Delta G^\ddagger$ for transmembrane diffusion of the solute. It does this by forming noncovalent interactions with the dehydrated solute to replace the hydrogen bonding with water and by providing a hydrophilic transmembrane pathway.

P3 Membrane proteins lower the activation energy for transport of polar compounds and ions by providing an alternative path across the membrane for specific solutes. Lowering the activation energy greatly increases the rate of transmembrane movement (recall Eqn 6-6, p. 182). Transporters are not enzymes in the usual sense; their "substrates" are moved from one compartment to another but are not chemically altered. Like enzymes, however, transporters bind their substrates with stereochemical specificity through multiple weak, noncovalent interactions. The negative free-energy change associated with these weak interactions, $\Delta G_{\text{binding}}$, counterbalances the positive free-energy change that accompanies loss of the water of hydration from the substrate, $\Delta G_{\text{dehydration}}$, thereby lowering $\Delta G^\ddagger$ for transmembrane passage (Fig. 11-29b). Transporter proteins span the lipid bilayer several times, forming a transmembrane pathway lined with hydrophilic amino acid side chains. The pathway provides an alternative route for a specific substrate to move across the lipid bilayer without its having to dissolve in the bilayer, further lowering $\Delta G^\ddagger$ for transmembrane diffusion. The result is an orders-of-magnitude increase in the substrate's rate of passage across the membrane.

Ion channels use a different mechanism than transporters to move inorganic ions across membranes. **P3** Ion channels speed the passage of ions across membranes by providing an aqueous path across the membrane through which inorganic ions can diffuse at very high rates. Most ion channels have a "gate" (**Fig. 11-30a**) regulated by a biological signal. When the gate is open, ions move across the membrane, through the channel, in the direction dictated by the ion's charge and the electrochemical gradient. Movement occurs at rates approaching the limit of unhindered diffusion (tens of millions of ions per second per channel—much higher than typical transporter rates). Ion channels typically show some specificity for an ion, but they are not saturable with their ion substrate. Flow through a channel stops either when the gating mechanism is closed (again, by a biological signal) or when there is no longer an electrochemical gradient providing the driving force for the movement. In contrast, transporters, which bind their "substrates" with high stereospecificity, catalyze transport at rates well below the limits of free diffusion, and they are saturable in the same sense as are enzymes: there is some substrate concentration above which further increases will not produce a greater rate of transport. Transporters have a gate on either side of the membrane, and the two gates are never open at the same time (Fig. 11-30b).

(a) Ion channel: single gate



(b) Transporter (pump): alternating gates

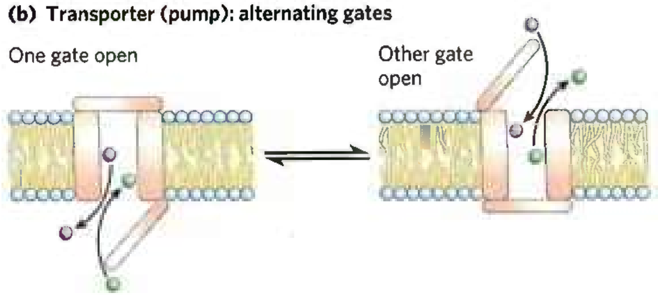


FIGURE 11-30 Differences between channels and transporters. (a) Ion channels have a transmembrane pore that is either open or closed, depending on the position of the single gate. When the gate is open, ions move through at a rate limited only by the maximum rate of diffusion. (b) Transporters have two gates, and both are never open at the same time. Movement of a substrate (an ion or a small molecule) through the membrane is therefore limited by the time needed for one gate to open and close (on one side of the membrane) and the second gate to open. Rates of movement through ion channels can be orders of magnitude greater than rates through transporters, but channels simply allow the ion to flow down the electrochemical gradient, whereas active transporters (pumps) can move a substrate against its concentration gradient. [Information from D. C. Gadsby, *Nat. Rev. Mol. Cell Biol.* 10:344, 2009, Fig. 1.]

Both transporters and ion channels constitute large families of proteins, defined not only by their primary sequences but also by their secondary structures. We next consider some well-studied representatives of the main transporter and channel families. You will also encounter some of these in Chapter 12 when we discuss transmembrane signaling, and some in later chapters in the context of the metabolic pathways in which they participate.

The Glucose Transporter of Erythrocytes Mediates Passive Transport

Energy-yielding metabolism in erythrocytes depends on a constant supply of glucose from the blood plasma, where the glucose concentration is maintained at about 4.5 to 5 mM. Glucose enters the erythrocyte by passive transport via a specific glucose transporter called GLUT1, at a rate about 50,000 times greater than it could cross the membrane unassisted.

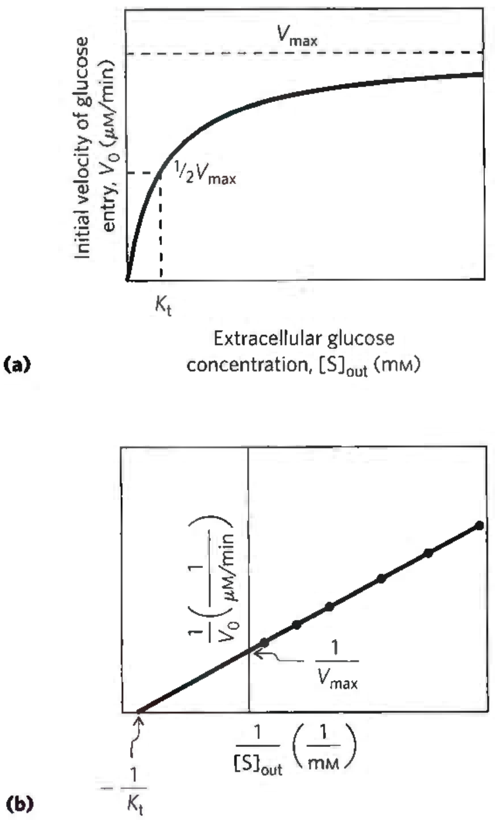


FIGURE 11-31 Kinetics of glucose transport into erythrocytes. (a) The initial rate of glucose entry into an erythrocyte, V_0, depends on the initial concentration of glucose on the outside, $[S]_{out}$. (b) Double-reciprocal plot of the data in (a). The kinetics of passive transport is analogous to the kinetics of an enzyme-catalyzed reaction. (Compare these plots with Figs 6-12 and 6-14.) K_t is analogous to K_m, the Michaelis constant.

P3 The process of glucose transport can be described by analogy with an enzymatic reaction in which the "substrate" is glucose outside the cell (S_{out}), the "product" is glucose inside the cell (S_{in}), and the "enzyme" is the transporter, T. When the initial rate of glucose uptake is measured as a function of external glucose concentration (**Fig. 11-31**), the resulting plot is hyperbolic: at high external glucose concentrations, the rate of uptake approaches V_{max}. Formally, such a transport process can be described by the set of equations

$$\begin{tikzcd}
S_{out} + T_1 \arrow[r, shift left, "k_1"] \arrow[d, shift right, "k_{-4}"'] & S_{out} \cdot T_1 \arrow[l, shift left, "k_{-1}"] \arrow[d, shift left, "k_2"] \\
S_{in} + T_2 \arrow[u, shift right, "k_4"'] \arrow[r, shift right, "k_{-3}"'] & S_{in} \cdot T_2 \arrow[l, shift right, "k_3"'] \arrow[u, shift left, "k_{-2}"]
\end{tikzcd}$$

in which k_1, k_{-1}, and so forth are the forward and reverse rate constants for each step; T_1 is the transporter conformation in which the glucose-binding site faces outward (in contact with blood plasma), and T_2 is the conformation in which it faces inward. Given that every step in this sequence is reversible, the transporter is, in principle, equally able to move glucose into or out of the cell. As for enzyme assays, this transporter assay measures the initial rate of uptake, when the product concentration (glucose concentration inside the cell) is zero, while the substrate concentration (glucose on the outside) is varied. In the living cell, GLUT1 accelerates the movement of glucose down its concentration gradient, which normally means *into* the cell. Glucose that enters a cell is generally metabolized immediately, and the intracellular glucose concentration is thereby kept low relative to its concentration in the blood.

The rate equations for glucose transport can be derived exactly as for enzyme-catalyzed reactions (Chapter 6), yielding an expression analogous to the Michaelis-Menten equation

$$V_0 = \frac{V_{\max}[\mathrm{S}]_{\mathrm{out}}}{K_{\mathrm{t}} + [\mathrm{S}]_{\mathrm{out}}} \tag{11-1}$$

in which V_0 is the initial velocity of accumulation of glucose inside the cell when its concentration in the surrounding medium is $[\mathrm{S}]_{\mathrm{out}}$, and $\boldsymbol{K_{\mathrm{t}}}$ **($\boldsymbol{K_{\mathrm{transport}}}$)** is a constant analogous to the Michaelis constant, a combination of rate constants that is characteristic of each transport system. This equation describes the *initial* velocity, the rate observed when $[\mathrm{S}]_{\mathrm{in}} = 0$. As is the case for enzyme-catalyzed reactions, the slope-intercept form of the equation describes a linear plot of $1/V_0$ against $1/[\mathrm{S}]_{\mathrm{out}}$, from which we can obtain values of K_{t} and $V_{\max}$ (Fig. 11-31b). When $[\mathrm{S}]_{\mathrm{out}} = K_{\mathrm{t}}$, the rate of uptake is $\frac{1}{2}V_{\max}$; the transport process is half-saturated. The concentration of glucose in blood, as noted above, is 4.5 to 5 mM, which is close enough to the K_{t} to ensure that GLUT1 is half-saturated with substrate and operates near one-half $V_{\max}$.

Because no chemical bonds are made or broken in the conversion of $\mathrm{S}_{\mathrm{out}}$ to S_{in}, neither "substrate" nor "product" is intrinsically more stable, and the process of entry is therefore fully reversible. As $[\mathrm{S}]_{\mathrm{in}}$ approaches $[\mathrm{S}]_{\mathrm{out}}$, the rates of entry and exit become equal. Such a system is therefore incapable of accumulating glucose within a cell at concentrations above that in the surrounding medium; it simply equilibrates glucose on the two sides of the membrane much faster than would occur in the absence of a specific transporter. GLUT1 is specific for D-glucose, with a K_{t} of about 6 mM. For the close analogs D-mannose and D-galactose, which differ only in the position of one hydroxyl group, the values of K_{t} are 20 mM and 30 mM, respectively, and for L-glucose, K_{t} exceeds 3,000 mM. Thus, GLUT1 shows the three hallmarks of passive transport: high rates of diffusion down a concentration gradient, saturability, and stereospecificity.

GLUT1 is an integral protein with 12 hydrophobic segments, each forming a membrane-spanning helix (**Fig. 11-32a**). The helices that line the transmembrane path for glucose are **amphipathic**; for each helix, the residues along one side are predominantly nonpolar, and those on the other side are mainly polar. This amphipathic structure is evident in a helical wheel diagram (Fig. 11-32b). A cluster of amphipathic helices are arranged so that their polar sides face each other and line a hydrophilic pore through which glucose can pass (Fig. 11-32c), while their hydrophobic sides interact with the surrounding membrane lipids such that the hydrophobic effect stabilizes the entire transporter structure.

Structural studies of mammalian GLUT1 and other GLUT transporters suggest that the protein cycles through a series of conformational changes, interconverting a form (T_1) with its glucose-binding site accessible only from the extracellular side, through a form in which the bound glucose is sequestered and inaccessible from either side, to a form (T_2) with the glucose-binding site open only to the intracellular side (**Fig. 11-33**).

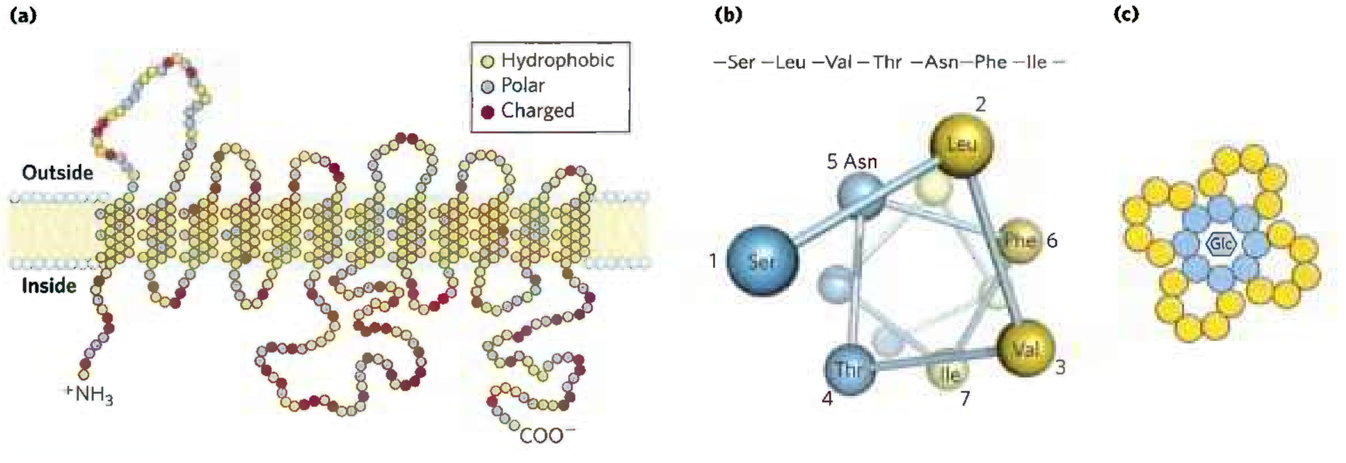


FIGURE 11-32 Membrane topology of the glucose transporter GLUT1. (a) Transmembrane helices are represented here as oblique (angled) rows of three or four amino acid residues, each row depicting one turn of the α helix. Of the 12 helices, 9 contain three or more polar or charged residues (blue or red), often separated by several hydrophobic residues (yellow). (b) A helical wheel diagram shows the distribution of polar and nonpolar residues on the surface of a helical segment. The helix is diagrammed as though observed along its axis from the amino terminus. Adjacent residues in the linear sequence are connected, and each residue is placed around the wheel in the position it occupies in the helix; recall that 3.6 residues are required to make one complete turn of the α helix. In this example, the polar residues (blue) are on one side of the helix, and the hydrophobic residues (yellow) are on the other. This is, by definition, an amphipathic helix. (c) Side-by-side association of amphipathic helices, each with its polar face oriented toward the central cavity, produces a transmembrane channel lined with polar (and charged) residues, available for interaction with glucose. [Information from (a, c) M. Mueckler, *Eur. J. Biochem.* 219:713, 1994.]

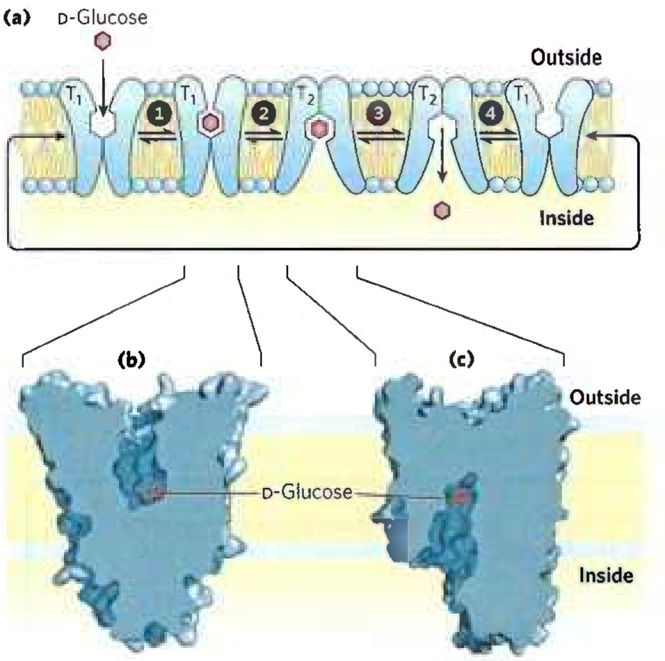


FIGURE 11-33 Model of glucose transport into erythrocytes by GLUT1. (a) The transporter exists in two extreme conformations: T_1, with the glucose-binding site exposed on the outer surface of the plasma membrane, and T_2, with the binding site exposed on the inner surface. Glucose transport occurs in four steps. ❶ Glucose in blood plasma binds to a stereospecific site on T_1; this lowers the activation energy for ❷ a conformational change from glucose$_{out}$ • T_1 to glucose$_{in}$ • T_2, effecting transmembrane passage of the glucose. ❸ Glucose is released from T_2 into the cytoplasm, and ❹ the transporter returns to the T_1 conformation, ready to transport another glucose molecule. Between the forms T_1 and T_2, there is an intermediate form (not shown here) in which glucose is sequestered within the transporter, with access to neither side. The structures of (b) human GLUT3 in the T_1 conformation and (c) human GLUT1 in the T_2 conformation, determined by x-ray crystallography, support the model shown in (a). [Data from (b) PDB ID 4ZWC, D. Deng et al., *Nature* 526:391, 2015; (c) PDB ID 4PYP, D. Deng et al., *Nature* 510:121, 2014.]

Twelve passive glucose transporters are encoded in the human genome, each with its unique kinetic properties, patterns of tissue distribution, and function (**Table 11-1**). GLUT1, in addition to supplying glucose to erythrocytes, also transports glucose across the blood-brain barrier, supplying the glucose that is essential for normal brain metabolism. The very rare individuals with defects in GLUT1 have a variety of brain-related symptoms, including seizures, movement and language disorders, and developmental delays. Standard care for such individuals includes a ketogenic diet, which provides the ketones that can serve as an alternative energy source for the brain. In the liver, GLUT2 transports glucose out of hepatocytes when liver glycogen is broken down to replenish blood glucose. GLUT2 has a large K_t ($\geq$17 mM) and can therefore respond to increased levels of intracellular glucose (produced by glycogen breakdown) by increasing outward transport. Skeletal and heart muscle and adipose tissue have yet another glucose transporter, GLUT4 (K_t = 5 mM), which is distinguished by its response to insulin: its activity increases when insulin signals a high blood glucose concentration, thus increasing the rate of glucose uptake into muscle and adipose tissue. **Box 11-1** describes the effect of insulin on this transporter. ■

The Chloride-Bicarbonate Exchanger Catalyzes Electroneutral Cotransport of Anions across the Plasma Membrane

The erythrocyte contains another passive transport system, an anion exchanger that is essential in CO_2 transport to the lungs from tissues such as skeletal muscle and liver. Waste CO_2 released from respiring tissues

TABLE 11-1 Glucose Transporters in Humans

Transporter	Tissue(s) where expressed	K_t (mM)	Role/characteristics[a]
GLUT1	Erythrocytes, blood-brain barrier, placenta, most tissues at a low level	3	Basal glucose uptake; defective in De Vivo disease
GLUT2	Liver, pancreatic islets, intestine, kidney	17	In liver and kidney, removal of excess glucose from blood; in pancreas, regulation of insulin release
GLUT3	Brain (neuron), testis (sperm)	1.4	Basal glucose uptake; high turnover number
GLUT4	Muscle, fat, heart	5	Activity increased by insulin
GLUT5	Intestine (primarily), testis, kidney	6[b]	Primarily fructose transport
GLUT6	Spleen, leukocytes, brain	>5	Possibly no transporter function
GLUT7	Small intestine, colon, testis, prostate	0.3	—
GLUT8	Testis, sperm acrosome	~2	—
GLUT9	Liver, kidney, intestine, lung, placenta	0.6	Urate and glucose transporter in liver, kidney
GLUT10	Heart, lung, brain, liver, muscle, pancreas, placenta, kidney	0.3[c]	Glucose and galactose transporter
GLUT11	Heart, skeletal muscle	0.16	Glucose and fructose transporter
GLUT12	Skeletal muscle, heart, prostate, placenta	—	—

Information on localization from M. Mueckler and B. Thorens, *Mol. Aspects Med.* 34:121, 2013. K_t values for glucose from R. Augustin, *IUBMB Life* 62:315, 2010.

[a] Dash indicates role uncertain.

[b] K_m for fructose.

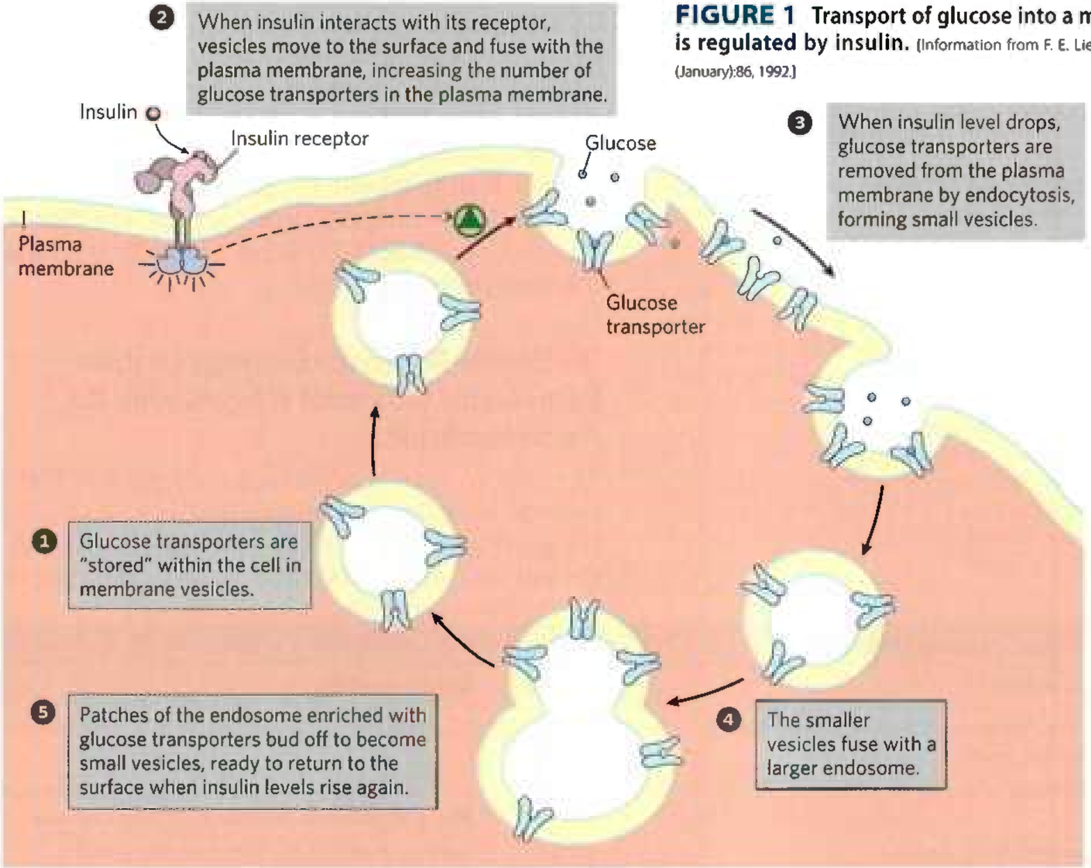
[c] K_m for 2-deoxyglucose.

BOX 11-1 **MEDICINE**

Defective Glucose Transport in Diabetes

When ingestion of a carbohydrate-rich meal causes blood glucose to exceed the usual concentration between meals (about 5 mM), excess glucose is taken up by the myocytes of cardiac and skeletal muscle (which store it as glycogen) and by adipocytes (which convert it to triacylglycerols). Glucose uptake into myocytes and adipocytes is mediated by the glucose transporter GLUT4. Between meals, some GLUT4 is present in the plasma membrane, but most (90%) is sequestered in the membranes of small intracellular vesicles (Fig. 1). Insulin is released from the pancreas in response to high blood glucose, and it triggers, within minutes, the movement of these vesicles to the plasma membrane. The vesicles fuse with the membrane, bringing most of the GLUT4 molecules to the cell surface (see Fig. 12-23) and increasing the rate of glucose uptake 15-fold or more. When blood glucose levels return to normal, insulin release slows and most GLUT4 molecules are removed from the plasma membrane by endocytosis and stored in vesicles.

In type 1 (insulin-dependent) diabetes mellitus, the inability to release insulin (and thus to mobilize glucose transporters) results in low rates of glucose uptake into muscle and adipose tissue. One consequence is a prolonged period of high blood glucose after a carbohydrate-rich meal. This condition is the basis for the glucose tolerance test used to diagnose diabetes (Chapter 23).



❷ When insulin interacts with its receptor, vesicles move to the surface and fuse with the plasma membrane, increasing the number of glucose transporters in the plasma membrane.

❸ When insulin level drops, glucose transporters are removed from the plasma membrane by endocytosis, forming small vesicles.

❶ Glucose transporters are "stored" within the cell in membrane vesicles.

❹ The smaller vesicles fuse with a larger endosome.

❺ Patches of the endosome enriched with glucose transporters bud off to become small vesicles, ready to return to the surface when insulin levels rise again.

FIGURE 1 **Transport of glucose into a myocyte by GLUT4 is regulated by insulin.** [Information from F. E. Lienhard et al., *Sci. Am.* 266 (January):86, 1992.]

into the blood plasma enters the erythrocyte, where it is converted to bicarbonate HCO_3^- by the enzyme carbonic anhydrase. (Recall that HCO_3^- is the primary buffer of blood pH; see Fig. 2-20.) The HCO_3^- reenters the blood plasma for transport to the lungs (**Fig. 11-34**). Because HCO_3^- is much more soluble in blood plasma than is CO_2, this roundabout route increases the capacity of the blood to carry carbon dioxide from the tissues to the lungs. In the lungs, HCO_3^- reenters the erythrocyte and is converted to CO_2, which is eventually released into the lung space and exhaled. To be effective, this shuttle requires very rapid movement of HCO_3^- across the erythrocyte membrane. As described in Chapter 5 (pp. 160–161), there is a second mechanism for moving CO_2 from tissue to lung, involving reversible binding of CO_2 to hemoglobin.)

The chloride-bicarbonate exchanger, also called the anion exchange (AE) protein, increases the rate of HCO_3^- transport across the erythrocyte membrane more than a millionfold. Like the glucose transporter, it is a dimeric integral protein that spans the membrane 14 times. This protein mediates the simultaneous movement of two anions: for every HCO_3^- ion that moves in one direction,

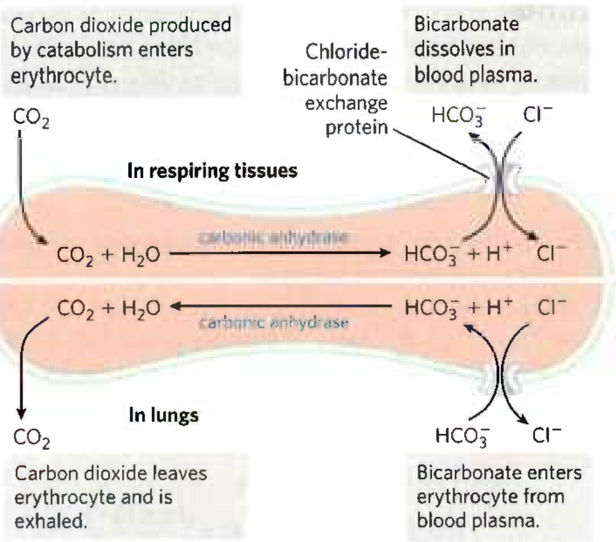

Carbon dioxide produced by catabolism enters erythrocyte.
Chloride-bicarbonate exchange protein
Bicarbonate dissolves in blood plasma.
CO_2
HCO_3^-
Cl^-
In respiring tissues
$CO_2 + H_2O$
carbonic anhydrase
$HCO_3^- + H^+$
Cl^-
$CO_2 + H_2O$
carbonic anhydrase
$HCO_3^- + H^+$
Cl^-
In lungs
CO_2
HCO_3^-
Cl^-
Carbon dioxide leaves erythrocyte and is exhaled.
Bicarbonate enters erythrocyte from blood plasma.

FIGURE 11-34 Chloride-bicarbonate exchanger of the erythrocyte membrane. This cotransport system allows the entry and exit of HCO_3^- without changing the membrane potential. Its role is to increase the CO_2-carrying capacity of the blood. The top half of the figure illustrates the events that take place in respiring tissues; the bottom half illustrates the events in the lungs.

one Cl^- ion moves in the opposite direction, with no net transfer of charge: the exchange is **electroneutral**. The coupling of Cl^- and HCO_3^- movements is obligatory; in the absence of chloride, bicarbonate transport stops. In this respect, the anion exchanger is typical of those systems, called **cotransport** systems, that simultaneously carry two solutes across a membrane (**Fig. 11-35**). When, as in this case, the two substrates move in opposite directions, the process is **antiport**. In **symport**, two substrates are moved simultaneously in the same direction. Transporters that carry only one substrate, such as the erythrocyte glucose transporter, are known as **uniport** systems.

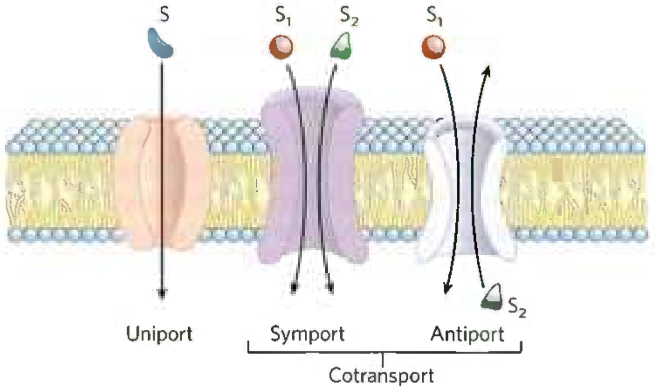

S
S_1
S_2
S_1
S_2
Uniport
Symport
Antiport
Cotransport

FIGURE 11-35 Three general classes of transport systems. Transporters differ in the number of solutes (substrates) transported and the direction in which each solute moves. Examples of all three types of transporter are discussed in the text. Note that this classification tells us nothing about whether these are energy-requiring (active transport) or energy-independent (passive transport) processes.

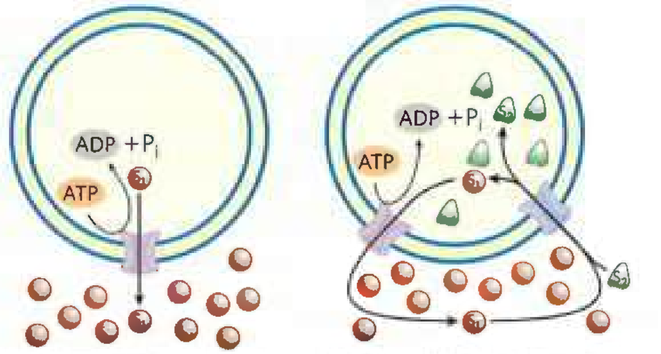
Active Transport Results in Solute Movement against a Concentration or Electrochemical Gradient

P3 In passive transport, the transported species always moves down its electrochemical gradient and is not accumulated above the equilibrium concentration. Active transport, by contrast, results in the accumulation of a solute above the equilibrium point. Active transport is essential when cells function in an environment in which key substrates are present outside the cell only at very low concentrations. For example, the bacterium *E. coli* can grow in a medium containing only 1 μM P_i (inorganic phosphate), but the cell must maintain internal P_i levels in the millimolar range. Worked Example 11-2 describes another such situation, which requires cells to pump Ca^{2+} outward across the plasma membrane. Active transport is thermodynamically unfavorable (endergonic) and takes place only when coupled, directly or indirectly, to an exergonic process such as the absorption of sunlight, an oxidation reaction, the breakdown of ATP, or the concomitant flow of some other chemical species down its electrochemical gradient. In **primary active transport**, solute accumulation is coupled directly to an exergonic chemical reaction, such as conversion of ATP to ADP + P_i (**Fig. 11-36**). **Secondary active transport** occurs when endergonic (uphill) transport of one solute is coupled to the exergonic (downhill) flow of a different solute that was originally pumped uphill by primary active transport.

The amount of energy needed for the transport of a solute against a gradient can be calculated from the initial concentration gradient. The general equation for the free-energy change in the chemical process that converts substrate (S) to product (P) is

$$\Delta G = \Delta G'^{\circ} + RT \ln([P]/[S]) \qquad (11\text{-}2)$$

where $\Delta G'^{\circ}$ is the standard free-energy change, R is the gas constant (8.315 J/mol•K), and T is the absolute


ADP + P_i
ATP
S_1
ADP + P_i
ATP
S_1
S_2

(a) Primary active transport **(b)** Secondary active transport

FIGURE 11-36 Two types of active transport. (a) In primary active transport, the energy released by ATP hydrolysis drives solute (S_1) movement against an electrochemical gradient. (b) In secondary active transport, a gradient of an ion (designated S_1; often Na^+) has been established by primary active transport. Movement of S_1 down its electrochemical gradient now provides the energy to drive cotransport of a second solute, S_2, against its electrochemical gradient.

temperature. When the "reaction" is simply transport of a solute from a region where its concentration is C_1 to a region where its concentration is C_2, no bonds are made or broken and $\Delta G'^\circ$ is zero. The free-energy change for transport, ΔG_t, is then

$$\Delta G_t = RT \ln (C_2/C_1) \qquad (11\text{-}3)$$

If there is, say, a 10-fold difference in concentration between two compartments, the cost of moving 1 mol of an uncharged solute at 25 °C uphill across a membrane separating the compartments is

$$\begin{aligned}\Delta G_t &= (8.315 \text{ J/mol} \cdot \text{K})(298 \text{ K}) \ln (10/1) = 5{,}700 \text{ J/mol}\\ &= 5.7 \text{ kJ/mol}\end{aligned}$$

Equation 11-3 holds for all uncharged solutes.

WORKED EXAMPLE 11-1 *Energy Cost of Pumping an Uncharged Solute*

Calculate the energy cost (free-energy change) of pumping an uncharged solute against a 10^4-fold concentration gradient at 25 °C.

SOLUTION: Begin with Equation 11-3. Substitute 1.0×10^4 for (C_2/C_1), 8.315 J/mol • K for *R*, and 298 K for *T*:

$$\begin{aligned}\Delta G_t &= RT \ln (C_2/C_1)\\ &= (8.315 \text{ J/mol} \cdot \text{K})(298 \text{ K})(1.0 \times 10^4)\\ &= 23 \text{ kJ/mol}\end{aligned}$$

When the solute is an *ion*, its movement without an accompanying counterion results in the endergonic separation of positive and negative charges, producing an electrical potential; such a transport process is said to be **electrogenic**. The energetic cost of moving an ion depends on the electrochemical potential (Fig. 11-25), the sum of the chemical and electrical gradients:

$$\Delta G_t = RT \ln (C_2/C_1) + ZF\,\Delta\psi \qquad (11\text{-}4)$$

where *Z* is the charge on the ion, *F* is the Faraday constant (96,480 J/V • mol), and $\Delta\psi$ is the transmembrane electrical potential (in volts). Eukaryotic cells typically have plasma membrane potentials of about 0.05 V (with the inside negative relative to the outside), so the second term on the right side of Equation 11-4 can make a significant contribution to the total free-energy change for transporting an ion. Most cells maintain more than a 10-fold difference in ion concentrations across their plasma or intracellular membranes, and for many cells and tissues active transport is therefore a major energy-consuming process.

WORKED EXAMPLE 11-2 *Energy Cost of Pumping a Charged Solute*

Calculate the energy cost (free-energy change) of pumping Ca^{2+} from the cytosol, where its concentration is about 1.0×10^{-7} M, to the extracellular fluid, where its concentration is about 1.0 mM. Assume a temperature of 37 °C (body temperature in a mammal) and a standard transmembrane potential of 50 mV (inside negative) for the plasma membrane.

SOLUTION: This is a case in which energy must be expended to counter two forces acting on the ion being transported: the membrane potential and the concentration difference across the membrane. These forces are expressed in the two terms on the right side of Equation 11-4:

$$\Delta G_t = RT \ln (C_2/C_1) + ZF\,\Delta\psi$$

in which the first term describes the chemical gradient and the second describes the electrical potential.

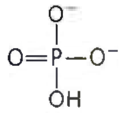
In Equation 11-4, substitute 8.315 J/mol • K for *R*, 310 K for *T*, 1.0×10^{-3} for C_2, 1.0×10^{-7} for C_1, +2 (the charge on a Ca^{2+} ion) for *Z*, 96,500 J/V • mol for *F*, and 0.050 V for $\Delta\psi$. Note that the transmembrane potential is 50 mV (inside negative), so the change in potential when an ion moves from inside to outside is 50 mV.

$$\begin{aligned}\Delta G_t &= RT \ln (C_2/C_1) + ZF\,\Delta\psi\\ &= (8.315 \text{ J/mol} \cdot \text{K})(310 \text{ K}) \ln \frac{(1.0 \times 10^{-3})}{(1.0 \times 10^{-7})}\\ &\quad + 2(96{,}500 \text{ J/V} \cdot \text{mol})(0.050 \text{ V})\\ &= 33 \text{ kJ/mol}\end{aligned}$$

The mechanism of active transport is of fundamental importance in biology. As we shall see in Chapters 19 and 20, ATP is formed in mitochondria and chloroplasts by a mechanism that is essentially ATP-driven ion transport operating in reverse. The energy made available by the spontaneous flow of protons across a membrane is calculable from Equation 11-4; remember that ΔG for flow *down* an electrochemical gradient has a negative value, and ΔG for transport of ions *against* an electrochemical gradient has a positive value.

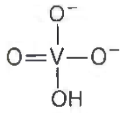
P-Type ATPases Undergo Phosphorylation during Their Catalytic Cycles

The family of active transporters called **P-type ATPases** are cation transporters that are reversibly phosphorylated by ATP (thus the name P-type) as part of the transport cycle. Phosphorylation forces a conformational change that is central to movement of the cation across the membrane. The human genome encodes at least 70 P-type ATPases that share similarities in amino acid sequence and topology, especially near the Asp residue that undergoes phosphorylation. All are integral proteins with 8 or 10 predicted membrane-spanning regions in a single polypeptide, and all are sensitive to inhibition by the transition-state analog **vanadate**, which mimics phosphate when under nucleophilic attack by a water molecule.



$$\mathrm{O{=}P(O^-)(OH){-}O^-}$$

Phosphate

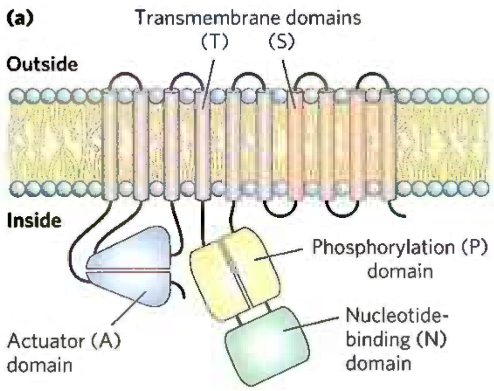


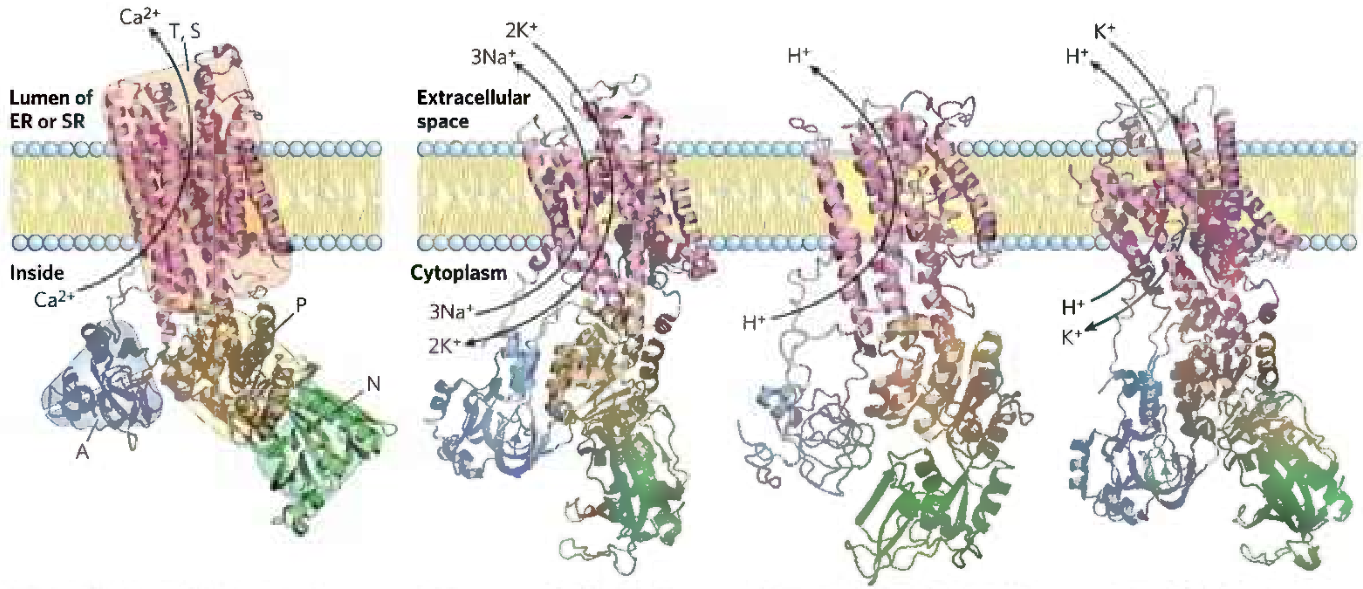
$$\mathrm{O{=}V(O^-)(OH){-}O^-}$$

Vanadate

The P-type ATPases are widespread in eukaryotes and bacteria. The Na^+K^+ ATPase of animal cells (an antiporter for Na^+ and K^+ ions) and the plasma membrane H^+ ATPase of plants and fungi set the transmembrane electrochemical potential in cells by establishing ion gradients across the plasma membrane. These gradients provide the driving force for secondary active transport and are also the basis for electrical signaling in neurons. In animal tissues, the **sarcoplasmic/endoplasmic reticulum *Ca^{2+}* ATPase (SERCA) pump** and the plasma membrane Ca^{2+} ATPase pump, which are uniporters for Ca^{2+} ions, together maintain the cytosolic level of Ca^{2+} below 1 μM. The SERCA pump moves Ca^{2+} from the cytosol into the lumen of the sarcoplasmic reticulum. Parietal cells in the lining of the mammalian stomach have a P-type ATPase that pumps H^+ and K^+ out of the cells and into the stomach, thereby acidifying the stomach contents. Lipid flippases, as we noted earlier, are structurally and functionally related to P-type transporters. Bacteria and eukaryotes use P-type ATPases to pump toxic heavy metal ions such as Cd^{2+} and Cu^{2+} out of cells.

All P-type pumps have similar structures (**Fig. 11-37**) and similar mechanisms. The mechanism postulated for P-type ATPases takes into account the large conformational changes and the phosphorylation-dephosphorylation of the critical Asp residue in the P (phosphorylation) domain that is known to occur during a catalytic cycle. For the SERCA pump (**Fig. 11-38**), each catalytic cycle moves two Ca^{2+} ions across the membrane and converts an ATP to ADP and P_i. The role of ATP binding and phosphoryl transfer to the enzyme is to bring about the interconversion of two conformations, E1 and E2, of the transporter. In the E1 conformation, the two Ca^{2+}-binding sites are exposed on the cytosolic side of the ER or sarcoplasmic reticulum and bind Ca^{2+} with high affinity. ATP binding and Asp phosphorylation drive a conformational change from E1 to E2 that exposes the Ca^{2+}-binding sites to the lumen and greatly reduces their affinity for Ca^{2+}, which releases the Ca^{2+} ions into the lumen. By this mechanism, the





(b) Ca^{2+} ATPase (SERCA pump) **(c)** Na^+K^+ ATPase Plasma membrane H^+ ATPase Gastric H^+K^+ ATPase

FIGURE 11-37 The general structure of the P-type ATPases. (a) P-type ATPases have three cytoplasmic domains (A, N, and P) and two transmembrane domains (T and S) consisting of multiple helices. The N (nucleotide-binding) domain binds ATP and Mg^{2+} and has protein kinase activity that phosphorylates a specific Asp residue in the P (phosphorylation) domain of all P-type ATPases. The A (actuator) domain has protein phosphatase activity and removes the phosphoryl group from the Asp residue with each catalytic cycle of the pump. A transport (T) domain with six transmembrane helices includes the ion-transporting structure, and four more transmembrane helices make up the support (S) domain, which provides physical support to the transport domain and may have other specialized functions in certain P-type ATPases. The binding sites for the ions to be transported are near the middle of the membrane, 40 to 50 Å from the phosphorylated Asp residue—thus, Asp phosphorylation-dephosphorylation does not *directly* affect ion binding. The A domain communicates movements of the N and P domains to the ion-binding sites. (b) A ribbon representation of the Ca^{2+} ATPase (SERCA pump). ATP binds to the N domain, and the Ca^{2+} ions to be transported bind to the T domain. (c) Other P-type ATPases have domain structures, and presumably mechanisms, like those of the SERCA pump; shown here are Na^+K^+ ATPase, the plasma membrane H^+ ATPase, and the gastric H^+K^+ ATPase. [(a) Information from M. Bublitz et al., *Curr. Opin. Struct. Biol.* 20:431, 2010, Fig. 1. Data from (b) PDB ID 1SU4, C. Toyoshima et al., *Nature* 405:647, 2000; (c) Na^+K^+ ATPase, PDB ID 3KDP, J. Preben Morth et al., *Nature* 450:1043, 2007; H^+ ATPase, PDB ID 3B8C, B. P. Pedersen et al., *Nature* 450:1111, 2007; H^+K^+ ATPase, PDB ID 3IXZ, K. Abe et al., *EMBO J.* 28:1637, 2009, and PDB ID 3B8E, J. Preben Morth et al., *Nature* 450:1043, 2007.]

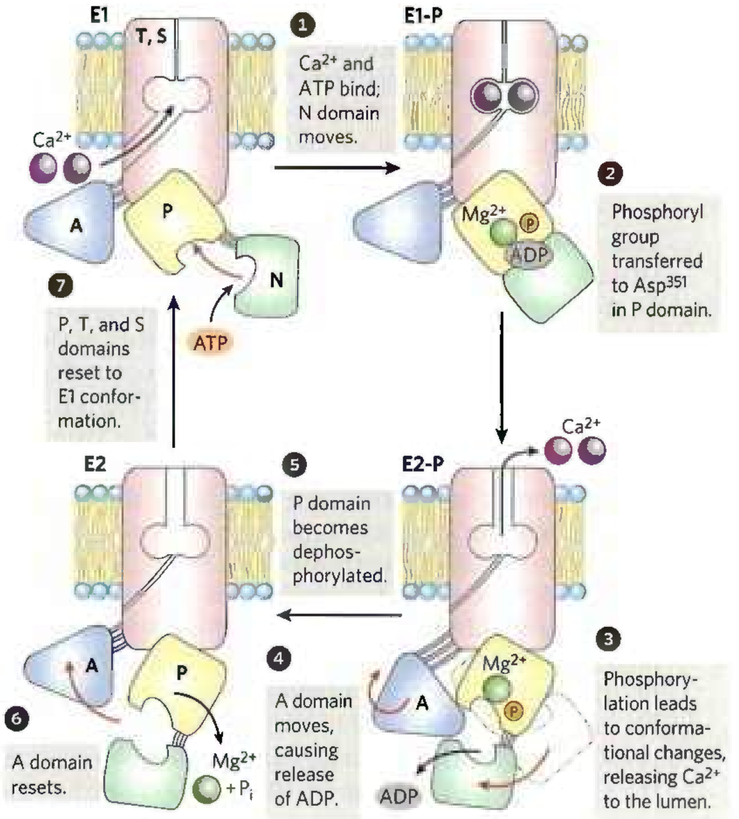

E1
T, S
Ca²⁺
A
P
N
ATP
① Ca²⁺ and ATP bind; N domain moves.
E1-P
Mg²⁺
P
ADP
② Phosphoryl group transferred to Asp³⁵¹ in P domain.
E2-P
Ca²⁺
③ Phosphorylation leads to conformational changes, releasing Ca²⁺ to the lumen.
④ A domain moves, causing release of ADP.
ADP
⑤ P domain becomes dephosphorylated.
E2
⑥ A domain resets.
Mg²⁺ + Pᵢ
⑦ P, T, and S domains reset to E1 conformation.

FIGURE 11-38 Postulated mechanism of the SERCA pump. The transport cycle begins with the protein in the E1 conformation, with the Ca^{2+}-binding sites facing the cytosol. ① Two Ca^{2+} ions bind, then ATP binds to the transporter and ② phosphorylates Asp^{351}, forming E1-P. ③ Phosphorylation favors the second conformation, E2-P, in which the Ca^{2+}-binding sites, now with a reduced affinity for Ca^{2+}, are accessible on the other side of the membrane (the lumen or extracellular space), and the released Ca^{2+} diffuses away. ④ ADP is released, and ⑤ E2-P is dephosphorylated. ⑥ The A domain resets and ⑦ the protein returns to the E1 conformation for another round of transport. [Information from W. Kühlbrandt, *Nat. Rev. Mol. Cell Biol.* 5:282, 2004.]

energy released by hydrolysis of ATP during one phosphorylation-dephosphorylation cycle drives Ca^{2+} across the membrane against a large electrochemical gradient.

A variation on this basic mechanism is seen in the **Na^+K^+ ATPase** of the plasma membrane. This cotransporter couples phosphorylation-dephosphorylation of the critical Asp residue to the simultaneous movement of both Na^+ and K^+ against their electrochemical gradients. The Na^+K^+ ATPase is responsible for maintaining low Na^+ and high K^+ concentrations in the cell relative to the extracellular fluid (**Fig. 11-39**). For each molecule of ATP converted to ADP and P_i, the transporter moves two K^+ ions inward and three Na^+ ions outward across the plasma membrane. Cotransport is therefore electrogenic, creating a net separation of charge across the membrane; in animals, this produces the membrane potential of –50 to –70 mV (inside negative relative to outside) that is characteristic of most cells and is essential to the conduction of action potentials in neurons. The central role of the Na^+K^+ ATPase is reflected in the energy invested in this single reaction: about 25% of the total energy consumption of a human at rest.

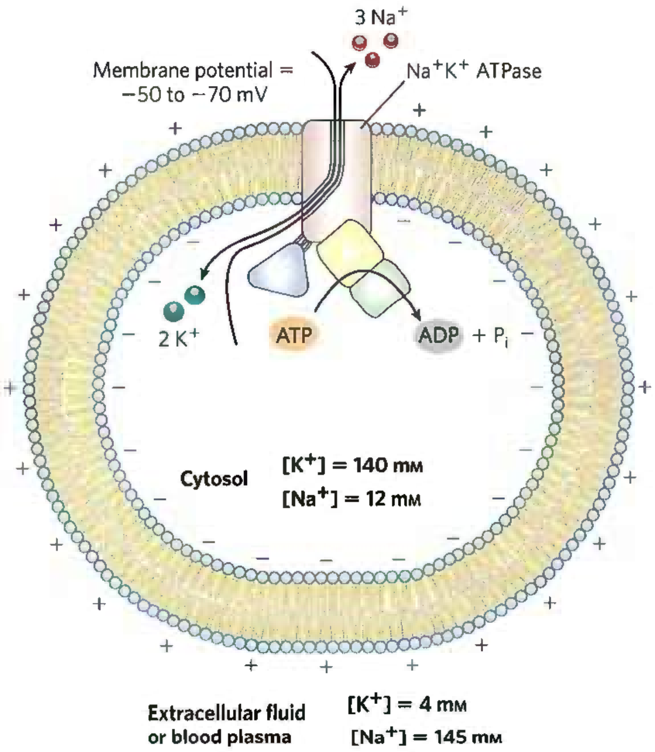

3 Na⁺
Membrane potential = −50 to −70 mV
Na⁺K⁺ ATPase
2 K⁺
ATP
ADP + Pᵢ
Cytosol
[K⁺] = 140 mM
[Na⁺] = 12 mM
Extracellular fluid or blood plasma
[K⁺] = 4 mM
[Na⁺] = 145 mM

FIGURE 11-39 Role of the Na^+K^+ ATPase in animal cells. This active transport system is primarily responsible for setting and maintaining the intracellular concentrations of Na^+ and K^+ in animal cells and for generating the membrane potential. It does this by moving three Na^+ ions out of the cell for every two K^+ ions it moves in. The electrical potential across the plasma membrane is central to electrical signaling in neurons, and the gradient of Na^+ is used to drive the uphill cotransport of solutes in many cell types.

V-Type and F-Type ATPases Are ATP-Driven Proton Pumps

V-type ATPases, a class of proton-transporting ATPases, are responsible for acidifying intracellular compartments in many organisms (thus, *V* for *v*acuolar). Proton pumps of this type maintain the vacuoles of fungi and higher plants at a pH between 3 and 6, well below that of the

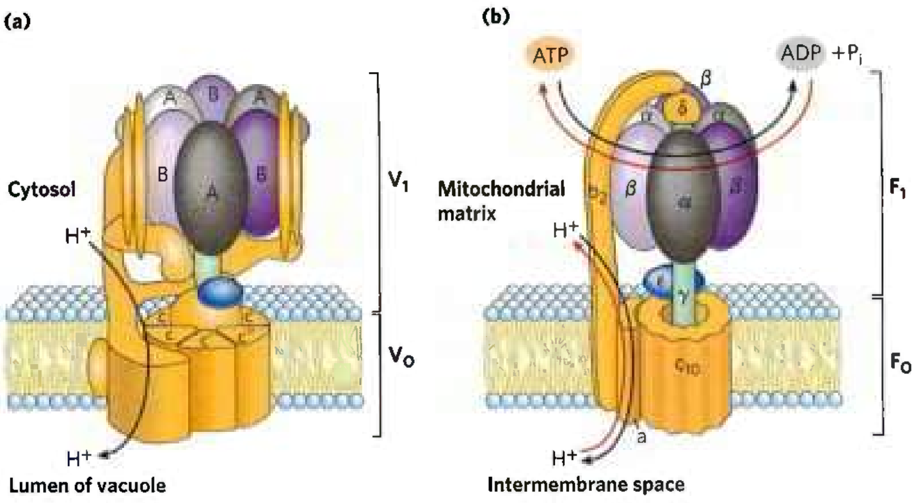


FIGURE 11-40 Two proton pumps with similar structures. (a) The V_0V_1 H^+ ATPase uses ATP to pump protons into vacuoles and lysosomes, creating their low internal pH. It has an integral (membrane-embedded) domain, V_0, that includes multiple identical c subunits, and a peripheral domain that projects into the cytosol and contains the ATP-hydrolyzing sites, on three identical B subunits (purple). (b) The F_0F_1 ATPase/ATP synthase of mitochondria has an integral domain, F_0, with multiple copies of the c subunit, and a peripheral domain, F_1, consisting of three α subunits, three β subunits, and a central shaft joined to the integral domain. F_0 and V_0 provide transmembrane channels through which protons are pumped as ATP is hydrolyzed on the β subunits of F_1 (B subunits of V_1). An ATP-driven proton transporter also can catalyze ATP synthesis (red arrows) as protons flow *down* their electrochemical gradient. This is the central reaction in the processes of oxidative phosphorylation and photophosphorylation.

surrounding cytosol (pH 7.5). V-type ATPases are also responsible for the acidification of lysosomes, endosomes, the Golgi complex, and secretory vesicles in animal cells. All V-type ATPases have a similar complex structure, with an integral (transmembrane) domain (V_0) that serves as a proton channel and a peripheral domain (V_1) that contains the ATP-binding site and the ATPase activity (**Fig. 11-40a**). The structure is similar to that of the well-characterized F-type ATPases.

F-type ATPase transporters catalyze the uphill transmembrane passage of protons, driven by ATP hydrolysis. The "F-type" designation derives from the identification of these ATPases as energy-coupling *f*actors. The F_0 integral membrane protein complex (Fig. 11-40b; subscript "o" denotes its inhibition by the drug oligomycin) provides a transmembrane pathway for protons, and the peripheral protein F_1 (subscript "1" indicating that this was the first of several factors isolated from mitochondria) uses the energy of ATP to drive protons uphill (into a region of higher H^+ concentration). The F_0F_1 organization of proton-pumping transporters must have developed very early in evolution. Bacteria such as *E. coli* use an F_0F_1 ATPase complex in their plasma membrane to pump protons outward, and archaea have a closely homologous proton pump, the A_0A_1 ATPase.

P3 Like all enzymes, F-type ATPases catalyze their reactions in both directions. Therefore, a sufficiently large proton gradient can supply the energy to drive the reverse reaction, ATP synthesis (Fig. 11-40b). When functioning in this direction, the F-type ATPases are more appropriately named **ATP synthases**. ATP synthases are central to ATP production in mitochondria during oxidative phosphorylation and in chloroplasts during photophosphorylation, as well as in bacteria and archaea. The proton gradient needed to drive ATP synthesis is produced by other types of proton pumps powered by substrate oxidation or sunlight. We provide a detailed description of these processes in Chapters 19 and 20.

ABC Transporters Use ATP to Drive the Active Transport of a Wide Variety of Substrates

ABC transporters constitute a large family of ATP-driven transporters that pump amino acids, peptides, proteins, metal ions, various lipids, bile salts, and many hydrophobic compounds, including drugs, across a membrane against a concentration gradient. Many ABC transporters are located in the plasma membrane, but some are also found in the ER and in the membranes of mitochondria and lysosomes. All members of this family have two ATP-binding domains ("cassettes") that give the family its name—*A*TP-*b*inding *c*assette transporters—and two transmembrane domains, each containing six transmembrane helices. In some cases, all these domains are in a single, long polypeptide; other ABC transporters have two subunits, each contributing a nucleotide-binding domain (NBD) and a domain with six transmembrane helices. The transport mechanism is believed to involve two forms of the transporter, one with its substrate-binding site facing the outside of the cell, the other open to substrate on the inside (**Fig. 11-41**). Substrates move across the membrane when the two forms interconvert, driven by ATP hydrolysis. The NBDs of all ABC proteins are similar in sequence and presumably in three-dimensional structure. They constitute

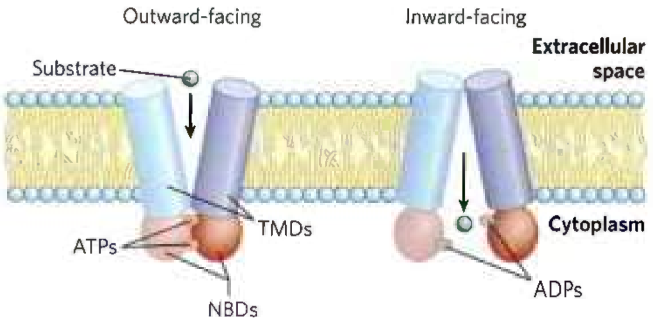


FIGURE 11-41 ABC transporters. The protein has two homologous halves, each with a six-helix transmembrane domain (TMD; blue), and a cytoplasmic nucleotide-binding domain (NBD; red). In the mechanism proposed for the coupling of ATP hydrolysis to transport, with ATP bound to the NBD sites, substrate binds to the transporter on the cytoplasmic side. Upon substrate binding and ATP hydrolysis to ADP, a conformational change exposes the substrate to the outside surface and lowers the transporter's affinity for its substrate; substrate diffuses away from the transporter into the extracellular space. This mechanism for the coupling of ATP hydrolysis to transport is based on structures of a number of ABC transporters crystallized under different conditions. Compare this process with the model of glucose transport in Figure 11-33.

the conserved molecular motor that can be coupled to a wide variety of transmembrane domains, each capable of pumping one specific substrate across a membrane. When coupled this way, the ATP-driven motor moves solutes against a concentration gradient, with a stoichiometry of about one ATP hydrolyzed per molecule of substrate transported.

The human genome contains at least 48 genes that encode ABC transporters; a number of these are presented in **Table 11-2**. Some of these transporters have very high specificity for a single substrate; others are more promiscuous, able to transport drugs that cells presumably did not encounter during their evolution. Many ABC transporters are involved in maintaining the composition of the lipid bilayer, such as the floppases that move membrane lipids from one leaflet of the bilayer to the other. Many others are needed to move sterols, sterol derivatives, and fatty acids into the bloodstream for transport throughout the body. For example, the cellular machinery for exporting excess cholesterol includes an ABC transporter (see Fig. 21-47). Mutations in the genes that encode some of these proteins contribute to genetic diseases, including liver failure, retinal degeneration, and Tangier disease. The cystic fibrosis transmembrane conductance regulator protein (CFTR) of the plasma membrane is an interesting case of an ABC protein that is an ion channel (for Cl^-), regulated by ATP hydrolysis, but without the pumping function characteristic of an active transporter (**Box 11-2**).

One human ABC transporter with very broad substrate specificity is the **multidrug transporter (MDR1)**, encoded by the *ABCB1* gene. MDR1 in the placental membrane and in the blood-brain barrier ejects toxic compounds that would damage the fetus or the brain. But it is also responsible for the striking resistance of certain tumors to some generally effective antitumor drugs. For example, MDR1 pumps the chemotherapeutic drugs doxorubicin and vinblastine out of cells, thus preventing their accumulation within a tumor and blocking their therapeutic effects. Overexpression of MDR1 is often associated with treatment failure in cancers of the liver, kidney, and colon. A related ABC transporter, BCRP (*b*reast *c*ancer *r*esistance *p*rotein, encoded by the *ABCG2* gene), is overexpressed in breast cancer cells, also conferring resistance to anticancer drugs. Highly selective inhibitors of these multidrug transporters are expected to enhance the effectiveness of antitumor drugs and are the objects of current drug discovery and design.

ABC transporters are also present in simpler animals and in plants and microorganisms. Yeast has 31 genes that encode ABC transporters, *Drosophila* has 56, and *E. coli* has 80, representing 2% of its entire genome. ABC transporters that are used by *E. coli* and other bacteria to import essentials such as vitamin B_{12} are the presumed evolutionary precursors of the MDRs of animal cells. The presence of ABC transporters that confer antibiotic resistance in pathogenic microbes (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *Neisseria gonorrhoeae*, and *Plasmodium falciparum*) is a serious public health concern and makes these transporters attractive targets for drug design. ■

TABLE 11-2 Some ABC Transporters in Humans

Gene(s)	Role/characteristics	Text reference
ABCA1	Reverse cholesterol transport; defect causes Tangier disease	Fig. 21-47
ABCA4	Only in visual receptors, recycling of all-*trans*-retinal	Fig. 12-19
ABCB1	Multidrug resistance P-glycoprotein 1; transport across blood-brain barrier	—
ABCB4	Multidrug resistance; transport of phosphatidylcholine in bile	—
ABCB11	Transports bile salts out of hepatocytes	Fig. 17-1
ABCC6	Sulfonylurea receptor; targeted by the drug glipizide in type 2 diabetes	Fig. 23-27
ABCG2	Breast cancer resistance protein (BCRP); major exporter of anticancer drugs	p. 396
ABCC7	CFTR (Cl^- channel); defect causes cystic fibrosis	Box 11-2

BOX 11-2 MEDICINE

A Defective Ion Channel in Cystic Fibrosis

Cystic fibrosis (CF) is a serious hereditary disease. In the United States, the frequency of CF is about 1 in 3,200 live births, and 1% to 4% (depending on ethnicity) are carriers, having one defective copy of the gene and one normal copy. Only individuals with two defective copies show the severe symptoms of the disease: obstruction of the gastrointestinal and respiratory tracts, commonly leading to bacterial infection of the airways.

The defective gene underlying CF was discovered in 1989. It encodes a membrane protein called cystic fibrosis transmembrane conductance regulator, or CFTR. This protein has two segments, each containing six transmembrane helices, two nucleotide-binding domains (NBDs), and a regulatory region that connects them (Fig. 1). CFTR is therefore very similar to other ABC transporter proteins, except that it functions as an *ion channel* (for Cl^-), not as a pump. The channel conducts Cl^- across the plasma membrane when both NBDs have bound ATP, and it closes when the ATP on one of the NBDs is broken down to ADP and P_i. The Cl^- channel is further regulated by phosphorylation of several Ser residues in the regulatory domain, catalyzed by cAMP-dependent protein kinase. When the regulatory domain is not phosphorylated, the Cl^- channel is closed.

The mutation responsible for CF in up to 90% of cases results in deletion of a Phe residue at position 508 (a mutation denoted F508del). The mutant protein folds incorrectly, causing it to be degraded in proteasomes. As a result, Cl^- movement is reduced across the plasma membranes of epithelial cells that line the airways, digestive tract, exocrine glands (pancreas, sweat glands), bile ducts, and vas deferens. Less-common mutations, such as G551D (Gly^{551} changed to Asp), lead to production of CFTR that is correctly folded and inserted into the membrane but is defective in Cl^- transfer.

Diminished export of Cl^- in individuals with CF is accompanied by diminished export of water from cells, causing the mucus on cell surfaces to become dehydrated, thick, and excessively sticky. In normal circumstances, cilia on the epithelial cells lining the inner surface of the lungs constantly sweep away bacteria that settle in this mucus, but the thick mucus in individuals with CF hinders this process, providing a haven in the lungs for pathogenic bacteria. Frequent infections by bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* cause progressive damage to the lungs and reduce respiratory efficiency, eventually resulting in death due to inadequate lung function.

Advances in therapy have raised the average life expectancy for people who have CF from just 10 years in 1960 to more than 40 years today. CFTR potentiators such as ivacaftor (VX-770) increase the function of the mutant G551D protein that is properly folded and in place in the plasma membrane. For individuals with the folding defect, F508del, CFTR

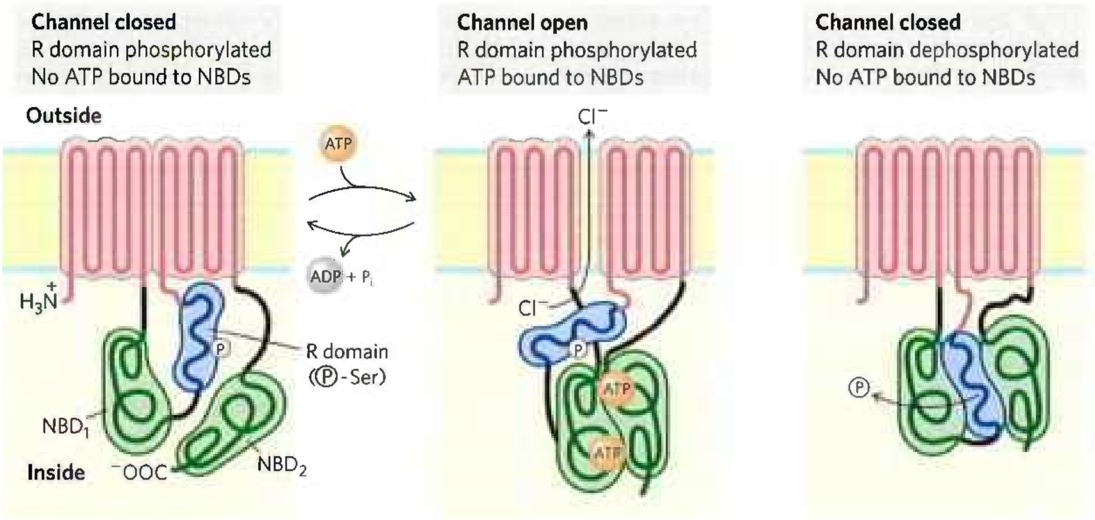


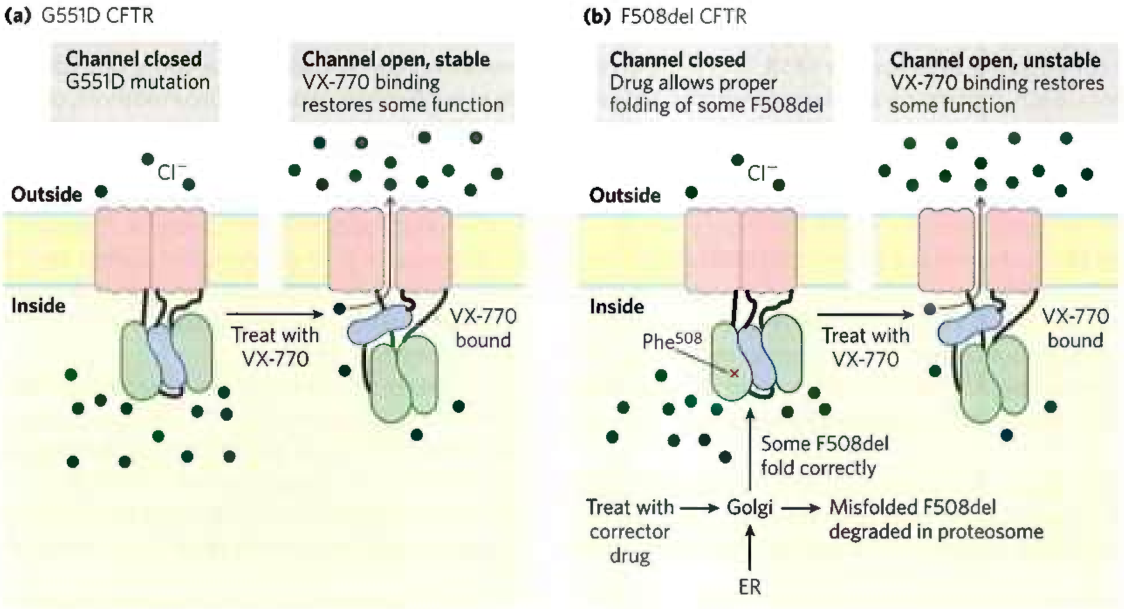
FIGURE 1 Three states of the CFTR protein. The protein has two segments, each with six transmembrane helices, and three functionally significant domains extend from the cytoplasmic surface: NBD_1 and NBD_2 (green) are nucleotide-binding domains that bind ATP, and the regulatory R domain (blue) is the site of phosphorylation by cAMP-dependent protein kinase. When this R domain is phosphorylated but no ATP is bound to the NBDs (left), the channel is closed. The binding of ATP opens the channel (middle) until the bound ATP is hydrolyzed. When the R domain is unphosphorylated (right), it binds the NBD domains and prevents ATP binding and channel opening. CFTR is a typical ABC transporter in all but two respects: most ABC transporters lack the regulatory domain, and CFTR acts as an ion channel (for Cl^-), not as a typical transporter.

(Continued on next page)

BOX 11-2 MEDICINE

A Defective Ion Channel in Cystic Fibrosis (*Continued*)

correctors improve the processing and delivery of the mutant protein to the cell surface; a combination of potentiator and corrector drugs is more effective than the corrector drug alone for these patients (Fig. 2). In 2019, clinicals trials of a combination of three drugs (ivacaftor, tezacaftor, and elexacaftor), acting as both potentiators and correctors, showed dramatic improvements in individuals with the most common mutation (F508del).



(a) G551D CFTR
Channel closed
G551D mutation
Channel open, stable
VX-770 binding restores some function
Cl^-
Outside
Inside
Treat with VX-770
VX-770 bound
(b) F508del CFTR
Channel closed
Drug allows proper folding of some F508del
Channel open, unstable
VX-770 binding restores some function
Cl^-
Outside
Inside
Phe^{508}
Treat with VX-770
VX-770 bound
Some F508del fold correctly
Treat with corrector drug
Golgi
Misfolded F508del degraded in proteosome
ER

FIGURE 2 (a) The CFTR mutation G551D (replacement of Gly^{551} with Asp) results in a protein that is inserted into the membrane correctly but is defective as a Cl^- channel. Addition of the potentiator drug VX-770 (ivacaftor) restores partial function to the Cl^- channel. (b) The more common mutation F508del (deletion of Phe^{508}) prevents proper folding of CFTR, causing it to be degraded in proteasomes. In the presence of a corrector drug, folding and membrane insertion can take place; addition of the potentiator drug results in partial restoration of Cl^- channel activity. The channel is unstable and is degraded over time. [Information from J. P. Clancy, *Sci. Transl. Med.* 6:1, 2014.]

Ion Gradients Provide the Energy for Secondary Active Transport

The ion gradients formed by primary transport of Na^+ or H^+ can, in turn, provide the driving force for cotransport of other solutes. Many cell types have transport systems that couple the spontaneous, downhill flow of these ions to the simultaneous uphill pumping of another ion, sugar, or amino acid.

In intestinal epithelial cells, glucose and certain amino acids are accumulated by symport with Na^+, down the Na^+ gradient established by the Na^+K^+ ATPase of the plasma membrane (**Fig. 11-42**). The apical surface of the intestinal epithelial cell (the surface that faces the intestinal contents) is covered with microvilli—long, thin projections of the plasma membrane that greatly increase the surface area exposed to the intestinal contents. The **Na^+-glucose symporter** in the apical plasma membrane takes up glucose from the intestine in a process driven by the downhill flow of Na^+:

$$2\text{Na}^+_{\text{out}} + \text{glucose}_{\text{out}} \longrightarrow 2\text{Na}^+_{\text{in}} + \text{glucose}_{\text{in}}$$

The energy required for this process comes from two sources: the greater concentration of Na^+ outside than inside the cell (the chemical potential) and the membrane (electrical) potential, which is inside negative and therefore draws Na^+ inward. P3 The strong thermodynamic tendency for Na^+ to move into the cell provides the energy needed for the transport of glucose into the cell, against its concentration gradient. An ion gradient created and sustained by energy-dependent ion pumping serves as the potential energy for cotransport of another species against its concentration gradient.

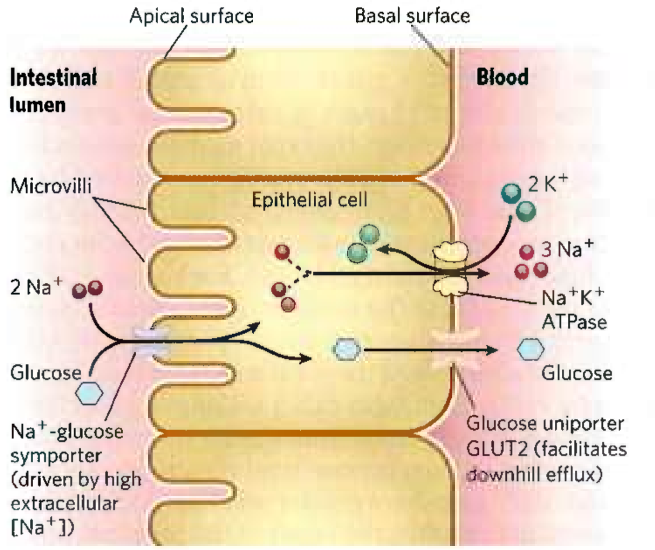


FIGURE 11-42 Glucose transport in intestinal epithelial cells. Glucose is cotransported with Na^+ across the apical plasma membrane into the epithelial cell. It moves through the cell to the basal surface, where it passes into the blood via GLUT2, a passive glucose uniporter. The Na^+K^+ ATPase continues to pump Na^+ outward to maintain the Na^+ gradient that drives glucose uptake.

WORKED EXAMPLE 11-3 *Energetics of Pumping by Symport*

Calculate the maximum $\frac{[\text{glucose}]_{\text{in}}}{[\text{glucose}]_{\text{out}}}$ ratio that can be achieved by the plasma membrane Na^+-glucose symporter of an epithelial cell when $[Na^+]_{\text{in}}$ is 12 mM, $[Na^+]_{\text{out}}$ is 145 mM, the membrane potential is −50 mV (inside negative), and the temperature is 37 °C.

SOLUTION: Using Equation 11-4 (p. 392), we can calculate the energy inherent in an electrochemical Na^+ gradient—that is, the cost of moving one Na^+ ion up this gradient:

$$\Delta G_t = RT \ln \frac{[Na^+]_{\text{out}}}{[Na^+]_{\text{in}}} + ZF\,\Delta\psi$$

We then substitute standard values for R, T, and F; the given values for $[Na^+]$ (expressed as molar concentrations); +1 for Z (because Na^+ has a positive charge); and 0.050 V for $\Delta\psi$. Note that the membrane potential is −50 mV (inside negative), so the change in potential when an ion moves from inside to outside is 50 mV.

$$\begin{aligned}\Delta G_t &= (8.315\ \text{J/mol}\cdot\text{K})(310\ \text{K})\ \ln\frac{(1.45\times10^{-1})}{(1.2\times10^{-2})}\\ &\quad + 1(96{,}500\ \text{J/V}\cdot\text{mol})(0.050\ \text{V})\\ &= 11.2\ \text{kJ/mol}\end{aligned}$$

When Na^+ reenters the cell, it releases the electrochemical potential created by pumping it out; ΔG for reentry is −11.2 kJ/mol of Na^+. This is the potential energy per mole of Na^+ that is available to pump glucose. Given that two Na^+ ions pass down their electrochemical gradient and into the cell for each glucose carried in by symport, the energy available to pump 1 mol of glucose is 2×11.2 kJ/mol $=22.4$ kJ/mol. We can now calculate the maximum concentration ratio of glucose that can be achieved by this pump (from Eqn 11-3, p. 392):

$$\Delta G_t = RT \ln \frac{[\text{glucose}]_{\text{in}}}{[\text{glucose}]_{\text{out}}}$$

Rearranging, then substituting the values of ΔG_t, R, and T, gives

$$\ln \frac{[\text{glucose}]_{\text{in}}}{[\text{glucose}]_{\text{out}}} = \frac{\Delta G_t}{RT} = \frac{22.4\ \text{kJ/mol}}{(8.315\ \text{J/mol}\cdot\text{K})(310\ \text{K})} = 8.69$$

$$\frac{[\text{glucose}]_{\text{in}}}{[\text{glucose}]_{\text{out}}} = e^{8.69} = 5.94\times10^{3}$$

Thus, the cotransporter can pump glucose inward until its concentration inside the epithelial cell is about 6,000 times the concentration outside (in the intestine). (This is the maximum theoretical ratio, assuming a perfectly efficient coupling of Na^+ reentry and glucose uptake.)

As glucose molecules are pumped from the intestine into the epithelial cell at the apical surface, glucose is simultaneously moved from the cell into the blood by passive transport through a glucose transporter (GLUT2) in the basal surface (Fig. 11-42). The crucial role of Na^+ in symport and antiport systems such as this requires the continued outward pumping of Na^+ to maintain the transmembrane Na^+ gradient.

In the kidney, a different Na^+-glucose symporter (SGLT2) is the target of drugs used to treat type 2 diabetes. Gliflozins are specific inhibitors of this Na^+-glucose symporter. They lower blood glucose by inhibiting glucose reabsorption in the kidney, thus preventing the damaging effects of elevated blood glucose. Glucose not reabsorbed in the kidney is cleared in the urine. This class of drugs, taken orally in combination with diet and exercise, lowers blood glucose significantly in individuals with type 2 diabetes.

Because of the essential role of ion gradients in active transport and energy conservation, compounds that collapse ion gradients across cellular membranes are effective poisons, and those that are specific for infectious microorganisms can serve as antibiotics. One such substance is valinomycin, a small cyclic peptide that neutralizes the K^+ charge by surrounding the ion with six carbonyl oxygens (**Fig. 11-43**). The hydrophobic peptide then acts as a shuttle, carrying K^+ across the membrane down its concentration gradient and deflating that gradient. Compounds that shuttle ions across membranes in this way are called **ionophores** ("ion bearers"). Both valinomycin and monensin (a Na^+-carrying ionophore) are antibiotics; they kill microbial cells by disrupting secondary transport processes and energy-conserving reactions. Monensin is widely used as an antifungal and antiparasitic agent. ■

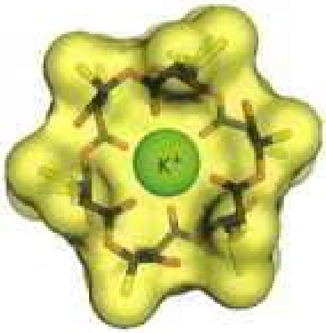


FIGURE 11-43 **Valinomycin, a peptide ionophore that binds K$^+$.** The central K$^+$ ion is surrounded by inward-facing polar and charged amino acid side chains, and the outside surface is covered with nonpolar side chains that make the whole structure hydrophobic enough to diffuse through the lipid bilayer, carrying K$^+$ down its concentration gradient. The resulting dissipation of the transmembrane ion gradient kills microbial cells, making valinomycin a potent antibiotic. [Information from P. Barak and E. A. Nater, http://virtual-museum.soils.wisc.edu, and K. Neupert-Laves and M. Dobler, *Helv. Chim. Acta* 58:432, 1975.]

Aquaporins Form Hydrophilic Transmembrane Channels for the Passage of Water

P3 A family of integral membrane proteins, the **aquaporins (AQPs)**, provide channels for rapid movement of water molecules across all plasma membranes. Aquaporins are found in all organisms, and multiple aquaporin genes are generally present, encoding similar but not identical proteins. Eleven aquaporins are known in mammals, each with a specific location and role (**Table 11-3**). The exocrine glands that produce sweat, saliva, and tears secrete water through aquaporins. Erythrocytes, which swell or shrink rapidly in response to abrupt changes in extracellular osmolarity as blood travels through the renal medulla, have a high density of aquaporin in their plasma membrane (2×10^5 copies of AQP1 per cell). Seven aquaporins play roles in urine production and water retention in the nephron (the functional unit of the kidney). Each renal AQP has a specific location in the nephron, and each has specific properties and regulatory features. For example, AQP2 in the epithelial cells of the renal collecting duct is regulated by vasopressin (also called antidiuretic hormone): more water is reabsorbed from the duct into the kidney tissues when the vasopressin level is high. Mutant mice with no AQP2 gene have greater urine output (polyuria) and more dilute urine, the result of the proximal tubule becoming less permeable to water. In humans, genetically defective AQPs are known to be responsible for a variety of diseases.

Water molecules flow through an AQP1 channel at a rate of about $10^9\ \text{s}^{-1}$. For comparison, the highest known turnover number for an enzyme is that for catalase, $4 \times 10^7\ \text{s}^{-1}$, and many enzymes have turnover numbers between $1\ \text{s}^{-1}$ and $10^4\ \text{s}^{-1}$ (see Table 6-7). The low activation energy for passage of water through aquaporin channels ($\Delta G^{\ddagger} < 15$ kJ/mol) suggests that water moves through the channels in a continuous stream, in the direction dictated by the osmotic gradient. (For a

TABLE 11-3 **Permeability Characteristics and Predominant Distribution of Known Mammalian Aquaporins**

Aquaporin	Permeant (permeability)	Tissue distribution	Primary subcellular distribution[a]
AQP0	Water (low)	Lens	Plasma membrane
AQP1	Water (high)	Erythrocyte, kidney, lung, vascular endothelium, brain, eye	Plasma membrane
AQP2	Water (high)	Kidney, vas deferens	Apical plasma membrane, intracellular vesicles
AQP3	Water (high), glycerol (high), urea (moderate)	Kidney, skin, lung, eye, colon	Basolateral plasma membrane
AQP4	Water (high)	Brain, muscle, kidney, lung, stomach, small intestine	Basolateral plasma membrane
AQP5	Water (high)	Salivary gland, lacrimal gland, sweat gland, lung, cornea	Apical plasma membrane
AQP6	Water (low), anions ($NO_3^- > Cl^-$)	Kidney	Intracellular vesicles
AQP7	Water (high), glycerol (high), urea (high)	Adipose tissue, kidney, testis	Plasma membrane
AQP8[b]	Water (high)	Testis, kidney, liver, pancreas, small intestine, colon	Plasma membrane, intracellular vesicles
AQP9	Water (low), glycerol (high), urea (high)	Liver, leukocyte, brain, testis	Plasma membrane
AQP10	Water (low), glycerol (high), urea (high)	Small intestine	Intracellular vesicles

Information from L. S. King et al., *Nat. Rev. Mol. Cell Biol.* 5:688, 2004.

[a] The apical plasma membrane faces the lumen of the gland or tissue; the basolateral plasma membrane is along the sides and base of the cell, not facing the lumen of the gland or tissue.

[b] AQP8 might also be permeated by urea.

discussion of osmosis, see p. 52.) Aquaporins do not allow passage of protons (hydronium ions, H_3O^+), which would collapse membrane electrochemical gradients.

Ion-Selective Channels Allow Rapid Movement of Ions across Membranes

Ion-selective channels—first recognized in neurons and now known to be present in the plasma membranes of all cells, as well as in the intracellular membranes of eukaryotes—provide another mechanism for moving inorganic ions across membranes. Ion channels, together with ion pumps such as the Na^+K^+ ATPase, determine a plasma membrane's permeability to specific ions and regulate the cytosolic concentration of ions and the membrane potential. In neurons, very rapid changes in the activity of ion channels cause the changes in membrane potential (action potentials) that carry signals from one end of a neuron to the other. In myocytes, rapid opening of Ca^{2+} channels in the sarcoplasmic reticulum releases the Ca^{2+} that triggers muscle contraction. We discuss the signaling functions of ion channels in Chapter 12. Here we describe the structural basis for ion-channel function, using as our example a well-studied K^+ channel.

Ion channels are distinct from ion transporters in at least three ways. First, the rate of flux through channels can be orders of magnitude greater than the turnover number for a transporter—10^7 to 10^8 ions/s for an ion channel, approaching the theoretical maximum for unrestricted diffusion. By contrast, the turnover rate of the Na^+K^+ ATPase is about 100 s^{-1}. Second, ion channels are not saturable: rates do not approach a maximum at high substrate concentration. Third, they are gated in response to some type of cellular event. In **ligand-gated channels** (which are generally oligomeric), binding of an extracellular or intracellular small molecule forces an allosteric transition in the protein, which opens or closes the channel. In **voltage-gated ion channels**, a change in transmembrane electrical potential (V_m) causes a charged protein domain to move relative to the membrane, opening or closing the channel. Both types of gating can be very fast. A channel typically opens in a fraction of a millisecond and may remain open for only milliseconds, making these molecular devices effective for very fast signal transmission in the nervous system.

Because a single ion channel typically remains open for only a few milliseconds, monitoring this process is beyond the limit of most biochemical measurements. Ion fluxes must therefore be measured electrically, either as changes in V_m (in the millivolt range) or as electric current I (in the microampere or picoampere range), using microelectrodes and appropriate amplifiers. In **patch-clamping**, very small currents are measured through a tiny region of the membrane surface containing only one or a few ion-channel molecules (**Fig. 11-44**). The researcher can measure the size and duration of the current that flows during one opening of an ion channel and can determine how often a channel opens and how that frequency is affected by membrane potential, regulatory ligands, toxins, and other agents. Patch-clamp studies

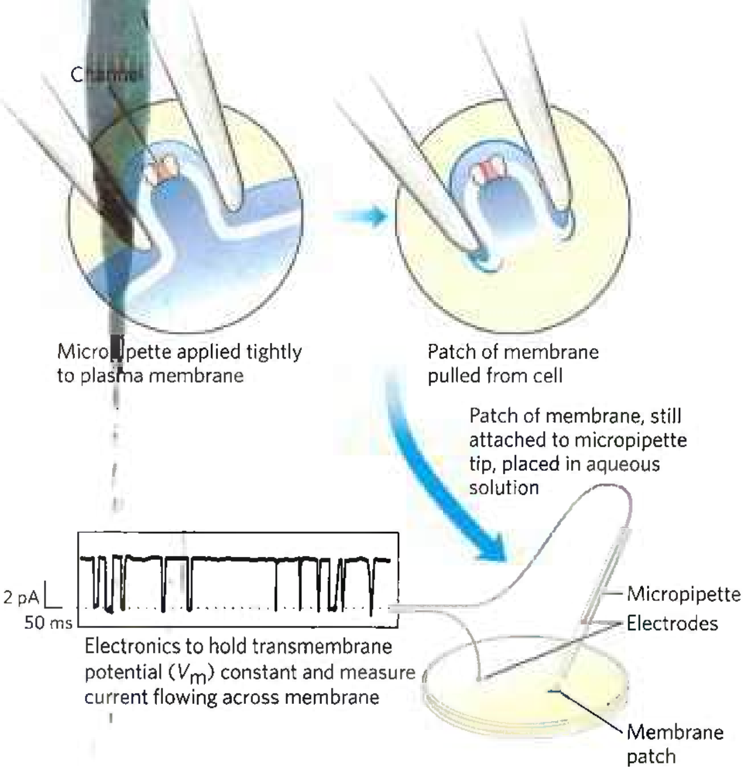

Channel
Micropipette applied tightly to plasma membrane
Patch of membrane pulled from cell
Patch of membrane, still attached to micropipette tip, placed in aqueous solution
2 pA
50 ms
Electronics to hold transmembrane potential (V_m) constant and measure current flowing across membrane
Micropipette
Electrodes
Membrane patch

FIGURE 11-44 Electrical measurements of ion-channel function. The "activity" of an ion channel is estimated by measuring the flow of ions through it, using the patch-clamp technique. A finely drawn-out pipette (micropipette) is pressed against the cell surface, and negative pressure in the pipette forms a pressure seal between pipette and membrane. As the pipette is pulled away from the cell, it pulls off a tiny patch of membrane (which may contain one or a few ion channels). After placing the pipette and attached patch in an aqueous solution, the researcher can measure channel activity as the electric current that flows between the contents of the pipette and the aqueous solution. In practice, a circuit is set up that "clamps" the transmembrane potential at a given value and measures the current that must flow to maintain this voltage. With highly sensitive detectors, researchers can measure the current flowing through a single ion channel, typically a few picoamperes. The trace shows the current through a single acetylcholine receptor channel as a function of time (in milliseconds), revealing how fast the channel opens and closes, how frequently it opens, and how long it stays open. Downward deflection represents channel opening. Clamping the V_m at different values permits determination of the effect of membrane potential on these parameters of channel function. [Information from V. Witzemann et al., *Proc. Natl. Acad. Sci. USA* 93:13,286, 1996.]

have revealed that as many as 10^4 ions can move through a single ion channel in 1 ms. Such an ion flux represents a huge amplification of the initial signal, which may be just one or two signaling molecules (neurotransmitters, for example).

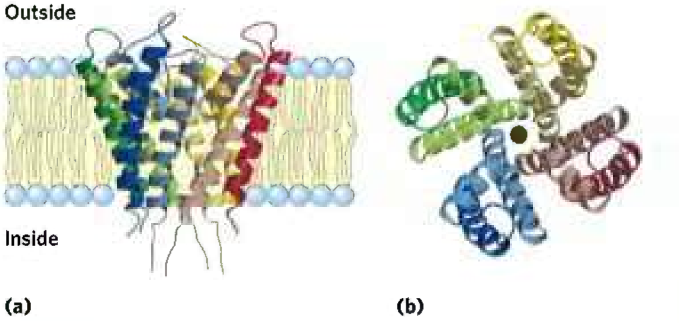
The Structure of a K$^+$ Channel Reveals the Basis for Its Specificity

The structure of a potassium channel from the bacterium *Streptomyces lividans* provides important insight into the way ion channels work. This bacterial ion channel is related in sequence to all other known K$^+$ channels and serves as the prototype for such channels, including the voltage-gated K$^+$ channel of neurons. Among the members of this protein family, the similarities in sequence are greatest in the "pore region," which contains the ion selectivity filter that allows K$^+$ (radius 1.33 Å) to pass 10^4 times more readily than Na$^+$ (radius 0.95 Å)—at a rate (about 10^8 ions/s) approaching the theoretical limit for unrestricted diffusion.

The K$^+$ channel consists of four identical subunits that span the membrane and form a cone within a cone surrounding the ion channel, with the wide end of the double cone facing the extracellular space (**Fig. 11-45a**). Each subunit has two transmembrane α helices and a third, shorter helix that contributes to the pore region. The outer cone is formed by one of the transmembrane helices of each subunit. The inner cone, formed by the other four transmembrane helices, surrounds the ion channel and cradles the ion selectivity filter. Viewed perpendicular to the plane of the membrane, the central channel is seen to be just wide enough to accommodate an unhydrated metal ion such as potassium (Fig. 11-45b).

Both the ion specificity and the high flux through the channel are understandable from what we know of the channel's structure (Fig. 11-45c). At the inner and outer plasma membrane surfaces, the entryways to the channel have several negatively charged amino acid residues, which presumably increase the local concentration of cations such as K$^+$ and Na$^+$. The ion path through the membrane begins (on the inner surface) as a wide, water-filled channel in which the ion can retain its hydration sphere. Further stabilization is provided by the short helices in the pore region of each subunit, with the partial negative charges of their electric dipoles pointed at K$^+$ in the channel. About two-thirds of the way through the membrane, this channel narrows in the region of the selectivity filter, forcing the ion to give up its hydrating water molecules. Carbonyl oxygen atoms in the backbone of the selectivity filter replace the water molecules in the hydration sphere, forming a series of perfect coordination shells through which the K$^+$ moves. This favorable interaction with the filter is not possible for Na$^+$, which is too small to make contact with all the potential oxygen ligands. The preferential stabilization of K$^+$ is the basis for the ion selectivity of the filter, and mutations that change residues in this part of the protein eliminate the channel's ion selectivity. The K$^+$-binding sites of the filter are flexible enough to collapse to fit any Na$^+$ that enters the channel, and this conformational change closes the channel.

There are four potential K$^+$-binding sites along the selectivity filter, each composed of an oxygen "cage" that provides ligands for the K$^+$ ions (Fig. 11-45c). In



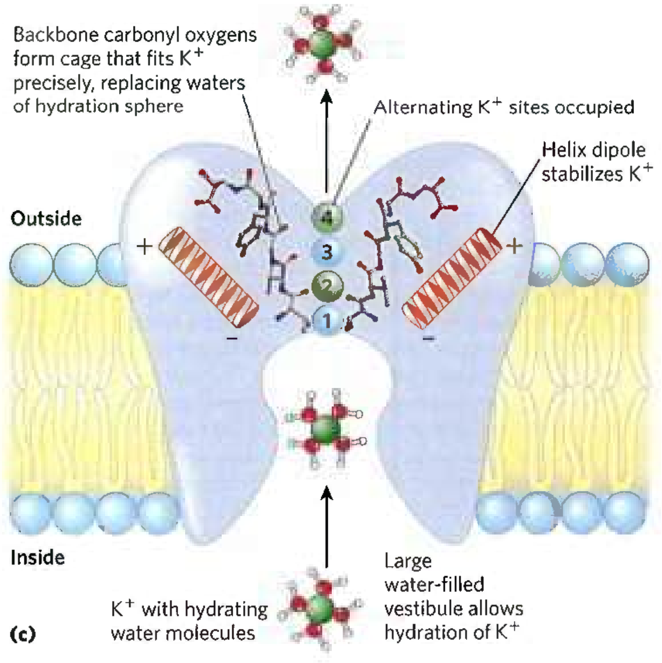

Backbone carbonyl oxygens form cage that fits K$^+$ precisely, replacing waters of hydration sphere
Alternating K$^+$ sites occupied
Helix dipole stabilizes K$^+$
Outside
4
3
2
1
+
+
–
–
Inside
K$^+$ with hydrating water molecules
Large water-filled vestibule allows hydration of K$^+$
(c)

FIGURE 11-45 The K$^+$ channel of *Streptomyces lividans*. (a) Viewed in the plane of the membrane, the channel consists of eight transmembrane helices (two from each of four identical subunits), forming a cone with its wide end toward the extracellular space. The inner helices of the cone (lighter colored) line the transmembrane channel, and the outer helices interact with the lipid bilayer. Short segments of each subunit converge in the open end of the cone to make a selectivity filter. (b) This view, perpendicular to the plane of the membrane, shows the four subunits arranged around a central channel just wide enough for a single K$^+$ ion to pass. (c) Diagram of a K$^+$ channel in cross section, showing the structural features critical to function. K$^+$ ions go through the channel in pairs, first in sites 1 and 3, then in sites 2 and 4. Carbonyl oxygens (red) of the peptide backbone in the selectivity filter protrude into the channel, interacting with and stabilizing the K$^+$ ions that are passing through. [Data from (a, b) PDB ID 1BL8, D. A. Doyle et al., *Science* 280:69, 1998; (c) G. Yellen, *Nature* 419:35, 2002, and PDB ID 1J95, M. Zhou et al., *Nature* 411:657, 2001.]

the crystal structure, two K^+ ions are visible within the selectivity filter, about 7.5 Å apart, and two water molecules occupy the unfilled positions. K^+ ions pass through the filter in single file; their mutual electrostatic repulsion probably just balances the interaction of each ion with the selectivity filter and keeps them moving. Movement of the two K^+ ions is concerted: first they occupy positions 1 and 3, then they hop to positions 2 and 4. The energetic difference between these two configurations (1, 3 and 2, 4) is very small; energetically, the selectivity pore is not a series of hills and valleys but a flat surface, which is ideal for rapid ion movement through the channel. The structure of the channel seems to have been optimized during evolution to give maximal flow rates and high specificity.

SUMMARY 11.3 *Solute Transport across Membranes*

- Some transporters simply facilitate passive diffusion of a solute across the membrane, from a higher concentration to a lower concentration. Others transport solutes against an electrochemical gradient; this requires a source of metabolic energy.
- Transporters move solutes across a membrane one or a few at a time, providing a binding site on each side of the membrane. The binding sites alternate between being accessible from the outside and from the inside. Ion channels provide a path across the membrane, which is either open or closed. When open, the channel allows the movement of large numbers of solute ion across the membrane at nearly the speed of unhindered diffusion.
- A family of glucose transporters in humans includes the passive transporter GLUT1, which is saturated at normal levels of glucose in the blood. GLUT1 facilitates movement of glucose from blood into erythrocytes.
- The chloride-bicarbonate exchanger of erythrocytes exchanges one Cl^- ion for one HCO_3^- ion across the erythrocyte plasma membrane, mediating the uptake of CO_2 in the tissues and its release in the lungs.
- Active transporters use energy to pump solutes against an electrochemical gradient.
- P-type ATPases, including the Na^+K^+ ATPase of the plasma membrane and the Ca^{2+} transporters of the sarcoplasmic/endoplasmic reticulum, couple phosphorylation and dephosphorylation of the transporter to alternate exposure of solute binding sites on the inside and the outside of the membrane. In animal cells, the Na^+K^+ ATPase maintains the differences in cytosolic and extracellular concentrations of Na^+ and K^+, and the resulting Na^+ gradient is used as the energy source for a variety of secondary active transport processes.
- V-type and F-type ATPases are active transporters that couple ATP cleavage to the uphill transport of H^+ ions. The same mechanism, working in reverse, allows the synthesis of ATP, driven by movement of protons down their electrochemical gradient.
- ABC transporters carry a variety of substrates (including many drugs) out of cells, using ATP as the energy source. The ATP-using domain is conserved in many ABC transporters, and it is coupled with various transmembrane domains that give substrate specificity.
- Some active cotransporters use the energy in an ion gradient generated catabolically to move a solute uphill. The Na^+-glucose cotransporter of the kidney and intestine is such a transporter.
- Water moves across membranes through aquaporins. Some aquaporins are regulated; some also transport glycerol or urea.
- Ion channels provide hydrophilic pores through which select ions can diffuse, moving down their electrical or chemical concentration gradients. Ion channels are unsaturable, have very high flux rates, and are highly specific for one ion.
- Structural studies of K^+ channels reveal the mechanism that allows great discrimination between K^+ and other ions like Na^+. The polar transmembrane passage precisely fits the K^+ ion, but allows neither larger ions nor smaller ions to pass.

KEY TERMS

Terms in bold are defined in the glossary.

micelle	368
bilayer	368
vesicle	368
fluid mosaic model	369
lipid transfer protein	371
integral proteins	372
peripheral proteins	372
amphitropic proteins	372
monotopic	372
bitopic	372
polytopic	372
hydropathy index	374
β barrel	374
porin	374
positive-inside rule	375
GPI-anchored protein	376
flippases	378
floppases	378
scramblases	379
FRAP	379
microdomains	380
rafts	380
caveolae	381
caveolin	381
BAR domain	382
septins	382
fusion protein	383
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membrane potential (V_m)	385
electrochemical gradient	385
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transporters	385
passive transport	385
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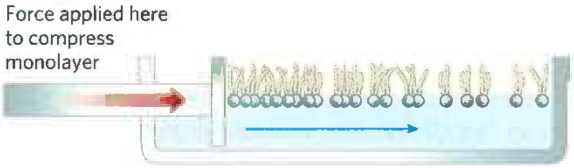
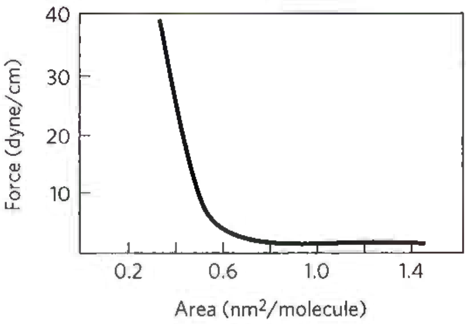
K_t ($K_{transport}$)	388
amphipathic	388
electroneutral	391
cotransport	391
antiport	391
symport	391
uniport	391
electrogenic	392
P-type ATPases	392
SERCA pump	393
Na^+K^+ATPase	394
V-type ATPases	394

F-type ATPases	395
ATP synthase	395
ABC transporters	395
multidrug transporters	396
Na$^+$-glucose symporter	398
ionophore	399
aquaporins (AQPs)	400
ligand-gated channel	401
voltage-gated ion channel	401
patch-clamping	401

PROBLEMS

1. Determining the Cross-Sectional Area of a Lipid Molecule When phospholipids are layered gently onto the surface of water, they orient at the air-water interface with their head groups in the water and their hydrophobic tails in the air. The experimental apparatus shown can be used to progressively reduce the surface area available to a layer of lipids. By measuring the force necessary to push the lipids together, we can determine when the molecules are packed tightly in a continuous monolayer; as that area is approached, the force needed to further reduce the surface area increases sharply, as shown in the graph. How would you use this apparatus to determine the average area occupied by a single lipid molecule in the monolayer?





2. Properties of Lipids and Lipid Bilayers Lipid bilayers form when phospholipids are suspended in water. The edges of these sheets close upon each other and undergo self-sealing to form vesicles (liposomes).

(a) What properties of lipids are responsible for this property of bilayers? Explain.

(b) What are the consequences of this property for the structure of biological membranes?

3. Length of a Fatty Acid Molecule The carbon–carbon bond distance for single-bonded carbons, such as those in a saturated fatty acyl chain, is about 1.5 Å. Estimate to one significant figure the length of a single molecule of palmitate in its fully extended form. If two molecules of palmitate were placed end to end, how would their total length compare with the thickness of the lipid bilayer in a biological membrane?

4. Membrane Proteins What are the three main categories of membrane proteins, and how are they distinguished experimentally?

5. Location of a Membrane Protein Treatment of disrupted erythrocyte membranes with a concentrated salt solution released an unknown membrane protein, X. Proteolytic enzymes cleaved X into fragments. In additional experiments, intact erythrocytes were treated with proteolytic enzymes, washed, then disrupted. Extraction of membrane components yielded intact X. What do these observations indicate about the location of X in the plasma membrane? Do the properties of X resemble those of an integral membrane protein or a peripheral membrane protein?

6. Predicting Membrane Protein Topology from Sequence You have cloned the gene for a human erythrocyte protein, which you suspect is a membrane protein. You deduce the amino acid sequence of the protein from the nucleotide sequence of the gene. From this sequence alone, how would you evaluate the possibility that the protein is an integral protein? Suppose the protein proves to be an integral protein with one transmembrane segment. Suggest biochemical or chemical experiments that might allow you to determine whether the protein is oriented with the amino terminus on the outside of the cell or on the inside of the cell.

7. Surface Density of a Membrane Protein *E. coli* can be induced to make about 10,000 copies of the lactose transporter (M_r 31,000) per cell. Assume that *E. coli* is a cylinder 1 μm in diameter and 2 μm long. The diameter of the lactose transporter is approximately 6 nm. What fraction of the plasma membrane surface is occupied by the lactose transporter molecules? Explain how you arrived at this conclusion.

8. Molecular Species in the Plasma Membrane The plasma membrane of *E. coli* is about 75% protein and 25% phospholipid by weight. How many molecules of membrane lipid are present for each molecule of membrane protein? Assume an average protein M_r of 50,000 and an average phospholipid M_r of 750. What more would you need to know to estimate the fraction of the membrane surface that is covered by lipids?

9. Temperature Dependence of Lateral Diffusion The experiment described in Figure 11-19 was performed at 37 °C. If the experiment were carried out at 10 °C, what effect would you expect on the rate of diffusion? Why?

10. Membrane Self-Sealing Cellular membranes are self-sealing—if they are punctured or disrupted mechanically, they quickly and automatically reseal. What properties of membranes are responsible for this important feature?

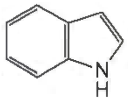
11. Lipid Melting Temperatures Membrane lipids in tissue samples obtained from different parts of a reindeer's leg have different fatty acid compositions. Membrane lipids from tissue near the hooves contain a larger proportion of unsaturated fatty acids than those from tissue in the upper leg. What is the significance of this observation?

12. Flip-Flop Diffusion What is the physical explanation for the very slow movement of membrane phospholipids from one leaflet of a biological membrane to the other? What factors influence this rate?

13. Bilayer Asymmetry The inner leaflet (monolayer) of the human erythrocyte membrane consists predominantly of phosphatidylethanolamine and phosphatidylserine. The outer leaflet consists predominantly of phosphatidylcholine and sphingomyelin. Although the phospholipid components of the membrane can diffuse in the fluid bilayer, this sidedness is preserved at all times. How?

14. Scramblase and Flippase Explain the difference between the scramblase enzymes and flippase enzymes based on the membranes with which they are associated, the symmetry of these membranes, and their energy requirements.

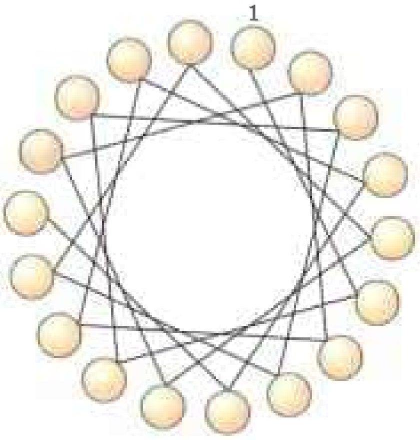
15. Membrane Permeability At pH 7, tryptophan crosses a lipid bilayer at about one-thousandth the rate of indole, a closely related compound:



Suggest an explanation for this observation.

16. Glucose Transporters A cell biologist working with cultured cells from intestinal epithelium finds that the cells take up glucose from the growth medium 10 times faster when the glucose concentration is 5 mM than when it is 0.2 mM. She also finds that glucose uptake requires Na^+ in the growth medium. What can you say about the glucose transporter in these cells?

17. Use of the Helical Wheel Diagram A helical wheel is a two-dimensional representation of a helix, a view along its central axis. Use the helical wheel diagram shown here to determine the distribution of amino acid residues in a helical segment with the sequence –Val–Asp–Arg–Val–Phe–Ser–Asn–Val–Cys–Thr–His–Leu–Lys–Thr–Leu–Gln–Asp–Lys–



What can you say about the surface properties of this helix? How would you expect the helix to be oriented in the tertiary structure of an integral membrane protein?

18. Synthesis of Gastric Juice: Energetics Parietal cells acidify gastric juice (pH 1.5) by pumping HCl from blood plasma (pH 7.4) into the stomach. How much ATP (in moles) is required to pump a mole of protons (H^+) against this concentration gradient? The free-energy change for ATP hydrolysis under cellular conditions is about –58 kJ/mol. Ignore the effects of the transmembrane electrical potential.

19. Electrogenic Transporters A single-cell organism, *Paramecium*, is large enough to allow the insertion of a microelectrode, permitting the measurement of the electrical potential between the inside of the cell and the surrounding medium (the membrane potential). The measured membrane potential is –50 mV (inside negative) in a living cell. What would happen if you added valinomycin to the surrounding medium, which contains K^+ and Na^+?

20. Energetics of the Na^+K^+ ATPase For a typical vertebrate cell with a membrane potential of –0.070 V (inside negative), what is the free-energy change for transporting 1 mol of Na^+ from the cell into the blood at 37 °C? Assume the Na^+ concentration is 12 mM inside the cell and 145 mM in blood plasma.

21. Action of Ouabain on Kidney Tissue Ouabain specifically inhibits the Na^+K^+ ATPase activity of animal tissues but is not known to inhibit any other enzyme. When ouabain is added to thin slices of living kidney tissue, it inhibits oxygen consumption by 66%. Why? What does this observation tell us about the use of respiratory energy by kidney tissue?

22. Digoxin to Inhibit Na^+K^+ ATPase The Na^+Ca^{2+} exchanger expressed in cardiac myocytes is a bidirectional antiporter protein that removes calcium from the cytoplasm by exchanging it with sodium. Cardiac myocytes also express the Na^+K^+ ATPase. Suppose that a Na^+K^+ ATPase inhibitor (digoxin) is added to cardiac myocytes. Using your knowledge of the relative concentrations of ions (intracellular versus extracellular) and the important role of the Na^+K^+ ATPase in maintaining the electrochemical gradient, what change would you expect in the intracellular [Ca^{2+}]? Why?

23. Energetics of Symport Suppose you determined experimentally that a cellular transport system for glucose, driven by symport of Na^+, could accumulate glucose to concentrations 25 times greater than in the external medium, while the external [Na^+] was only 10 times greater than the intracellular [Na^+]. Would this violate the laws of thermodynamics? If not, how could you explain this observation?

24. Labeling the Lactose Transporter A bacterial lactose transporter, which is highly specific for lactose, contains a Cys residue that is essential to its transport activity. Covalent reaction of *N*-ethylmaleimide (NEM) with this Cys residue irreversibly inactivates the transporter. A high concentration of lactose in the medium prevents inactivation by NEM, presumably by sterically protecting the Cys residue, which is in or near the lactose-binding site. You know nothing else about the transporter protein. Suggest an experiment that might allow you to determine the M_r of this Cys-containing transporter polypeptide.

25. Transport Types You have just discovered a new L-alanine transporter in liver cells (hepatocytes). Poisoning hepatocytes with cyanide (which blocks ATP synthesis) reduces alanine transport by 90%. Tenfold reduction in extracellular [Na^+] has no immediate effect on alanine transport. How would you use these observations to decide whether the alanine transporter is passive or active, primary or secondary?

26. Intestinal Uptake of Leucine You are studying the uptake of L-leucine by epithelial cells of the mouse intestine. Measurements of the rates of uptake of L-leucine and several of its analogs, with and without Na^+ in the assay buffer, yield the results given in the table. What can you conclude about the properties and mechanism of the leucine transporter? Would you expect L-leucine uptake to be inhibited by ouabain (see problem 21)?

	Uptake in presence of Na^+		**Uptake in absence of Na^+**	
Substrate	V_{max}	K_t(mM)	V_{max}	K_t(mM)
L-Leucine	420	0.24	23	0.2
D-Leucine	310	4.7	5	4.7
L-Valine	225	0.31	19	0.31

27. Ion Channel Selectivity Potassium channels consist of four subunits that form a channel just wide enough for K^+ ions to pass through. Although Na^+ ions are smaller (M_r 23, radius 0.95 Å) than K^+ ions (M_r 39, radius 1.33 Å), the potassium channels in the bacterium *Streptomyces lividans* transport 10^4 times more K^+ ions than Na^+ ions. What prevents Na^+ ions from passing through potassium channels?

28. Effect of an Ionophore on Active Transport Consider the leucine transporter described in Problem 26. Would V_{max} and/or K_t change if you added a Na^+ ionophore to the assay solution containing Na^+? Explain.

BIOCHEMISTRY ONLINE

29. Predicting Membrane Protein Topology I Online bioinformatics tools make hydropathy analysis easy if you know the amino acid sequence of a protein. At the Protein Data Bank (www.rcsb.org), the Protein Feature View displays additional information about a protein gleaned from other databases, such as UniProt and SCOP2. A simple graphical view of a hydropathy plot created using a window of 15 residues shows hydrophobic regions in red and hydrophilic regions in blue.

(a) Looking only at the displayed hydropathy plots in the Protein Feature View, what predictions would you make about the membrane topology of these proteins: glycophorin A (PDB ID 1AFO), myoglobin (PDB ID 1MBO), and aquaporin (PDB ID 2B6O)?

(b) Now, refine your information using the ProtScale tools at the ExPASy bioinformatics resource portal. Each of the PDB Protein Feature Views was created with a UniProt Knowledgebase ID. For glycophorin A, the UniProtKB ID is P02724; for myoglobin, P02185; and for aquaporin, Q6J8I9. Go to the ExPASy portal (http://web.expasy.org/protscale) and select the Kyte & Doolittle hydropathy analysis option, with a window of 7 amino acids. Enter the UniProtKB ID for aquaporin (Q6J8I9, which you can also get from the PDB's Protein Feature View page), then select the option to analyze the complete chain (residues 1 to 263). Use the default values for the other options and click Submit to get a hydropathy plot. Save a GIF image of this plot. Now repeat the analysis using a window of 15 amino acids. Compare the results for the 7-residue and 15-residue window analyses. Which window size gives you a better signal-to-noise ratio?

(c) Under what circumstances would it be important to use a narrower window?

30. Predicting Membrane Protein Topology II The epinephrine receptor in animal cells is an integral membrane protein (M_r 64,000) that is believed to have seven transmembrane α-helical regions.

(a) Show that a protein of this size is capable of spanning the membrane seven times.

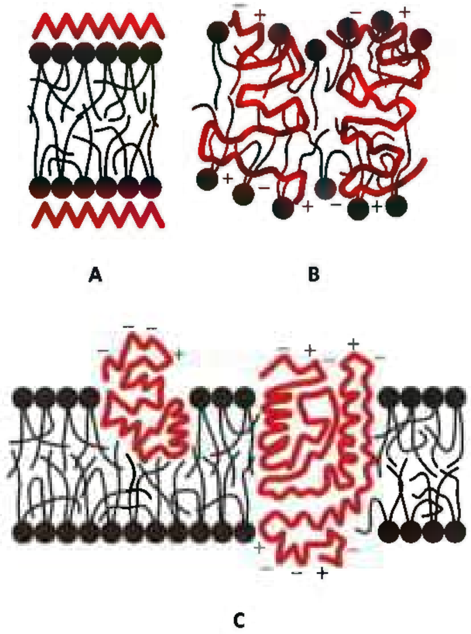
(b) Given the amino acid sequence of this protein, how would you predict which regions of the protein form the membrane-spanning helices?

(c) Go to the Protein Data Bank (www.rcsb.org). Use the PDB identifier 1DEP to retrieve the data page for a portion of the β-adrenergic receptor (one type of epinephrine receptor) isolated from turkey. Using JSmol to explore the structure, predict whether this portion of the receptor is located within the membrane or at the membrane surface. Explain your answer. Now use the Protein Feature View to see the hydrophobicity analysis of the sequence. Does this support your answer?

(d) Retrieve the data for a portion of another receptor, the acetylcholine receptor of neurons and myocytes, using the PDB identifier 1A11. As in (c), predict where this portion of the receptor is located and explain your answer.

DATA ANALYSIS PROBLEM

31. The Fluid Mosaic Model of Biological Membrane Structure Figure 11-3 shows the currently accepted fluid mosaic model of biological membrane structure. This model was presented in detail in a review article by S. J. Singer in 1971. In the article, Singer presented the three models of membrane structure that had been proposed up to that time:



A. The Davson-Danielli-Robertson Model. This was the most widely accepted model in 1971, when Singer's review was published. In this model, the phospholipids are arranged as a bilayer. Proteins are found on both surfaces of the bilayer, attached to it by ionic interactions between the charged head groups of the phospholipids and charged groups of the proteins. Crucially, there is no protein in the interior of the bilayer.

B. The Benson Lipoprotein Subunit Model. Here the proteins are globular and the membrane is a protein-lipid mixture. The hydrophobic tails of the lipids are embedded in the hydrophobic parts of the proteins. The lipid head groups are exposed to the solvent. There is no lipid bilayer.

C. The Lipid–Globular Protein Mosaic Model. This is the model shown in Figure 11-3. The lipids form a bilayer and proteins are embedded in it, some extending through the bilayer and others not. Proteins are anchored in the bilayer by interactions between the hydrophobic tails of the lipids and hydrophobic portions of the protein.

For the data given below, consider how each piece of information aligns with each of the three models of membrane structure. Which model(s) are supported, which are not supported, and what reservations do you have about the data or their interpretation? Explain your reasoning.

(a) When cells were fixed, stained with osmium tetroxide, and examined in the electron microscope, the membranes showed a "railroad track" appearance, with two dark-staining lines separated by a light space.

(b) The thickness of membranes in cells fixed and stained in the same way was found to be 5 to 9 nm. The thickness of a "naked" phospholipid bilayer, without proteins, was 4 to 4.5 nm. The thickness of a single monolayer of proteins was about 1 nm.

(c) Singer wrote in his article: "The average amino acid composition of membrane proteins is not distinguishable from that of soluble proteins. In particular, a substantial fraction of the residues is hydrophobic" (p. 165).

(d) As described in Problems 1 and 2 of this chapter, researchers had extracted membranes from cells, extracted the lipids, and compared the area of the lipid monolayer with the area of the original cell membrane. The interpretation of the results was complicated by the issue illustrated in the graph of Problem 1: the area of the monolayer depended on how hard it was pushed. With very light pressures, the ratio of monolayer area to cell membrane area was about 2.0. At higher pressures—thought to be more like those found in cells—the ratio was substantially lower.

(e) Circular dichroism spectroscopy uses changes in polarization of UV light to make inferences about protein secondary structure (see Fig. 4-9). On average, this technique showed that membrane proteins have a large amount of α helix and little or no β sheet. This finding was consistent with most membrane proteins having a globular structure.

(f) Phospholipase C is an enzyme that removes the polar head group (including the phosphate) from phospholipids. In several studies, treatment of intact membranes with phospholipase C removed about 70% of the head groups without disrupting the "railroad track" structure of the membrane.

(g) Singer described in his article a study in which "a glycoprotein of molecular weight about 31,000 in human red blood cell membranes is cleaved by tryptic treatment of the membranes into soluble glycopeptides of about 10,000 molecular weight, while the remaining portions are quite hydrophobic" (p. 199). Trypsin treatment did not cause gross changes in the membranes, which remained intact.

Singer's review also included many more studies in this area. In the end, though, the data available in 1971 did not conclusively prove Model C was correct. As more data have accumulated, this model of membrane structure has been accepted by the scientific community.

Reference

Singer, S.J. 1971. The molecular organization of biological membranes. In *Structure and Function of Biological Membranes* (L.I. Rothfield, ed.), pp. 145–222. New York: Academic Press.

Chapter 12

BIOCHEMICAL SIGNALING

12.1 General Features of Signal Transduction *409*
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The ability of cells to receive and act on signals from outside the plasma membrane is fundamental to life. Cells and organisms constantly sample the surrounding medium for nutrients, oxygen, light, and sexual partners, and for the presence of noxious chemicals, predators, or competitors for food. These signals elicit appropriate responses, such as motion toward food or away from toxic substances. In multicellular organisms, cells exchange hundreds of signals—neurotransmitters, hormones, key metabolites—which trigger appropriate responses in such cellular activities as metabolism, cell division, embryonic growth and development, movement, and defense. In all these cases, the signal represents *information* that is detected by specific receptors and converted to a cellular response, which always involves a *chemical* process. This conversion of information into a chemical change, **signal transduction**, is a universal property of living cells.

In this chapter, we present examples of specific biological signaling systems from which we have acquired our current understanding of the biochemistry of signal transduction in animals. We emphasize the following principles:

P1 **Cells respond to external signals through receptor-mediated processes that amplify the signal, integrate it with input from other receptors, transmit the signal to the appropriate effectors, and eventually end the response.**

P2 **There is a high degree of evolutionary conservation of signaling proteins and transduction mechanisms across the animal phyla.** At least a billion years of evolution have passed since the plant and animal branches of the eukaryotes diverged, which is reflected in the differences in signaling mechanisms used in the two kingdoms. We focus here on the animal kingdom.

P3 **In multicellular animals, GPCRs with seven transmembrane helices are the largest group of plasma membrane receptors.** These *G P*rotein–*C*oupled *R*eceptors act through G proteins, which are turned on when they bind guanosine triphosphate (GTP). Animals have hundreds of different GPCRs, each able to respond to a specific signal.

P4 **Plasma membrane receptors with an intracellular tyrosine kinase activity act through cascades of protein kinases to transduce signals about the metabolic state, including growth factors.**

P5 **Phosphorylation of intrinsically disordered regions of signaling proteins acts as a switch, toggling enzyme activity, or creating binding sites for other molecules.** Signal responses are integrated by multiprotein signaling complexes with modular domains that bind phosphorylated Tyr, Ser, or Thr residues.

P3 **Ion channels gated by membrane potential or ligands are central to signaling in all cells, including bacteria, plants, and animals.**

P7 **Some hormone signals act inside the cell, not at the plasma membrane, forming complexes with specific receptor proteins that regulate gene expression.**

P8 **Cells receive extracellular signals that determine progress through the cell division cycle, or trigger cell death—processes regulated by phosphorylation and dephosphorylation of key regulatory proteins.**

P9 **Defective signaling proteins or defective regulation of their synthesis and breakdown can disrupt cell cycle regulation and lead to tumor formation (cancer).**

12.1 General Features of Signal Transduction

Signal transductions are remarkably specific and exquisitely sensitive. **Specificity** is achieved by precise molecular complementarity between the signal and receptor molecules (**Fig. 12-1a**), mediated by the same kinds of weak (noncovalent) forces that mediate enzyme-substrate and antigen-antibody interactions. Multicellular organisms have an additional level of specificity, because the receptors for a given signal, or the intracellular targets of a given signal pathway, are present only in certain cell types. Thyrotropin-releasing hormone, for example, triggers responses in the cells of the anterior pituitary but not in hepatocytes, which lack receptors for this hormone. Epinephrine alters glycogen metabolism in hepatocytes but not in adipocytes; in this case, both cell types have receptors for the hormone, but whereas hepatocytes contain glycogen and the glycogen-metabolizing enzyme that is stimulated by epinephrine, adipocytes contain neither.

Signal-Transducing Systems Share Common Features

The extraordinary **sensitivity** of signal transduction results from the high affinity of signal receptors for their ligands (Fig. 12-1b). This affinity can be expressed as the dissociation constant, K_d, for the receptor-ligand interaction. K_d is commonly 10^{-7} M or less, meaning that the receptor detects micromolar to nanomolar concentrations of a signaling ligand. In some cases, cooperativity in receptor-ligand interactions results in relatively large changes in receptor activation with small changes in ligand concentration, further increasing the sensitivity of signal detection.

P1 **Amplification** results when an enzyme is activated by a signal receptor and, in turn, catalyzes the activation of many molecules of a second enzyme, each of which activates many molecules of a third enzyme and so on, in an **enzyme cascade** (Fig. 12-1c). Such cascades can produce amplifications of several orders of magnitude within milliseconds. The response to a signal must also be terminated, such that the downstream effects are in proportion to the strength of the original stimulus.

Interacting signaling proteins are **modular**. Many signaling proteins have multiple domains that recognize specific features in several other proteins, or in the cytoskeleton or plasma membrane. P5 This modularity allows a cell to mix and match a set of signaling molecules to create a wide variety of multienzyme complexes with different functions or cellular locations. One common theme in these interactions is the binding of one modular signaling protein to *phosphorylated* residues in another protein; the resulting interaction can be regulated by phosphorylation or dephosphorylation of the protein partner (Fig. 12-1d). Nonenzymatic **scaffold proteins** with affinity for several enzymes that interact in cascades bring these enzymes together, ensuring that they interact at specific cellular locations and at specific times. Many of the regions involved in protein-protein interactions are intrinsically disordered (see Fig. 4-20), capable of folding differently depending on which protein they interact with. As a result, a single protein can have multiple functions in signaling pathways.

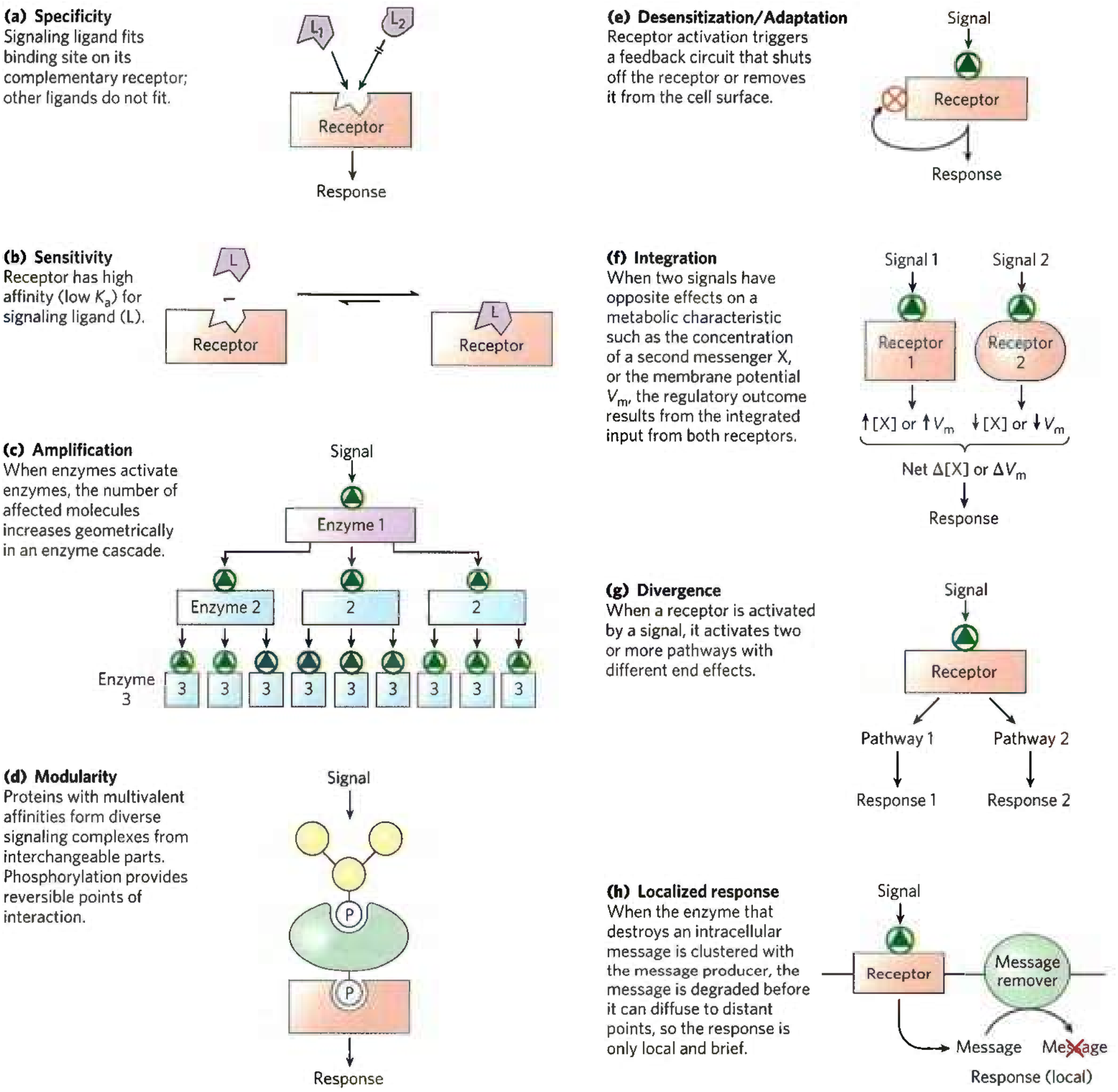
The sensitivity of receptor systems is subject to modification. When a signal is present continuously, the receptor system becomes **desensitized** (Fig. 12-1e), so that it no longer responds to the signal. When the stimulus falls below a certain threshold, the system again becomes sensitive. Think of what happens to your visual transduction system when you walk from bright sunlight into a darkened room or from darkness into the light.

Signal **integration** (Fig. 12-1f) is the ability of the system to receive multiple signals and produce a unified response appropriate to the combined needs of the cell or organism. Different signaling pathways converse with each other at several levels, generating complex cross talk that maintains homeostasis in the cell and the organism.

Signaling pathways are often **divergent**—branched rather than linear; one stimulus acts through one receptor, which activates two or more pathways with different downstream targets and responses (Fig. 12-1g).

A final noteworthy feature of signal-transducing systems is **response localization** within a cell (Fig. 12-1h). When the components of a signaling system are confined to a specific subcellular structure (a raft in the plasma membrane, for example), a cell can regulate a process locally, without affecting distant regions of the cell.

One of the revelations of research on signaling is the remarkable degree to which signaling mechanisms have been conserved during evolution. P5 Although the number of different biological signals to which cells respond is probably in the thousands (**Table 12-1** lists a few important types), and the kinds of responses elicited



(a) Specificity Signaling ligand fits binding site on its complementary receptor; other ligands do not fit.

(b) Sensitivity Receptor has high affinity (low K_a) for signaling ligand (L).

(c) Amplification When enzymes activate enzymes, the number of affected molecules increases geometrically in an enzyme cascade.

(d) Modularity Proteins with multivalent affinities form diverse signaling complexes from interchangeable parts. Phosphorylation provides reversible points of interaction.

(e) Desensitization/Adaptation Receptor activation triggers a feedback circuit that shuts off the receptor or removes it from the cell surface.

(f) Integration When two signals have opposite effects on a metabolic characteristic such as the concentration of a second messenger X, or the membrane potential V_m, the regulatory outcome results from the integrated input from both receptors.

(g) Divergence When a receptor is activated by a signal, it activates two or more pathways with different end effects.

(h) Localized response When the enzyme that destroys an intracellular message is clustered with the message producer, the message is degraded before it can diffuse to distant points, so the response is only local and brief.

L_1
L_2
Receptor
Response
L
Receptor
Receptor
Signal
Enzyme 1
Enzyme 2
2
2
Enzyme 3
3
Signal
P
P
Response
Signal
Receptor
Response
Signal 1
Signal 2
Receptor 1
Receptor 2
↑[X] or ↑V_m
↓[X] or ↓V_m
Net Δ[X] or ΔV_m
Response
Signal
Receptor
Pathway 1
Pathway 2
Response 1
Response 2
Signal
Receptor
Message remover
Message
Message
Response (local)

FIGURE 12-1 Eight features of signal-transducing systems.

TABLE 12-1 Some Signals to Which Cells Respond

Antigens	Mechanical touch
Cell surface glycoproteins, oligosaccharides	Microbial, insect pathogens
Developmental signals	Neurotransmitters
Extracellular matrix components	Nutrients
Growth factors	Odorants
Hormones	Pheromones
Hypoxia	Tastants
Light	

by these signals are comparably numerous, the machinery for transducing all of these signals is built from about 10 basic types of protein components (**Table 12-2**).

The General Process of Signal Transduction in Animals Is Universal

In this chapter we consider the molecular details of several representative signal-transduction systems, classified according to the type of receptor. The trigger for each system is different, but P1 the general features of signal transduction are common to all: a signal (ligand) interacts with a receptor; the activated receptor

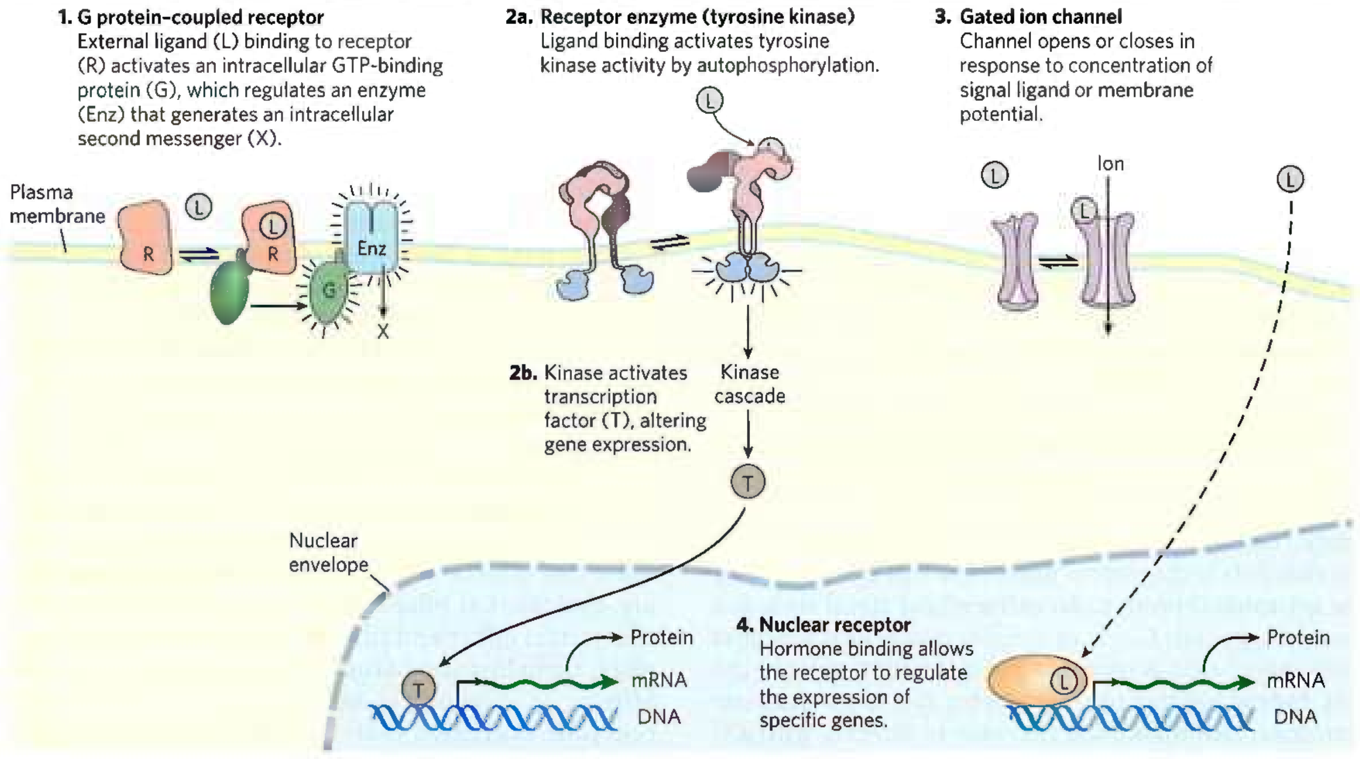
TABLE 12-2 **Some Conserved Elements of Animal Signaling Systems**

Plasma membrane receptors with 7 transmembrane (7tm) helices
G proteins that bind GTP or GDP and interface with membrane receptors
Membrane enzymes with cyclic nucleotides as substrates or products
Protein kinases that phosphorylate GPCR receptors
Membrane protein tyrosine kinases
Cyclic nucleotide–dependent protein kinases
Ca^{2+}-binding proteins
Ca^{2+}-dependent protein kinases
Protein kinases that are activated during cell division
Nonenzymic protein scaffolds that bring modules together

interacts with cellular machinery, producing a second signal or a change in the activity of a cellular protein; the metabolic activity of the target cell undergoes a change; and finally, the transduction event ends. To illustrate these general features of signaling systems, we will look at examples of four basic receptor types (**Fig. 12-2**).

1. *G protein–coupled receptors* that *indirectly* activate (through GTP-binding proteins, or G proteins) enzymes that generate intracellular second messengers. Examples include the β-adrenergic receptor system that responds to epinephrine (Section 12.2) and the vision, olfaction, and gustation systems (Section 12.3).
2. *Receptor enzymes* in the plasma membrane that have an enzymatic activity on the cytoplasmic side, triggered by ligand binding on the extracellular side. Receptors with tyrosine kinase activity, for example, catalyze the phosphorylation of Tyr residues in specific intracellular target proteins. Examples include the insulin receptor (Section 12.4) and receptor guanylyl cyclases (see Box 12-2).
3. *Gated ion channels* of the plasma membrane that open and close (hence the term "gated") in response to the binding of chemical ligands or changes in transmembrane potential. These are the simplest signal transducers (Section 12.6).
4. *Nuclear receptors* that bind specific ligands (such as the hormone estrogen) and alter the rate at which specific genes are transcribed and translated into cellular proteins. Because steroid hormones function through mechanisms intimately related to the regulation of gene expression, we consider them briefly in Section 12.7 and defer a detailed discussion of their action until Chapters 23 and 28.

As we begin this discussion of biological signaling, a word about the nomenclature of signaling proteins is in order. These proteins are typically discovered in one context and named accordingly, then prove to be involved in


1. G protein-coupled receptor
External ligand (L) binding to receptor (R) activates an intracellular GTP-binding protein (G), which regulates an enzyme (Enz) that generates an intracellular second messenger (X).

2a. Receptor enzyme (tyrosine kinase)
Ligand binding activates tyrosine kinase activity by autophosphorylation.

2b. Kinase activates transcription factor (T), altering gene expression.

3. Gated ion channel
Channel opens or closes in response to concentration of signal ligand or membrane potential.

4. Nuclear receptor
Hormone binding allows the receptor to regulate the expression of specific genes.

FIGURE 12-2 **Four general types of signal transducers.**

a broader range of biological functions for which the original name is not helpful. For example, the retinoblastoma protein, pRb, was initially identified as the product of a mutation that contributes to cancer of the retina (retinoblastoma), but it is now known to function in many pathways essential to cell division in all cells, not just those of the retina. Some genes and proteins are given noncommittal names: the tumor suppressor protein p53, for example, is a *p*rotein of *53* kDa, but its name gives no clue to its great importance in the regulation of cell division and the development of cancer. In this chapter we generally define these protein names as we encounter them, introducing the names that are commonly used by researchers in the field.

SUMMARY 12.1 *General Features of Signal Transduction*

- All cells have specific and highly sensitive signal-transducing mechanisms, which have been conserved during evolution.
- A wide variety of stimuli act through specific protein receptors in the plasma membrane.
- In all types of signal transduction, receptors bind the signal ligand and initiate a process that amplifies the signal, integrates it with input from other receptors, and transmits the information throughout the cell. If the signal persists, receptor desensitization reduces or ends the response.
- Multicellular organisms have four general types of signaling mechanisms: plasma membrane proteins that act through G proteins, receptors with internal enzyme activity (such as tyrosine kinase), gated ion channels, and nuclear receptors that bind steroids and alter gene expression.

12.2 G Protein–Coupled Receptors and Second Messengers

As their name implies, **G protein–coupled receptors (GPCRs)** are receptors that act through a member of the **guanosine nucleotide–binding protein**, or **G protein**, family. P3 Three essential components define signal transduction through GPCRs: a plasma membrane receptor with seven transmembrane helical segments, a G protein that cycles between active (guanosine triphosphate (GTP)-bound) and inactive (guanosine diphosphate (GDP)-bound) forms, and an effector enzyme (or ion channel) in the plasma membrane that is regulated by the activated G protein. An extracellular signal such as a hormone, growth factor, or neurotransmitter is the "first messenger" that activates a receptor from outside the cell. Ligand binding to the receptor forces an allosteric transition that allows the receptor to interact with a G protein, causing it to exchange its bound GDP for a GTP from the cytosol. The G protein then dissociates from the activated receptor and binds to the nearby effector enzyme, altering its activity. The effector enzyme then causes a change in the cytosolic concentration of a low molecular weight metabolite (such as 3′,5′-cyclic AMP) or inorganic ion (Ca^{2+}), which acts as a **second messenger** to activate or inhibit one or more downstream targets, often protein kinases.

The human genome encodes just over 800 GPCRs, about 350 for detecting hormones, growth factors, and other endogenous ligands, and up to 500 that serve as olfactory (smell) and gustatory (taste) receptors. The largest superfamily of proteins encoded in the human genome, GPCRs have been implicated in many common medical conditions, including allergies, depression, blindness, diabetes, and various cardiovascular defects. GPCR mutations are also found in 20% of all cancers. In the United States, more than a third of *all* pharmaceuticals on the market target a GPCR. For example, the β-adrenergic receptor, which mediates the effects of epinephrine, is the target of the "beta blockers," prescribed for such diverse conditions as hypertension, cardiac arrhythmia, glaucoma, anxiety, and migraine headache. More than 100 of the GPCRs found in the human genome are still "orphan receptors," meaning that their natural ligands are not yet identified, and so we know little about their biology. The β-adrenergic receptor, with well-understood biology and pharmacology, is the prototype for all GPCRs, and our discussion of signal-transducing systems begins there. ■

The β-Adrenergic Receptor System Acts through the Second Messenger cAMP

Epinephrine released from the adrenal glands sounds the alarm when a threat requires an animal to mobilize its energy-generating machinery; it signals the need to fight or flee. Epinephrine action begins when the hormone binds to a protein receptor in the plasma membrane of an epinephrine-sensitive cell (a myocyte in muscle, for example). **Adrenergic receptors** ("adrenergic" reflects the alternative name for epinephrine, adrenaline) are of four general types, α_1, α_2, β_1, and β_2, defined by differences in their affinities and responses to a group of agonists and antagonists. **Agonists** are molecules (natural ligands or their structural analogs) that bind a receptor and produce the effects of the natural ligand; **antagonists** are analogs that bind the receptor without triggering the normal effect and thereby block the effects of agonists, including the natural ligand. In some cases, the affinity of a synthetic agonist or antagonist for the receptor is greater than that of the natural agonist

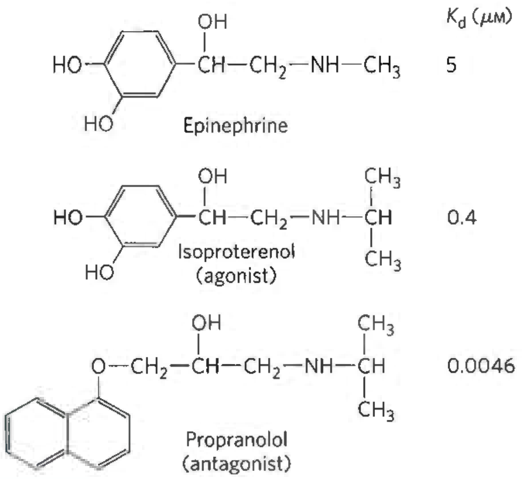


FIGURE 12-3 Epinephrine and its synthetic analogs. Epinephrine regulates energy-yielding metabolism in muscle, liver, and adipose tissue. Its affinity for its receptor is expressed as a dissociation constant for the receptor-ligand complex. Isoproterenol and propranolol are synthetic analogs, one an agonist with an affinity for the receptor that is higher than that of epinephrine, and the other an antagonist with extremely high affinity.

(Fig. 12-3). The four types of adrenergic receptors are found in different target tissues and mediate different responses to epinephrine. Here we focus on the **β-adrenergic receptors** of muscle, liver, and adipose tissue. These receptors mediate changes in fuel metabolism, as described in Chapter 23, including the increased breakdown of glycogen and fat. Adrenergic receptors of the β_1 and β_2 subtypes act through the same mechanism, so in our discussion, "β-adrenergic" applies to both types.

P3 Like all GPCRs, the β-adrenergic receptor is an integral protein with seven hydrophobic, helical regions of 20 to 28 amino acid residues that span the plasma membrane seven times, thus the alternative names for GPCRs: **seven-transmembrane (7tm)** or **heptahelical receptors**. GPCRs effect signal transduction through interaction with **heterotrimeric G proteins**, a conserved family of signaling proteins with three subunits, α, β, and γ. The binding site for GDP or GTP is on the α subunit. When GDP is bound, the G protein is in its trimeric, inactive form.

GPCRs are allosteric proteins. When epinephrine binds to the β-adrenergic receptor (Fig. 12-4a, step ❶), allosteric transitions in the receptor and its associated G protein favor the replacement of GDP with GTP. Thus the hormone-bound GPCR acts as a **guanosine nucleotide–exchange factor (GEF)**. In this active form, the G protein (step ❷) can transmit the signal from the activated receptor to the downstream effector protein, adenylyl cyclase. Because this G protein stimulates its effector, it is referred to as a **stimulatory G protein**, or **G_s**. In the active form, the β and γ subunits of G_s dissociate from the α subunit as a $\beta\gamma$ dimer, and $G_{s\alpha}$, with its bound GTP, moves in the plane of the membrane from the receptor to a nearby molecule of adenylyl cyclase (step ❸).

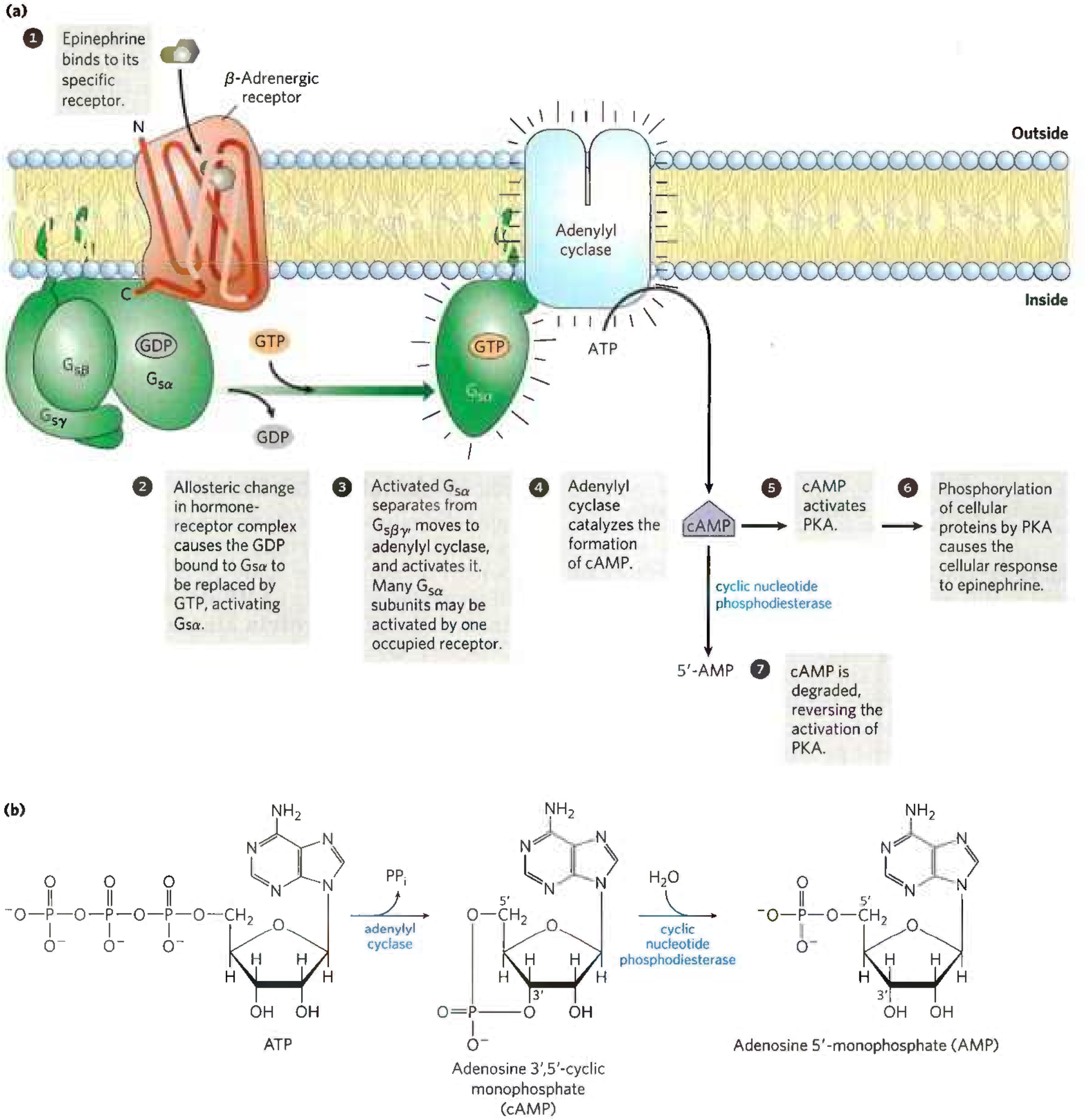
Adenylyl cyclase is an integral protein of the plasma membrane, with its active site on the cytoplasmic face. The association of active $G_{s\alpha}$ with adenylyl cyclase stimulates the cyclase to catalyze the synthesis of second messenger cAMP from ATP (Fig. 12-4a, step ❹; Fig. 12-4b), raising the cytosolic [cAMP]. The interaction between $G_{s\alpha}$ and adenylyl cyclase occurs only when $G_{s\alpha}$ is bound to GTP.

The stimulation by $G_{s\alpha}$ is self-limiting; *$G_{s\alpha}$ has intrinsic GTPase activity that switches $G_{s\alpha}$ to its inactive form* by converting its bound GTP to GDP (Fig. 12-5). The inactive $G_{s\alpha}$ dissociates from adenylyl cyclase, rendering the cyclase inactive. $G_{s\alpha}$ reassociates with the $\beta\gamma$ dimer ($G_{s\beta\gamma}$), and inactive G_s is again available to interact with a hormone-bound receptor.

Cyclic AMP Activates Protein Kinase A

Epinephrine exerts its downstream effects through the increase in [cAMP] that results from activation of adenylyl cyclase. Cyclic AMP, the second messenger, allosterically activates **cAMP-dependent protein kinase**, also called **protein kinase A** or **PKA** (Fig. 12-4a, step ❺), which catalyzes the phosphorylation of specific Ser or Thr residues of targeted proteins, such as glycogen phosphorylase *b* kinase. The latter enzyme is active when phosphorylated and can begin the process of mobilizing glycogen stores in muscle and liver in anticipation of the need for energy, as signaled by epinephrine.

The inactive form of PKA has two identical catalytic subunits (C) and two identical regulatory subunits (R) (Fig. 12-6a). The tetrameric R_2C_2 complex is catalytically inactive, because an autoinhibitory domain of each R subunit occupies the substrate-binding cleft of each C subunit. The autoinhibitory domain is an intrinsically disordered region that can fold to fit any of several protein partners. Cyclic AMP is an allosteric activator of PKA. When cAMP binds to the R subunits, they undergo a conformational change that moves the autoinhibitory domain of R out of the catalytic domain of C, and the R_2C_2 complex dissociates to yield two free, catalytically active C subunits. P5 This same basic mechanism — displacement of an autoinhibitory domain — mediates the allosteric activation of many types of protein kinases by their second messengers (as in Figs 12-21 and 12-29, for example). The structure of the substrate-binding cleft in PKA is the prototype for all known protein kinases (Fig. 12-6b); certain residues in this cleft region have identical counterparts in all of the 544 protein kinases encoded in the human genome. The ATP-binding site of each catalytic subunit positions ATP perfectly for the transfer of its


(a)
β-Adrenergic receptor
N
C
Outside
Inside
Adenylyl cyclase
$G_{s\beta}$
$G_{s\gamma}$
$G_{s\alpha}$
GDP
GTP
GTP
GDP
$G_{s\alpha}$
ATP
cAMP
cyclic nucleotide phosphodiesterase
5′-AMP
(b)
ATP
PP_i
adenylyl cyclase
Adenosine 3′,5′-cyclic monophosphate (cAMP)
H_2O
cyclic nucleotide phosphodiesterase
Adenosine 5′-monophosphate (AMP)
5′
3′

① Epinephrine binds to its specific receptor.

② Allosteric change in hormone-receptor complex causes the GDP bound to Gsα to be replaced by GTP, activating Gsα.

③ Activated $G_{s\alpha}$ separates from $G_{s\beta\gamma}$, moves to adenylyl cyclase, and activates it. Many $G_{s\alpha}$ subunits may be activated by one occupied receptor.

④ Adenylyl cyclase catalyzes the formation of cAMP.

⑤ cAMP activates PKA.

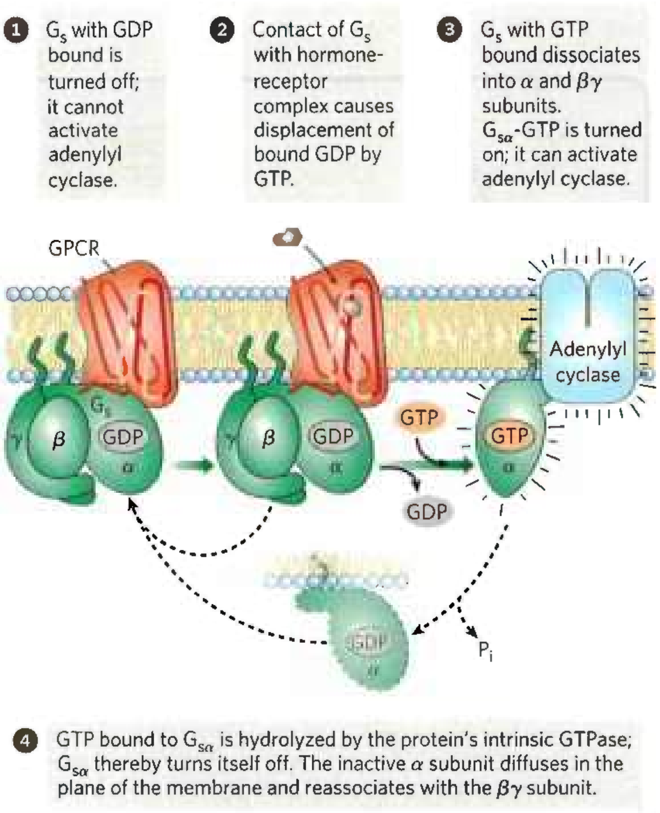
⑥ Phosphorylation of cellular proteins by PKA causes the cellular response to epinephrine.

⑦ cAMP is degraded, reversing the activation of PKA.

FIGURE 12-4 Transduction of the epinephrine signal: the β-adrenergic pathway. (a) The mechanism that couples binding of epinephrine to its receptor with activation of adenylyl cyclase; the seven steps are discussed in the text. The same adenylyl cyclase molecule in the plasma membrane may be regulated by a stimulatory G protein (G_s), as shown, or by an inhibitory G protein (G_i, not shown). G_s and G_i are under the influence of different hormones. Hormones that induce GTP binding to G_i cause *inhibition* of adenylyl cyclase, resulting in lower cellular [cAMP]. (b) The combined action of the enzymes that catalyze steps ④ and ⑦, synthesis and hydrolysis of cAMP by adenylyl cyclase and cAMP phosphodiesterase, respectively.

terminal (γ) phosphoryl group to the —OH in the side chain of a Ser or Thr residue in the target protein.

As indicated in Figure 12-4a (step ⑥), PKA regulates many enzymes downstream in the signaling pathway. Although these downstream targets have diverse functions, they share a region of sequence similarity around the Ser or Thr residue that undergoes phosphorylation, a sequence that marks them for regulation by PKA. The substrate-binding cleft of PKA recognizes these sequences and phosphorylates their Thr or Ser residue. Comparison of the sequences of various protein substrates for PKA has yielded the short **consensus**


GPCR
G_s
γ
β
GDP
α
β
GDP
α
GTP
GDP
Adenylyl cyclase
GTP
α
GDP
α
P_i

① G_s with GDP bound is turned off; it cannot activate adenylyl cyclase.

② Contact of G_s with hormone-receptor complex causes displacement of bound GDP by GTP.

③ G_s with GTP bound dissociates into α and βγ subunits. $G_{s\alpha}$-GTP is turned on; it can activate adenylyl cyclase.

④ GTP bound to $G_{s\alpha}$ is hydrolyzed by the protein's intrinsic GTPase; $G_{s\alpha}$ thereby turns itself off. The inactive α subunit diffuses in the plane of the membrane and reassociates with the βγ subunit.

FIGURE 12-5 The GTPase switch. G proteins cycle between GDP-bound (off) and GTP-bound (on). The protein's GTPase activity, in many cases stimulated by RGS proteins (regulators of G-protein signaling; see Fig. 12-8), determines how quickly bound GTP is hydrolyzed to GDP and thus how long the G protein remains active.

sequence in which the target for phosphorylation (Ser or Thr) is most commonly embedded. For PKA, the consensus sequence is xR[RK]x[ST]B, where x can be any residue, R is Arg, [RK] can be either Arg or Lys, [ST] is either Ser or Thr (which is the residue phosphorylated), and B is any basic residue. The consensus sequences of a number of protein kinases are shown in Table 6-10; they define the targets of the hundreds of protein kinases in the eukaryotic cell.

How do we know that two proteins interact, or where in the cell they interact? Changes in the state of association of two proteins (such as the R and C subunits of PKA) can be seen by measuring the nonradiative transfer of energy between fluorescent probes attached to each protein, a technique called fluorescence resonance energy transfer (FRET), which we describe in **Box 12-1**.

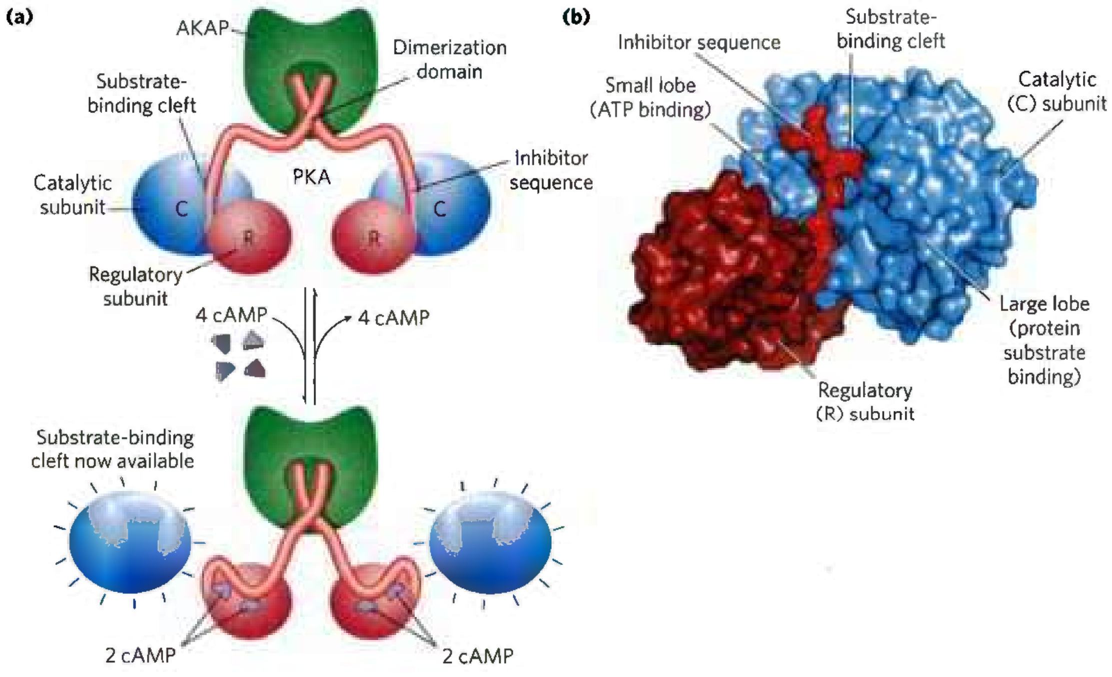

(a)
AKAP
Dimerization domain
Substrate-binding cleft
Catalytic subunit
PKA
C
R
R
C
Inhibitor sequence
Regulatory subunit
4 cAMP
4 cAMP
Substrate-binding cleft now available
2 cAMP
2 cAMP
(b)
Inhibitor sequence
Substrate-binding cleft
Small lobe (ATP binding)
Catalytic (C) subunit
Large lobe (protein substrate binding)
Regulatory (R) subunit

FIGURE 12-6 Activation of cAMP-dependent protein kinase (PKA). (a) When [cAMP] is low, the two identical regulatory subunits (R; red) associate with the two identical catalytic subunits (C; blue). In this R_2C_2 complex, the inhibitor sequences of the R subunits lie in the substrate-binding cleft of the C subunits and prevent binding of protein substrates; the complex is therefore catalytically inactive. The amino-terminal sequences of the R subunits interact to form an R_2 dimer, the site of binding to an A kinase anchoring protein (AKAP; Fig. 12-11). When [cAMP] rises in response to a hormonal signal, each R subunit binds two cAMP molecules and undergoes a dramatic reorganization that pulls its inhibitory sequence away from the C subunit, opening up the substrate-binding cleft and releasing each C subunit in its catalytically active form. (b) A crystal structure showing part of the R_2C_2 complex — one C subunit and part of one R subunit. The amino-terminal dimerization region of the R subunit is omitted for simplicity. The small lobe of C contains the ATP-binding site, and the large lobe surrounds and defines the cleft where the protein substrate binds and undergoes phosphorylation at a Ser or Thr residue, with a phosphoryl group transferred from ATP. In this inactive form, the inhibitor sequence of R blocks the substrate-binding cleft of C, inactivating it. [(b) Data from PDB ID 3FHI, C. Kim et al., *Science* 307:690, 2005.]

BOX 12-1

FRET: Biochemistry in a Living Cell

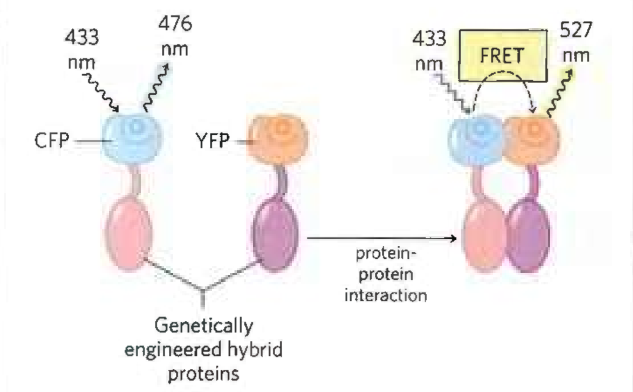

433 nm
476 nm
CFP
YFP
Genetically engineered hybrid proteins
protein-protein interaction
433 nm
FRET
527 nm

FIGURE 1 When the donor protein (CFP) is excited with monochromatic light of wavelength 433 nm, it emits fluorescent light at 476 nm (left). When the (red) protein fused with CFP interacts with the (purple) protein fused with YFP, that interaction brings CFP and YFP close enough to allow fluorescence resonance energy transfer (FRET) between them. Now, when CFP absorbs light of 433 nm, instead of fluorescing at 476 nm, it transfers energy directly to YFP, which then fluoresces at its characteristic emission wavelength, 527 nm. The ratio of light emission at 527 nm and 476 nm is therefore a measure of the extent of interaction between the red and purple proteins.

Fluorescent probes are commonly used to detect biochemical changes in single living cells on a nanosecond time scale. In one widely used procedure, **green fluorescent protein (GFP)** and variants with different fluorescence spectra, described in Chapter 9 (see Fig. 9-16), are genetically fused with another protein. These fluorescent hybrid proteins act as spectroscopic rulers for measuring distances between interacting proteins within a cell. They can be used indirectly as measures of local concentrations of compounds that change the distance between two proteins.

An excited fluorescent molecule such as GFP or YFP (yellow fluorescent protein) can dispose of the energy from the absorbed photon in either of two ways: (1) by fluorescence, emitting a photon of slightly longer wavelength (lower energy) than the exciting light, or (2) by nonradiative **fluorescence resonance energy transfer (FRET)**, in which the energy of the excited molecule (the donor) passes directly to a nearby molecule (the acceptor) *without emission of a photon*, exciting the acceptor (Fig. 1). The acceptor can now decay to its ground state by fluorescence; the emitted photon has a longer wavelength (lower energy) than both the original exciting light and the fluorescence emission of the donor. This second mode of decay (FRET) is possible only when donor and acceptor are close to each other (within 1 to 50 Å); the efficiency of FRET is inversely proportional to the *sixth power* of the distance between donor and acceptor. Thus, very small changes in the distance between donor and acceptor register as very large changes in FRET, measured as the fluorescence of the acceptor molecule when the donor is excited. With sufficiently sensitive light detectors, this fluorescence signal can be located to specific regions of a single, living cell.

FRET has been used to measure [cAMP] in living cells. The gene for BFP (blue fluorescent protein) is fused with that for the regulatory subunit (R) of cAMP-dependent protein kinase (PKA), and the gene for GFP is fused with that for the catalytic subunit (C) (Fig. 2). When these two hybrid proteins are expressed in a cell, BFP (donor) and GFP (acceptor) in the inactive PKA (R_2C_2 tetramer) are close enough to undergo FRET. Wherever in the cell [cAMP] increases, the R_2C_2 complex dissociates into R_2 and 2 C and the FRET signal is lost, because donor and acceptor are now too far apart for efficient FRET. Viewed in the fluorescence microscope, the region of higher [cAMP] has a minimal GFP signal and higher BFP signal. Measuring the ratio of emission at 460 nm and 545 nm gives a sensitive measure of the change in [cAMP]. By determining this ratio for all regions of the cell, the investigator can generate a false color image of the cell in which the ratio, or relative [cAMP], is represented by

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P1 As in many signaling pathways, signal transduction by the β-adrenergic receptor and adenylyl cyclase entails several steps that *amplify* the original hormone signal (**Fig. 12-7**). First, the binding of one hormone molecule to one receptor molecule catalytically activates many G_s molecules that associate with the activated receptor, one after the other. Next, by activating one molecule of adenylyl cyclase, each active $G_{s\alpha}$ molecule stimulates the catalytic synthesis of *many* molecules of cAMP. The second messenger cAMP now activates PKA, each molecule of which catalyzes the phosphorylation of *many* molecules of the target protein—phosphorylase *b* kinase in Figure 12-7. This kinase activates glycogen phosphorylase *b*, which leads to the rapid mobilization of glucose from glycogen. The net effect of the cascade is amplification of the hormonal signal by orders of magnitude, which accounts for the very low concentration of epinephrine (or any other hormone) required for hormone activity. This signaling pathway is also rapid: the signal leads to intracellular changes within fractions of a second.

Several Mechanisms Cause Termination of the β-Adrenergic Response

P1 To be useful, a signal-transducing system has to *turn off* after the hormonal or other stimulus has ended, and mechanisms for shutting off the signal are part of all signaling systems. Most systems also adapt to the continued presence of the signal by becoming less sensitive to

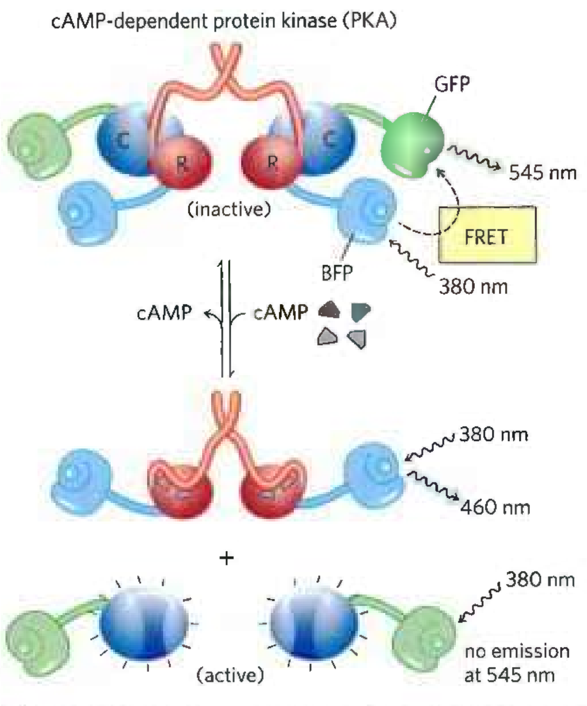


FIGURE 2 Measuring [cAMP] with FRET. Gene fusion creates hybrid proteins that exhibit FRET when the PKA regulatory (R) and catalytic (C) subunits are associated (low [cAMP]). When [cAMP] rises, the subunits dissociate and FRET ceases. The ratio of emission at 460 nm (dissociated) and 545 nm (complexed) thus offers a sensitive measure of [cAMP].

the intensity of the color. Images recorded at timed intervals reveal changes in [cAMP] over time.

A variation of this technology has been used to measure the activity of PKA in a living cell (Fig. 3). Researchers create a phosphorylation target for PKA by producing a hybrid protein containing four elements: YFP (acceptor); a short peptide with a Ser residue surrounded by the consensus sequence for PKA; Ⓟ–Ser-binding domain (called 14-3-3); and CFP (donor). When the Ser residue is not phosphorylated, 14-3-3 has no affinity for the Ser residue and the hybrid protein exists in an extended form, with the donor and acceptor too far apart to generate a FRET signal. Wherever PKA is active in the cell, it phosphorylates the Ser residue of the hybrid protein, and 14-3-3 binds to the Ⓟ–Ser. In doing so, it draws YFP and CFP together and a FRET signal is detected with the fluorescence microscope, revealing the presence of active PKA.

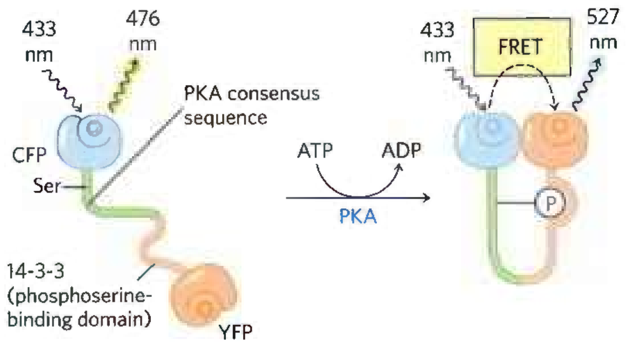


FIGURE 3 Measuring the activity of PKA with FRET. An engineered protein links YFP and CFP via a peptide that contains (1) a Ser residue surrounded by the consensus sequence for phosphorylation by PKA and (2) the 14-3-3 Ⓟ–Ser-binding domain. Active PKA phosphorylates the Ser residue, which docks with the 14-3-3 binding domain, bringing the fluorescence proteins close enough to allow FRET, revealing the presence of active PKA.

With more sophisticated microscopic equipment, the FRET signal from a single protein molecule can be detected with much higher spatial and temporal resolution than is available in FRET with an ensemble of molecules.

it, by *desensitizing*. The β-adrenergic system illustrates both. Here, our focus is on termination.

The response to β-adrenergic stimulation will end when the concentration of the ligand (epinephrine) in the blood drops below the K_d for its receptor. The epinephrine then dissociates from the receptor, and the latter reassumes its inactive conformation, in which it can no longer activate G_s.

A second means of ending the response is the hydrolysis of GTP bound to the G_α subunit, catalyzed by the GTPase activity of the G protein. Conversion of bound GTP to GDP favors the return of G_α to the conformation in which it binds the $G_{\beta\gamma}$ subunits—the conformation in which the G protein is unable to interact with or stimulate adenylyl cyclase. This ends the production of cAMP. The rate of inactivation of G_s depends on the GTPase activity, which for G_α alone is very feeble. However, **GTPase activator proteins (GAPs)** strongly stimulate this GTPase activity, causing more-rapid inactivation of the G protein (**Fig. 12-8**). GAPs can themselves be regulated by other factors, providing a fine-tuning of the response to β-adrenergic stimulation. A third mechanism for terminating the response is to remove the second messenger: cAMP is hydrolyzed to 5′-AMP (which is not active as a second messenger) by **cyclic nucleotide phosphodiesterase** (Fig. 12-4a, step ❼; 12-4b).

Finally, at the end of the signaling pathway, the metabolic effects that result from phosphorylation of target enzymes by PKA are reversed by the action of

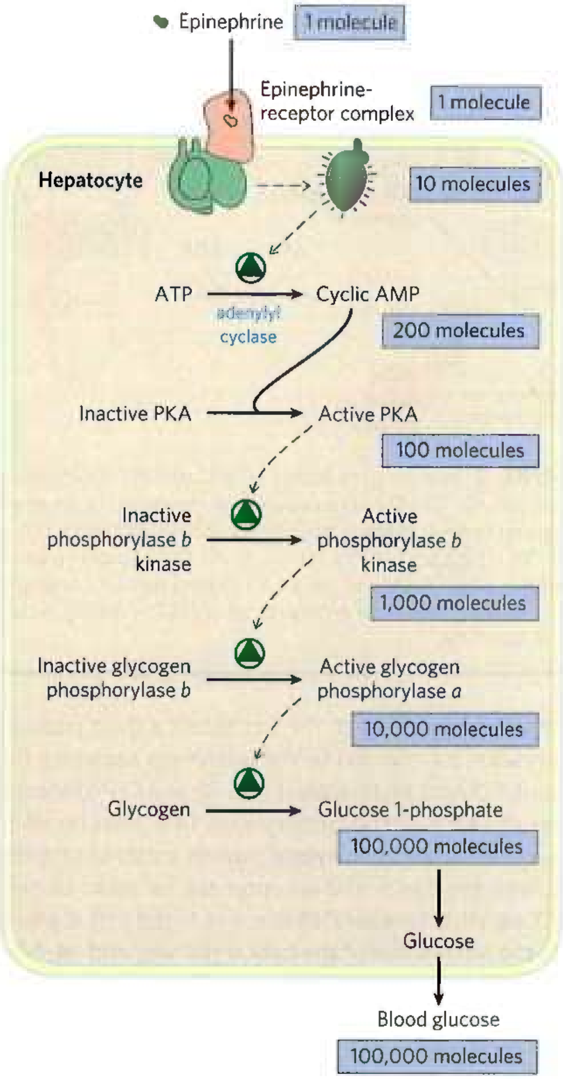

Epinephrine
1 molecule
Epinephrine-receptor complex
1 molecule
Hepatocyte
10 molecules
ATP
adenylyl cyclase
Cyclic AMP
200 molecules
Inactive PKA
Active PKA
100 molecules
Inactive phosphorylase b kinase
Active phosphorylase b kinase
1,000 molecules
Inactive glycogen phosphorylase b
Active glycogen phosphorylase a
10,000 molecules
Glycogen
Glucose 1-phosphate
100,000 molecules
Glucose
Blood glucose
100,000 molecules

FIGURE 12-7 Epinephrine cascade. Epinephrine triggers a series of reactions in hepatocytes in which catalysts activate catalysts, resulting in great amplification of the original hormone signal. The numbers of molecules shown are simply to illustrate amplification and are almost certainly gross underestimates. Binding of one molecule of epinephrine to one β-adrenergic receptor on the cell surface activates many (possibly hundreds of) G proteins, one after another, each of which goes on to activate a molecule of the enzyme adenylyl cyclase. Adenylyl cyclase acts catalytically, producing many molecules of cAMP for each activated adenylyl cyclase. (Because two molecules of cAMP are required to activate one PKA catalytic subunit, this step does not amplify the signal.)

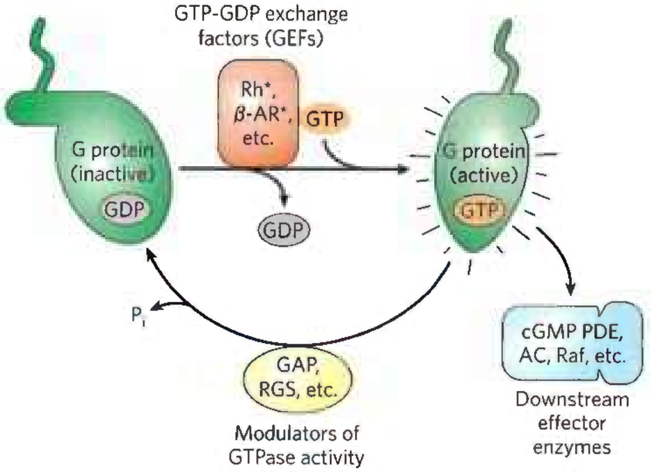

GTP-GDP exchange factors (GEFs)
Rh*, β-AR*, etc.
GTP
G protein (inactive)
GDP
GDP
G protein (active)
GTP
P$_i$
GAP, RGS, etc.
Modulators of GTPase activity
cGMP PDE, AC, Raf, etc.
Downstream effector enzymes

FIGURE 12-8 Factors that regulate G-protein activity. Inactive G proteins, both small G proteins such as Ras and heterotrimeric G proteins such as G_s, interact with upstream GTP-GDP exchange factors (red). Often these exchange factors are activated (*) receptors such as rhodopsin (Rh) and β-adrenergic receptors (AR). The G proteins are activated by GTP binding, and in the GTP-bound form, activate downstream effector enzymes (blue), such as cGMP phosphodiesterase (PDE), adenylyl cyclase (AC), and Raf. By modulating the GTPase activity of G proteins, GTPase activator proteins (GAPs, in the case of small G proteins) and regulators of G-protein signaling (RGSs) (yellow), determine how long the G protein will remain active.

phosphoprotein phosphatases, which hydrolyze phosphorylated Tyr, Ser, or Thr residues, releasing inorganic phosphate (P_i). About 190 genes in the human genome encode phosphoprotein phosphatases, fewer than the number (about 540) that encode protein kinases, reflecting the relative promiscuity of the phosphoprotein phosphatases. A single **phosphoprotein phosphatase** (PP1) dephosphorylates some 200 different phosphoprotein targets, including the β-adrenergic receptor and other GPCRs. Some phosphatases are known to be regulated; others may be constantly active. When [cAMP] drops and PKA returns to its inactive form (step ⑦ in Fig. 12-4a), the balance between phosphorylation and dephosphorylation is tipped toward dephosphorylation by these phosphatases.

The β-Adrenergic Receptor Is Desensitized by Phosphorylation and by Association with Arrestin

The mechanisms for signal termination described above take effect when the stimulus ends. A different mechanism, desensitization, damps the response *even while the signal persists*. When the receptor remains occupied with epinephrine, **β-adrenergic receptor kinase**, or **βARK**, phosphorylates several Ser residues near the receptor's carboxyl terminus, which is on the cytoplasmic side of the plasma membrane (**Fig. 12-9**). βARK is drawn to the plasma membrane by its association with the $G_{s\beta\gamma}$ subunits and is thus positioned to phosphorylate the receptor. Receptor phosphorylation creates a binding site for the protein **β-arrestin**, or **βarr** (also called arrestin 2), and binding of β-arrestin blocks the sites in the receptor that interact with the G protein (**Fig. 12-10**). The binding of β-arrestin also facilitates the sequestration of receptor molecules and their removal from the plasma membrane by endocytosis into small intracellular vesicles (endosomes). The arrestin-receptor complex recruits clathrin and other proteins involved in vesicle formation, which initiate membrane invagination, leading to the formation of endosomes containing the adrenergic receptor. In this state, the receptors are inaccessible to epinephrine and

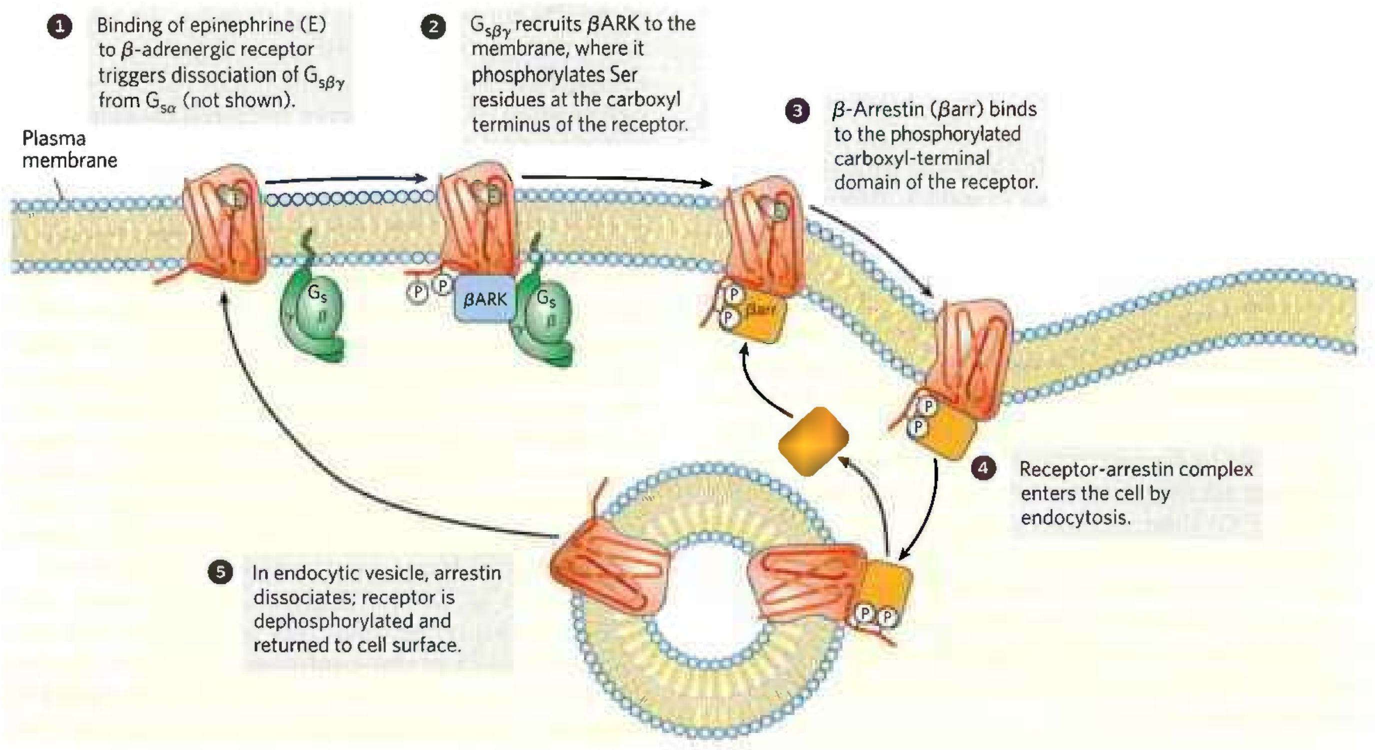

① Binding of epinephrine (E) to β-adrenergic receptor triggers dissociation of $G_{s\beta\gamma}$ from $G_{s\alpha}$ (not shown).
② $G_{s\beta\gamma}$ recruits βARK to the membrane, where it phosphorylates Ser residues at the carboxyl terminus of the receptor.
③ β-Arrestin (βarr) binds to the phosphorylated carboxyl-terminal domain of the receptor.
④ Receptor-arrestin complex enters the cell by endocytosis.
⑤ In endocytic vesicle, arrestin dissociates; receptor is dephosphorylated and returned to cell surface.
Plasma membrane
E
G_s
P
βARK
βarr

FIGURE 12-9 Desensitization of the β-adrenergic receptor in the continued presence of epinephrine. This process is mediated by two proteins: β-adrenergic protein kinase (βARK) and β-arrestin (βarr). Not shown here is the phosphorylation and activation of βARK by PKA. PKA is activated by the rise in [cAMP] in response to the initial signal (epinephrine).

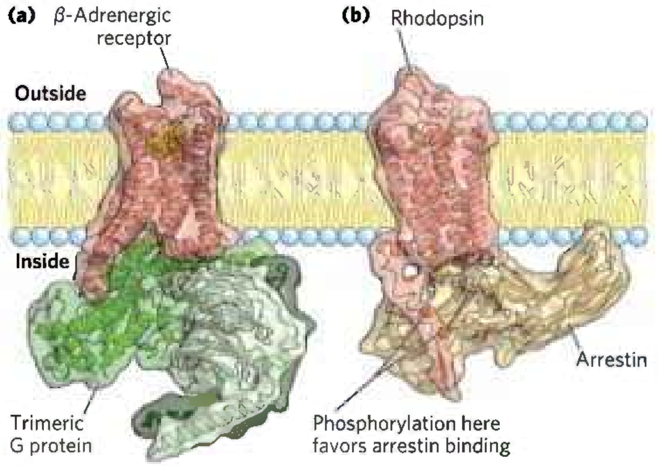

(a) β-Adrenergic receptor
(b) Rhodopsin
Outside
Inside
Trimeric G protein
Phosphorylation here favors arrestin binding
Arrestin

FIGURE 12-10 Mutual exclusion of trimeric G protein and arrestin in their interaction with a GPCR. (a) The β-adrenergic receptor complexed with its trimeric G protein, G_s. (b) Another GPCR, the rhodopsin receptor, phosphorylated near its carboxyl terminus, and bound to arrestin. Binding of arrestin blocks binding and further activation of the G protein and ends the response to the initial signal. [Data from (a) PDB ID 3SN6, S. G. F. Rasmussen et al., *Nature* 477:549, 2011; (b) PDB ID 4ZWJ, Y. Kang et al., *Nature* 523:561, 2015.]

therefore inactive. These receptor molecules are eventually dephosphorylated and returned to the plasma membrane, completing the circuit and resensitizing the system to epinephrine.

β-Adrenergic receptor kinase is a member of a family of **G protein–coupled receptor kinases (GRKs)**, all of which phosphorylate GPCRs on their carboxyl-terminal cytoplasmic domains and play roles similar to that of βARK in desensitization and resensitization of their receptors. Seven GRKs and four different arrestins are encoded in the human genome; each GRK is capable of desensitizing a particular subset of GPCRs, and each arrestin can interact with many different types of phosphorylated receptors.

⚕ The receptor-arrestin complex has another important role: it initiates signaling by a second pathway, the MAPK cascade described below. Thus, acting through a single GPCR, epinephrine triggers two divergent signaling pathways. The two pathways, one initiated by the receptor's interaction with a G protein and the other initiated by its interaction with arrestin, can be differentially affected by the agonist; in some cases, one agonist favors the G-protein pathway and another favors the arrestin pathway. This bias is an important consideration in the development of a medication that acts through a GPCR. For example, the most addictive of the opioid drugs of abuse act more strongly through G-protein signaling than through arrestin. An ideal opioid pain medication would act through the branch of the pathway that has therapeutic effects and not through the pathway that leads to addiction. ■

Cyclic AMP Acts as a Second Messenger for Many Regulatory Molecules

P2 Epinephrine is just one of many hormones, growth factors, and other regulatory molecules that act by changing the intracellular [cAMP] and thus the activity of PKA. **Table 12-3** lists a few examples. Glucagon binds to its receptors in the plasma membrane of adipocytes, activating (via a G_s protein) adenylyl cyclase. PKA, stimulated by the resulting rise in [cAMP], phosphorylates and activates two proteins critical to the mobilization of the fatty acids of stored fats (see Fig. 17-2). Similarly, the peptide hormone ACTH (adrenocorticotropic hormone, also called corticotropin), produced by the anterior pituitary, binds to specific receptors in the adrenal cortex, activating adenylyl cyclase and raising the intracellular [cAMP]. PKA then phosphorylates and activates several of the enzymes required for the synthesis of cortisol and other steroid hormones. In many cell types, the catalytic subunit of PKA can also move into the nucleus, where it phosphorylates the **cAMP response element binding protein (CREB)**, which alters the expression of specific genes regulated by cAMP.

Some hormones act by *inhibiting* adenylyl cyclase, thus *lowering* [cAMP] and *suppressing* protein phosphorylation by PKA. For example, the binding of somatostatin to its receptor in the pancreas leads to activation of an **inhibitory G protein**, or $\mathbf{G_i}$, structurally homologous to G_s, that inhibits adenylyl cyclase and lowers [cAMP]. In this way, somatostatin inhibits the secretion of several hormones, including glucagon. In adipose tissue, prostaglandin E_2 (PGE_2; see Fig. 10-17) inhibits adenylyl cyclase, thus lowering [cAMP] and slowing the mobilization of lipid reserves triggered by epinephrine and glucagon. In certain other tissues, PGE_2 stimulates cAMP synthesis: its receptors are coupled to adenylyl cyclase through a stimulatory G protein, G_s. In tissues with α_2-adrenergic receptors, epinephrine lowers [cAMP]; in this case, the receptors are coupled to adenylyl cyclase through an inhibitory G protein, G_i. In short, an extracellular signal such as epinephrine or PGE_2 can have different effects on different tissues or cell types, depending on three factors: the type of receptor in the tissue, the type of G protein (G_s or G_i) with which the receptor is coupled, and the set of PKA target enzymes in the cell. By summing the influences that tend to increase and decrease [cAMP], a cell achieves the integration of signals that is a general feature of signal-transducing mechanisms (Fig. 12-1f).

TABLE 12-3 **Some Signals That Use cAMP as Second Messenger**

Corticotropin (ACTH)
Corticotropin-releasing hormone (CRH)
Dopamine [D_1, D_2]
Epinephrine (β-adrenergic)
Follicle-stimulating hormone (FSH)
Glucagon
Histamine [H_2]
Luteinizing hormone (LH)
Melanocyte-stimulating hormone (MSH)
Odorants (many)
Parathyroid hormone
Prostaglandins E_1, E_2 (PGE_1, PGE_2)
Serotonin [5-HT_1, 5-HT_4]
Somatostatin
Tastants (sweet, bitter)
Thyroid-stimulating hormone (TSH)

Note: Receptor subtypes in square brackets. Subtypes may have different transduction mechanisms. For example, serotonin is detected in some tissues by receptor subtypes 5-HT_1 and 5-HT_4, which act through adenylyl cyclase and cAMP, and in other tissues by receptor subtype 5-HT_2, acting through the phospholipase C-IP_3 mechanism (see Table 12-4).

Another factor that explains how so many types of signals can be mediated by a single second messenger (cAMP) is the confinement of the signaling process to a specific region of the cell by **adaptor proteins**—noncatalytic proteins that hold together other protein molecules that function in concert (further described below). **P5** **AKAPs (A kinase anchoring proteins)** have multiple, distinct protein-binding domains, often intrinsically disordered regions; they are multivalent adaptor proteins. One domain binds to the R subunits of PKA and another binds to a specific structure in the cell, confining the PKA to the vicinity of that structure. For example, specific AKAPs bind PKA to microtubules, actin filaments, ion channels, mitochondria, or the nucleus. Different types of cells have different complements of AKAPs, so cAMP might stimulate phosphorylation of mitochondrial proteins in one cell and stimulate phosphorylation of actin filaments in another. In some cases, an AKAP connects PKA with the enzyme that triggers PKA activation (adenylyl cyclase) or terminates PKA action (cAMP phosphodiesterase or phosphoprotein phosphatase) (**Fig. 12-11**). The very close proximity of these activating and inactivating enzymes presumably achieves a highly localized, and very brief, response.

In a process analogous with the cAMP-PKA pathway, a cyclic derivative of GTP (3′,5′-cyclic GMP; cGMP) is generated in response to an extracellular signal. The cGMP activates a cGMP-dependent protein kinase (PKG), which phosphorylates specific protein substrates, changing their activities in response to the initial signal (**Box 12-2**).

G Proteins Act as Self-Limiting Switches in Many Processes

Proteins sensitive to the binding of either GTP or GDP play critical roles in many cellular processes, including sensory perception, signaling for cell division, growth

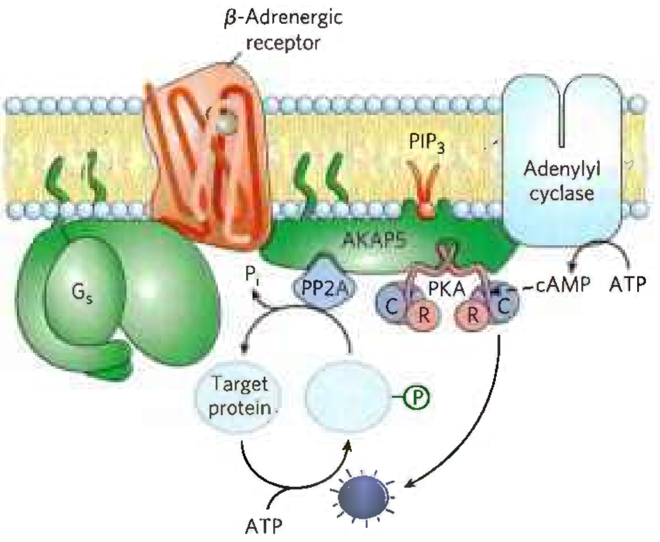


FIGURE 12-11 Nucleation of supramolecular complexes by A kinase anchoring proteins (AKAPs). AKAP5 is one of a family of proteins that act as multivalent scaffolds, holding PKA catalytic subunits—through interaction of the AKAP with the PKA regulatory subunits—in proximity to a particular region or structure in the cell. AKAP5 is targeted to rafts in the cytoplasmic face of the plasma membrane by two covalently attached palmitoyl groups and a site that binds phosphatidylinositol 3,4,5-trisphosphate (PIP_3) in the membrane. AKAP5 also has binding sites for the β-adrenergic receptor, adenylyl cyclase, PKA, and a phosphoprotein phosphatase (PP2A), bringing them all together in the plane of the membrane. When epinephrine binds to the β-adrenergic receptor, adenylyl cyclase produces cAMP, which reaches the nearby PKA quickly and with very little dilution. PKA phosphorylates its target protein, altering its activity, until the phosphoprotein phosphatase removes the phosphoryl group and returns the target protein to its prestimulus state. The AKAPs in this and other cases bring about a high local concentration of enzymes and second messengers, so that the signaling circuit remains highly localized and the duration of the signal is limited.

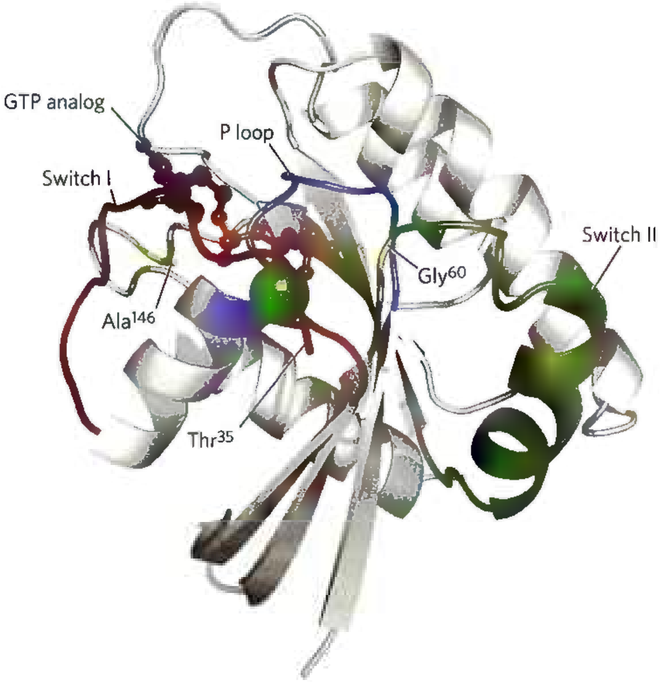


FIGURE 12-12 Ras, the G-protein prototype. Mg^{2+}-GTP is held by critical residues in the phosphate-binding P loop (blue) and by Thr^{35} in the switch I region (red) and Gly^{60} in the switch II region (green). Ala^{146} gives specificity for GTP over ATP. In the structure shown here, the nonhydrolyzable GTP analog Gpp(NH)p is in the GTP-binding site. [Data from PDB ID 5P21, E. F. Pai et al., *EMBO J.* 9:2351, 1990.]

and differentiation, intracellular movements of proteins and membrane vesicles, and protein synthesis. The human genome encodes nearly 200 of these proteins, which differ in size and subunit structure, intracellular location, and function. But all G proteins share a common feature: they can become activated by binding GTP and then, after a brief period, can inactivate themselves with their GTPase activity, thereby serving as molecular binary switches with built-in timers. This superfamily of proteins includes the trimeric G proteins involved in β-adrenergic signaling (G_s or G_i) and vision (transducin); small, monomeric G proteins such as that involved in insulin signaling (Ras; see below) and others that function in vesicle trafficking (ARF, RAC1, and Rab), transport into and out of the nucleus (Ran), and timing of the cell cycle (Rho); and several proteins involved in protein synthesis (initiation factor IF2 and elongation factors EF-Tu and EF-G; see Chapter 27). Among the trimeric G proteins, G_α subunits have covalently linked lipids: the amino terminus is palmitoylated, and some are also myristoylated. G_γ and the monomeric Ras protein have an isoprenyl lipid at their carboxyl termini. The attached lipids keep them anchored to the plasma membrane, restricting their action to that two-dimensional plane.

All G proteins have the same core structure and use the same mechanism for switching between an inactive conformation, favored when GDP is bound, and an active conformation, favored when GTP is bound. We can use **Ras** (~20 kDa, a minimal signaling unit) as a prototype for all members of this superfamily (**Fig. 12-12**).

In the nucleotide-binding site of Ras, Ala^{146} hydrogen bonds to the guanine oxygen, allowing GTP, but not ATP, to bind. In the GTP-bound conformation, the G protein exposes previously buried regions (called **switch I** and **switch II**) that interact with proteins downstream in the signaling pathway until the G protein inactivates itself by hydrolyzing its bound GTP to GDP. The critical determinant of G-protein conformation is the γ phosphate of GTP, which interacts with a region called the **P loop** (*p*hosphate-binding). In Ras, the γ phosphate of GTP binds to a Lys residue in the P loop and to two critical residues, Thr^{35} in switch I and Gly^{60} in switch II, that hydrogen-bond with the oxygens of the γ phosphate of GTP. These hydrogen bonds act like a pair of springs holding the protein in its active

BOX 12-2 MEDICINE

Receptor Guanylyl Cyclases, cGMP, and Protein Kinase G

Guanylyl cyclases (Fig. 1) are receptor enzymes that, when activated, convert GTP to the second messenger **guanosine 3′,5′-cyclic monophosphate (cGMP)** (Fig. 2). Many of the actions of cGMP in animals are mediated by **cGMP-dependent protein kinase**, also called **protein kinase G (PKG)**. On activation by cGMP, PKG phosphorylates Ser and Thr residues in target proteins. The catalytic and regulatory domains of this enzyme are in a single polypeptide (M_r ~80,000). Part of the regulatory domain fits snugly in the substrate-binding cleft. Binding of cGMP forces this activation loop out of the binding site, opening the site to PKG target proteins.

Cyclic GMP carries different messages in different tissues. In cardiac muscle (a type of smooth muscle), it signals relaxation. In the kidney and the intestine, it triggers changes in ion transport and water retention. Guanylyl cyclase in the kidney is activated by the peptide hormone **atrial natriuretic factor** (**ANF**; Fig. 1a), which is released by cells in the cardiac atrium when the heart is stretched by increased blood volume. Carried in the blood to the kidney, ANF activates guanylyl cyclase in cells of the collecting ducts. The resulting rise in [cGMP] triggers increased renal excretion of Na^+ and consequently of water, driven by the change in osmotic pressure.

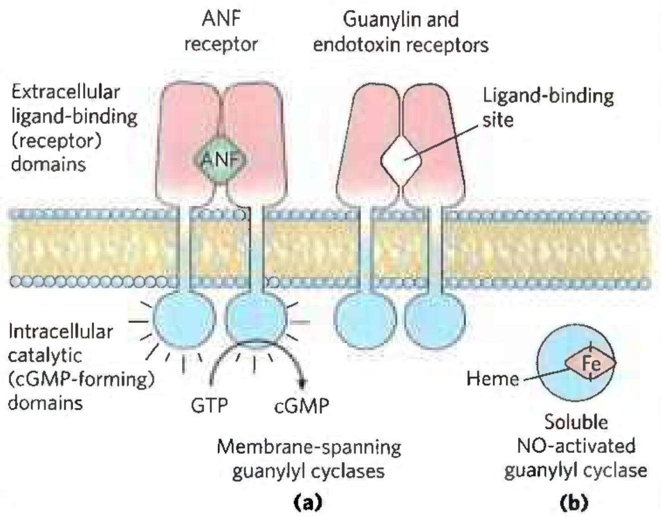

ANF receptor
Guanylin and endotoxin receptors
Extracellular ligand-binding (receptor) domains
Ligand-binding site
ANF
Intracellular catalytic (cGMP-forming) domains
GTP
cGMP
Heme
Fe
Membrane-spanning guanylyl cyclases
(a)
Soluble NO-activated guanylyl cyclase
(b)

FIGURE 1 Two types of signal-transducing guanylyl cyclase. (a) Membrane-spanning guanylyl cyclases such as the ANF and guanylin receptors are homodimers with an extracellular ligand-binding domain and an intracellular guanylyl cyclase domain. (b) A soluble heme-containing guanylyl cyclase is activated by intracellular NO.

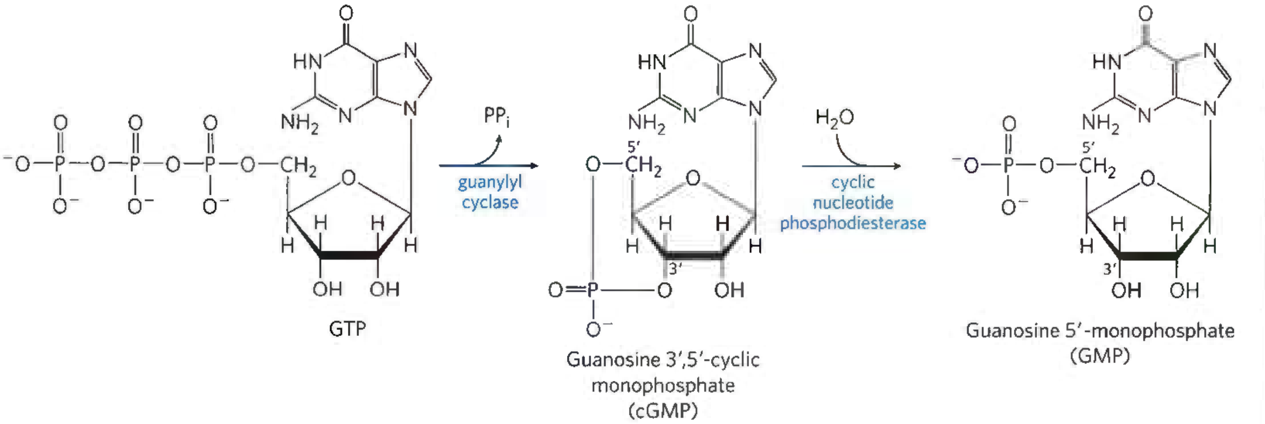

PPi
guanylyl cyclase
H2O
cyclic nucleotide phosphodiesterase
GTP
Guanosine 3′,5′-cyclic monophosphate (cGMP)
Guanosine 5′-monophosphate (GMP)

FIGURE 2 Synthesis of cGMP by guanylyl cyclase and its hydrolysis by cGMP phosphodiesterase.

conformation (**Fig. 12-13**). When GTP is cleaved to GDP, and P_i is released, these hydrogen bonds are lost; the protein then relaxes into its inactive conformation, burying switch I and II so they are no longer available to interact with other partners.

The GTPase activity of most G proteins is very weak, but it is increased up to 10^5-fold by **GTPase activator proteins (GAPs)**, also called, in the case of heterotrimeric G proteins, **regulators of G-protein signaling** (**RGSs**; Fig. 12-8). GAPs and RGSs thus determine how long the G-protein switch remains on. There are about 40 different RGS proteins associated with a variety of processes and expressed in most tissues. They have a critical Arg residue that reaches into the G-protein GTPase active site and contributes to catalysis. The intrinsically slow process of replacing bound GDP with GTP, switching the protein on, is catalyzed by **guanosine nucleotide–exchange factors** (**GEFs**, such as the β-adrenergic receptor) associated with the G protein. The ligand-bound β-adrenergic receptor is one of many GEFs, and a broad

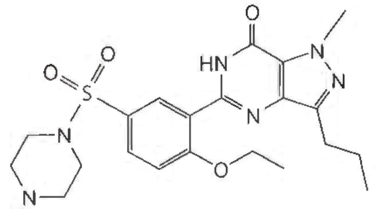
Water loss reduces the blood volume, countering the stimulus that initially led to ANF secretion. Vascular smooth muscle also has an ANF receptor–guanylyl cyclase; on binding to this receptor, ANF causes relaxation (vasodilation) of the blood vessels, which increases blood flow while decreasing blood pressure.

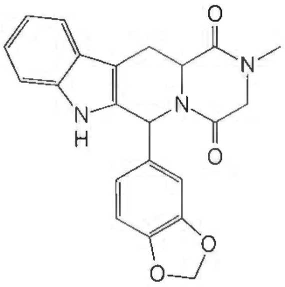
A similar receptor guanylyl cyclase in the plasma membrane of epithelial cells lining the intestine is activated by the peptide **guanylin** (Fig. 1a), which regulates Cl^- secretion in the intestine. This receptor is also the target of a heat-stable peptide endotoxin produced by *Escherichia coli* and other gram-negative bacteria. The elevation in [cGMP] caused by the endotoxin increases Cl^- secretion and consequently decreases reabsorption of water by the intestinal epithelium, producing diarrhea.

A distinctly different type of guanylyl cyclase is a soluble cytosolic protein with a tightly associated heme group (Fig. 1b), an enzyme activated by nitric oxide (NO). Nitric oxide is produced from arginine by Ca^{2+}-dependent **NO synthase**, present in many mammalian tissues, and diffuses from its cell of origin into nearby cells (see Chapter 22).

In the target cell, NO binds to the heme group of guanylyl cyclase and activates cGMP production. In the heart, cGMP-dependent protein kinase reduces the forcefulness of contractions by stimulating the ion pump(s) that remove Ca^{2+} from the cytosol. NO-induced relaxation of cardiac muscle is the same response brought about by nitroglycerin and other nitrovasodilators taken to relieve **angina pectoris**, the pain caused by contraction of a heart deprived of O_2 because of blocked coronary arteries. Nitric oxide is unstable and its action is brief; within seconds of its formation, it undergoes oxidation to nitrite or nitrate. Nitrovasodilator drugs produce long-lasting relaxation of cardiac muscle because they break down over several hours, yielding a steady stream of NO. The value of nitroglycerin as a treatment for angina was discovered serendipitously in factories producing nitroglycerin as an explosive in the 1860s. Workers with angina reported that their condition was much improved during the workweek but worsened on weekends. The physicians treating these workers heard this story so often that they made the connection, and a drug was born.

The effects of increased cGMP synthesis diminish after the stimulus ceases, because a specific phosphodiesterase (cGMP PDE) converts cGMP to the inactive 5′-GMP (see Fig. 2). Humans have several isoforms of cGMP PDE, with different tissue distributions. The isoform in the blood vessels of the penis is inhibited by the drugs sildenafil (Viagra) and tadalafil (Cialis), which therefore cause [cGMP] to remain elevated once raised by an appropriate stimulus, accounting for the usefulness of this drug in the treatment of erectile dysfunction.


Sildenafil (Viagra)


Tadalafil (Cialis)

range of proteins act as GAPs. Their combined effects set the level of GTP-bound G proteins, and thus the strength of the response to signals that arrive at the receptors.

P9 Because G proteins play crucial roles in so many signaling processes, it is not surprising that defects in G proteins lead to a variety of diseases. In about 25% of all human cancers (and in a much higher proportion of certain types of cancer), a mutation in a Ras protein—typically in one of the critical residues around the GTP-binding site or in the P loop—virtually eliminates its GTPase activity. Once activated by GTP binding, this Ras protein remains constantly active, promoting cell division in cells that should not divide. The tumor suppressor gene *NF1* encodes a GAP that enhances the GTPase activity of normal Ras. Mutations in *NF1* that result in a nonfunctioning GAP leave Ras with only its GTPase activity, which is very weak (that is, has a very low turnover number); once activated by GTP binding, Ras stays active for an extended period, continuing to send the signal: divide.

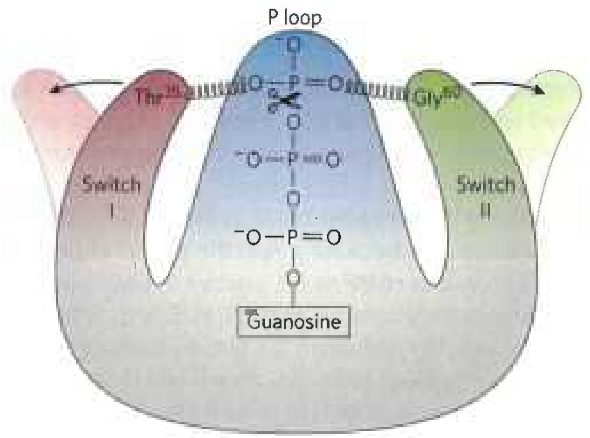
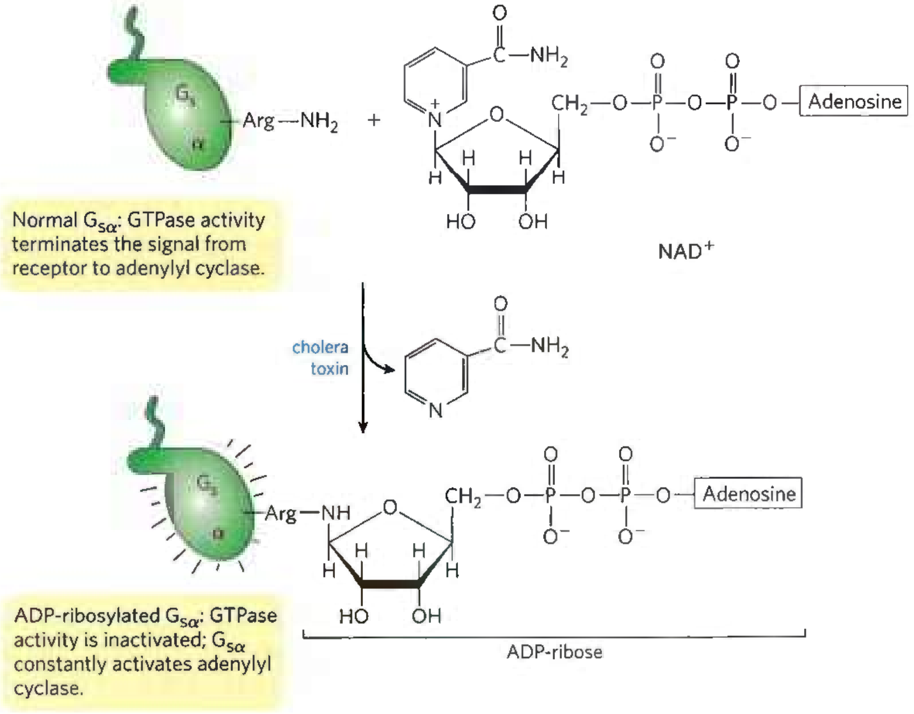


FIGURE 12-13 GTP hydrolysis flips the switches in Ras. When bound GTP is hydrolyzed by the GTPase activities of Ras and its GTPase activator protein (GAP), loss of hydrogen bonds to Thr^{35} and Gly^{60} allows the switch I and switch II regions to relax into a conformation in which they are no longer available to interact with downstream targets. [Information from I. R. Vetter and A. Wittinghofer, *Science* 294:1299, 2001, Fig. 3.]

Defective heterotrimeric G proteins can also lead to disease. Mutations in the gene that encodes the α subunit of G_s (which mediates changes in [cAMP] in response to hormonal stimuli) may result in a G_α that is permanently active or permanently inactive. "Activating" mutations generally occur in residues crucial to GTPase activity; they lead to a continuously elevated [cAMP], with significant downstream consequences, including undesirable cell proliferation. Such mutations are found in about 40% of pituitary tumors (adenomas). Individuals with "inactivating" mutations in G_α are unresponsive to hormones (such as thyroid hormone) that act through cAMP. Mutation in the gene for the transducin α subunit (T_α), which is involved in visual signaling, leads to a type of night blindness, apparently due to defective interaction between the activated T_α subunit and the phosphodiesterase of the rod outer segment (see Fig. 12-19). A sequence variation in the gene encoding the β subunit of a heterotrimeric G protein is commonly found in individuals with hypertension (high blood pressure), and this variant gene is suspected of involvement in obesity and atherosclerosis.

The pathogenic bacterium that causes cholera produces a toxin that targets a G protein, interfering with normal signaling in host cells. **Cholera toxin**, secreted by *Vibrio cholerae* in the intestine of an infected person, is a heterodimeric protein. Subunit B recognizes and binds to specific gangliosides on the surface of intestinal epithelial cells and provides a route for subunit A to enter these cells. After entry, subunit A is broken into two fragments, A1 and A2. A1 associates with the host cell's ADP-ribosylation factor ARF6, a small G protein, through residues in its switch I and switch II regions—which are accessible only when ARF6 is in its active (GTP-bound) form. This association with ARF6 activates A1, which catalyzes the transfer of ADP-ribose from NAD$^+$ to the critical Arg residue in the P loop of the α subunit of G_s (**Fig. 12-14**). ADP-ribosylation



Normal $G_{s\alpha}$: GTPase activity terminates the signal from receptor to adenylyl cyclase.

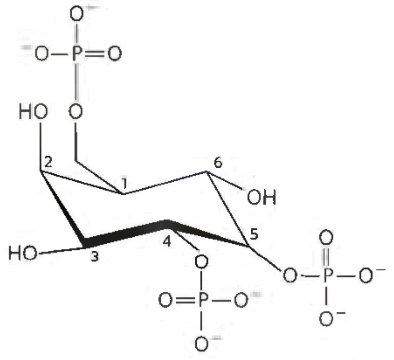
ADP-ribosylated $G_{s\alpha}$: GTPase activity is inactivated; $G_{s\alpha}$ constantly activates adenylyl cyclase.

FIGURE 12-14 ADP-ribosylation locks $G_{s\alpha}$ in the active conformation. The bacterial toxin that causes cholera is an enzyme that catalyzes transfer of the ADP-ribose moiety of NAD$^+$ (nicotinamide adenine dinucleotide) to an Arg residue of $G_{s\alpha}$. The G proteins thus modified fail to respond to normal hormonal stimuli. The pathology of cholera results from defective regulation of adenylyl cyclase and overproduction of cAMP.

blocks the GTPase activity of G_s and thereby renders G_s permanently active. This results in continuous activation of the adenylyl cyclase of intestinal epithelial cells, chronically high [cAMP], and chronically active PKA. PKA phosphorylates the CFTR Cl^- channel (see Box 11-2) and a Na^+-H^+ exchanger in the intestinal epithelial cells. The resultant efflux of NaCl triggers massive water loss through the intestine as cells respond to the ensuing osmotic imbalance. Severe dehydration and electrolyte loss are the major pathologies in cholera. These can be fatal in the absence of prompt rehydration therapy. ■

Diacylglycerol, Inositol Trisphosphate, and Ca^{2+} Have Related Roles as Second Messengers

A second broad class of GPCRs is coupled through a G protein to a plasma membrane **phospholipase C (PLC)** that catalyzes cleavage of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate, or PIP_2 (see Fig. 10-14). When one of the agonists (hormone, neurotransmitter, growth factor; **Table 12-4**) that act by this mechanism binds its specific receptor in the plasma membrane (**Fig. 12-15**, step ①), the receptor-hormone complex catalyzes GTP-GDP exchange on an associated trimeric G protein, $\mathbf{G_q}$ (step ②). This activates the G_q proteins in much the same way that the β-adrenergic receptor activates G_s (Fig. 12-4). The activated G_q activates the PIP_2-specific PLC (Fig. 12-15, step ③), which catalyzes the production of two potent second messengers (step ④), **diacylglycerol** and **inositol 1,4,5-trisphosphate**, or $\mathbf{IP_3}$ (not to be confused with PIP_3, p. 435 or Fig. 12-24).



Inositol 1,4,5-trisphosphate (IP_3)

Inositol trisphosphate, a water-soluble compound, diffuses from the plasma membrane to the endoplasmic reticulum (ER), where it binds to the $\mathbf{IP_3}$**-gated** $\mathbf{Ca^{2+}}$ **channel**, causing the channel to open. The action of the SERCA pump (pp. 393–394) ensures that $[Ca^{2+}]$ in the ER is orders of magnitude higher than that in the cytosol, so when these gated Ca^{2+} channels open, Ca^{2+} rushes into the cytosol (Fig. 12-15, step ⑤), and the cytosolic $[Ca^{2+}]$ rises sharply to about 10^{-6} M. One effect of elevated $[Ca^{2+}]$ is the activation of **protein kinase C** (**PKC**; *C* for Ca^{2+}). Diacylglycerol cooperates with Ca^{2+} in activating PKC, thus also acting as a second messenger (step ⑥). Activation involves the movement of a PKC domain (the pseudosubstrate domain) away from its location in the substrate-binding region of the enzyme, allowing the enzyme to bind and phosphorylate proteins that contain a PKC consensus sequence—Ser or Thr residues embedded in an amino acid sequence recognized by PKC (step ⑦). **Figure 12-16** shows the structure of the IP_3 receptor and a proposed mechanism of its action as a gated Ca^{2+} channel.

Several isozymes of PKC are known, each with a characteristic tissue distribution, target protein specificity, and role. PLCβ is activated by GPCRs; another isoform, PLCγ, is activated by receptor tyrosine kinases, as described below. The ultimate PKC targets include cytoskeletal proteins, enzymes, and nuclear proteins that regulate gene expression. Taken together, this family of enzymes has a wide range of cellular actions, affecting neuronal and immune function and the regulation of cell division. Compounds that lead to overexpression of PKC or that increase its activity to abnormal levels act as tumor promoters; animals exposed to these substances have increased rates of cancer.

Calcium Is a Second Messenger That Is Limited in Space and Time

There are many variations on this basic scheme for Ca^{2+} signaling. In many cell types that respond to extracellular signals, Ca^{2+} serves as a second messenger that triggers intracellular responses, such as exocytosis in neurons and endocrine cells, contraction in skeletal muscle, and cytoskeletal rearrangements during amoeboid movement. In unstimulated cells, cytosolic $[Ca^{2+}]$ is kept very low

TABLE 12-4 Some Signals That Act through Phospholipase C, IP_3, and Ca^{2+}

Acetylcholine [muscarinic M_1]	Gastrin-releasing peptide	Platelet-derived growth factor (PDGF)
α_1-Adrenergic agonists	Glutamate	Serotonin [5-HT_2]
Angiogenin	Gonadotropin-releasing hormone (GRH)	Thyrotropin-releasing hormone (TRH)
Angiotensin II	Histamine [H_1]	Vasopressin
ATP [P_{2x}, P_{2y}]	Light (*Drosophila*)	
Auxin	Oxytocin	

Note: Receptor subtypes are in square brackets; see footnote to Table 12-3.

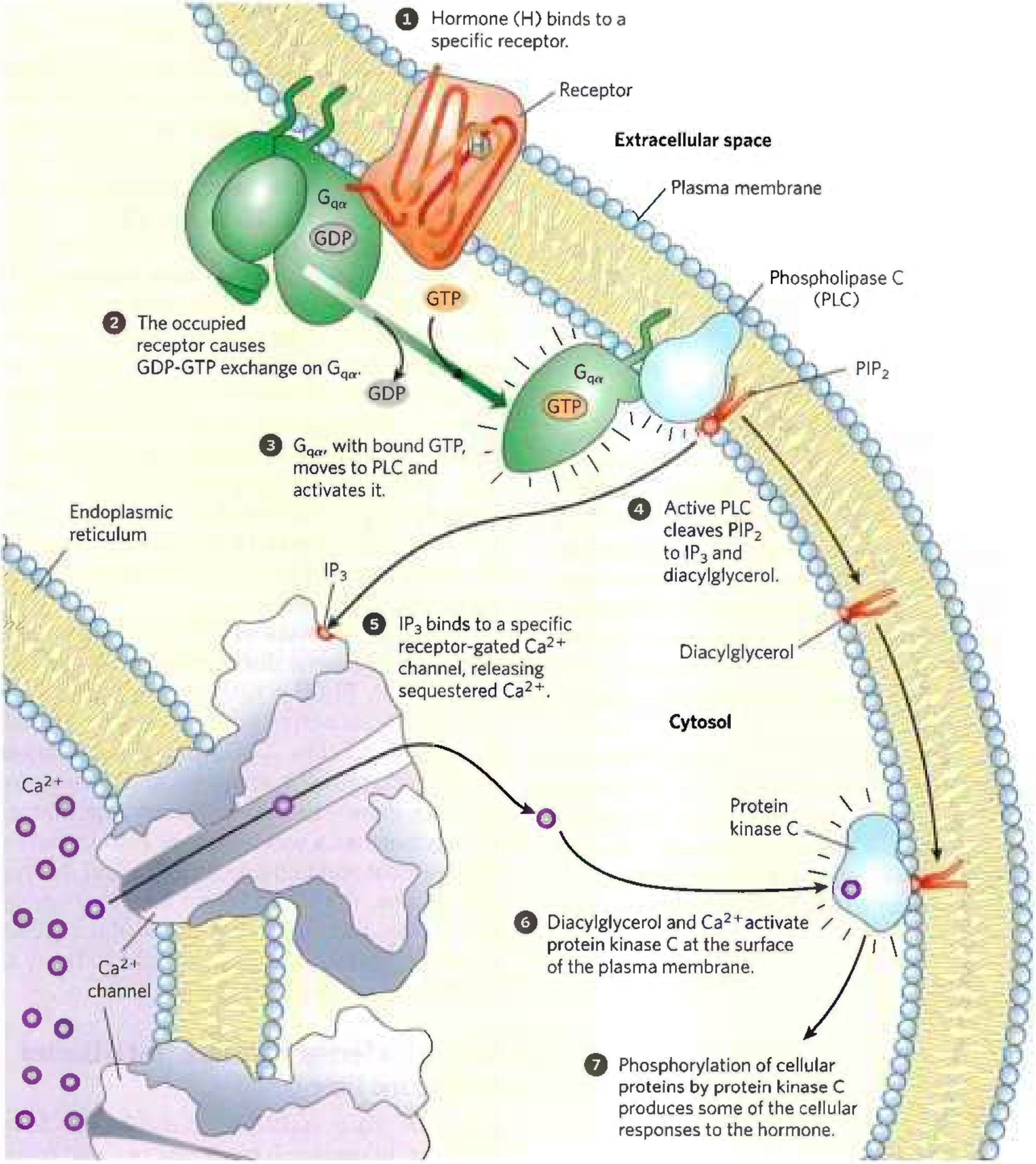

① Hormone (H) binds to a specific receptor.
Receptor
H
Extracellular space
Plasma membrane
$G_{q\alpha}$
GDP
GTP
Phospholipase C (PLC)
② The occupied receptor causes GDP-GTP exchange on $G_{q\alpha}$.
GDP
$G_{q\alpha}$
GTP
PIP_2
③ $G_{q\alpha}$, with bound GTP, moves to PLC and activates it.
Endoplasmic reticulum
④ Active PLC cleaves PIP_2 to IP_3 and diacylglycerol.
IP_3
⑤ IP_3 binds to a specific receptor-gated Ca^{2+} channel, releasing sequestered Ca^{2+}.
Diacylglycerol
Cytosol
Ca^{2+}
Protein kinase C
Ca^{2+} channel
⑥ Diacylglycerol and Ca^{2+} activate protein kinase C at the surface of the plasma membrane.
⑦ Phosphorylation of cellular proteins by protein kinase C produces some of the cellular responses to the hormone.

FIGURE 12-15 Hormone-activated phospholipase C and IP_3. Two intracellular second messengers are produced in the hormone-sensitive phosphatidylinositol system: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol are cleaved from phosphatidylinositol 4,5-bisphosphate (PIP_2). Both contribute to the activation of protein kinase C. By raising cytosolic [Ca^{2+}], IP_3 also activates other Ca^{2+}-dependent enzymes; thus Ca^{2+} also acts as a second messenger.

($<10^{-7}$ M) by the action of Ca^{2+} pumps in the ER, mitochondria, and plasma membrane (as further discussed below). **P6** Hormonal, neural, or other stimuli cause either an influx of Ca^{2+} into the cell through specific Ca^{2+} channels in the plasma membrane or the release of sequestered Ca^{2+} from the ER or mitochondria, in either case raising the cytosolic [Ca^{2+}] and triggering a cellular response.

Changes in intracellular [Ca^{2+}] are detected by Ca^{2+}-binding proteins that regulate a variety of Ca^{2+}-dependent enzymes. **Calmodulin** (**CaM**; M_r 17,000) is an acidic protein with four high-affinity Ca^{2+}-binding sites ($K_d \approx 0.1$ to 1 μM) (**Fig. 12-17**). When intracellular [Ca^{2+}] rises to about 10^{-6} M (1 μM), the binding of Ca^{2+} to calmodulin drives a conformational change in the protein. Calmodulin associates with a variety of proteins and, in its Ca^{2+}-bound state, modulates their activities. It is a member of a family of Ca^{2+}-binding proteins that also includes troponin (see Fig. 5-30), which triggers skeletal muscle contraction in response to increased [Ca^{2+}]. Members of this family share a characteristic Ca^{2+}-binding structure, the EF hand.

Calmodulin is a subunit of the **Ca^{2+}/calmodulin-dependent protein kinases** (**CaM kinases**, types I through IV). When intracellular [Ca^{2+}] increases in response to a stimulus, calmodulin binds Ca^{2+}, undergoes a change in conformation, and activates the CaM kinase. The kinase

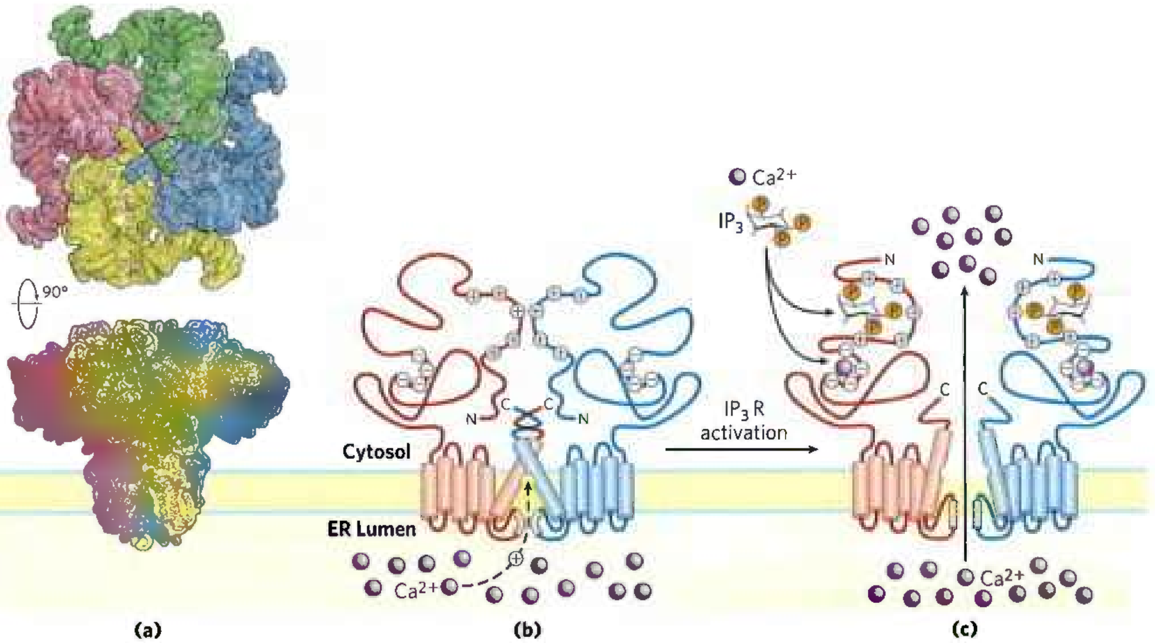


FIGURE 12-16 Proposed mechanism of action of the IP_3-gated Ca^{2+} channel. (a) The IP_3 receptor in its closed conformation, determined by cryo-EM. The 1.3 MDa tetramer has 24 transmembrane helices that surround a central pore. The bulk of the protein protrudes out of the ER into the cytosol and contains the IP_3-binding sites. The central pore is the channel for Ca^{2+} movement, which does not conduct Ca^{2+} in the absence of IP_3. (b, c) Model for receptor activation by IP_3, showing only two of the four identical subunits (b) in the absence of IP_3, and (c) with one IP_3 bound to each subunit. According to this model, IP_3 binding near the amino-terminal end of a subunit causes a major rearrangement of the α helix at the carboxyl-terminal end, opening the Ca^{2+} channel. [(a) Data from PDB ID 6MU2, G. Fan et al., *Cell Res.* 28:1158, 2018. (b, c) Information from M. J. Berridge, *Physiol. Rev.* 96:1261, 2016, Fig. 3.]

then phosphorylates target enzymes, regulating their activities. Calmodulin is also a regulatory subunit of phosphorylase *b* kinase of muscle, which is activated by Ca^{2+}. Thus Ca^{2+} triggers ATP-requiring muscle contractions while also activating glycogen breakdown, providing fuel for ATP synthesis. Many other enzymes are known to be modulated by Ca^{2+} through calmodulin (**Table 12-5**). The activity of the second messenger Ca^{2+}, like that of cAMP, can be spatially restricted; after its release triggers a local response, Ca^{2+} is generally removed before it can diffuse to distant parts of the cell.

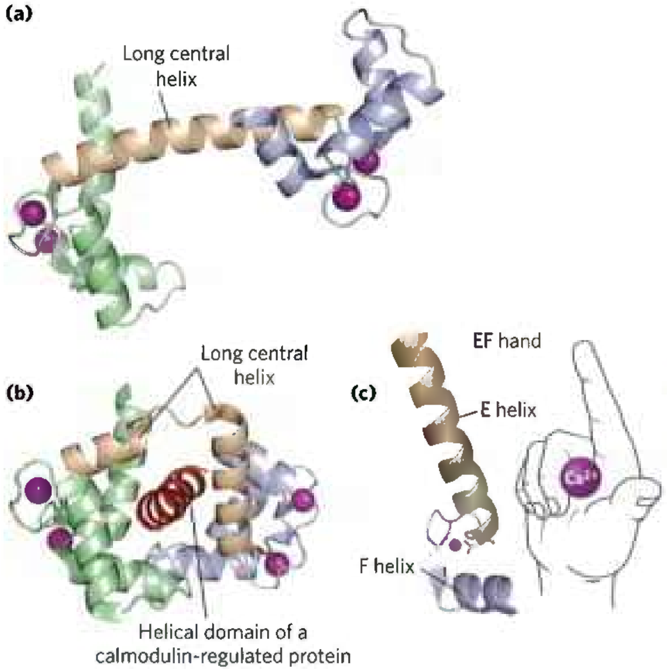


FIGURE 12-17 Calmodulin, the protein mediator of many Ca^{2+}-stimulated enzymatic reactions. (a) In this ribbon model of the crystal structure of calmodulin, the four high-affinity Ca^{2+}-binding sites are occupied by Ca^{2+} (purple). The amino-terminal domain is on the left; the carboxyl-terminal domain on the right. (b) Calmodulin associated with a helical domain of one of the many enzymes it regulates, calmodulin-dependent protein kinase II. Notice that the long central α helix of calmodulin visible in (a) has bent back on itself in binding to the helical substrate domain. The central helix of calmodulin is clearly more flexible in solution than in the crystal. (c) Each of the four Ca^{2+}-binding sites occurs in a helix-loop-helix motif called the EF hand, found in many Ca^{2+}-binding proteins. [Data from (a) PDB ID 1CLL, R. Chattopadhyaya et al., *J. Mol. Biol.* 228:1177, 1992; (b, c) PDB ID 1CDL, W. E. Meador et al., *Science* 257:1251, 1992.]

TABLE 12-5 Some Proteins Regulated by Ca^{2+} and Calmodulin

Adenylyl cyclase (brain)
Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases I to IV)
Ca^{2+}-dependent Na^+ channel (*Paramecium*)
Ca^{2+}-release channel of sarcoplasmic reticulum
Calcineurin (phosphoprotein phosphatase 2B)
cAMP phosphodiesterase
cAMP-gated olfactory channel
cGMP-gated Na^+, Ca^{2+} channels (rod and cone cells)
Glutamate decarboxylase
Myosin light-chain kinases
NAD^+ kinase
Nitric oxide synthase
Phosphatidylinositol 3-kinase
Plasma membrane Ca^{2+} ATPase (Ca^{2+} pump)
RNA helicase (p68)

Commonly, Ca^{2+} level does not simply rise and then fall, but rather oscillates with a period of a few seconds (**Fig. 12-18**) — even when the extracellular concentration of the triggering hormone remains constant. The mechanism underlying [Ca^{2+}] oscillations presumably entails feedback regulation by Ca^{2+} on some part of the Ca^{2+}-release process. Whatever the mechanism, the effect is that one kind of signal (hormone concentration, for example) is converted into another (frequency and amplitude of intracellular [Ca^{2+}] "spikes"). The Ca^{2+} signal diminishes as Ca^{2+} diffuses away from the initial source (the Ca^{2+} channel), is sequestered in the ER, or is pumped out of the cell.

There is significant cross talk between the Ca^{2+} and cAMP signaling systems. In some tissues, both the enzyme that produces cAMP (adenylyl cyclase) and the enzyme that degrades cAMP (phosphodiesterase) are stimulated by Ca^{2+}. Temporal and spatial changes in [Ca^{2+}] can therefore produce transient, localized changes in [cAMP]. We have noted already that PKA, the enzyme that responds to cAMP, is often part of a highly localized supramolecular complex assembled on scaffold proteins such as AKAPs. This subcellular localization of target enzymes, combined with temporal and spatial gradients in [Ca^{2+}] and [cAMP], allows a cell to respond to one or several signals with subtly nuanced metabolic changes.

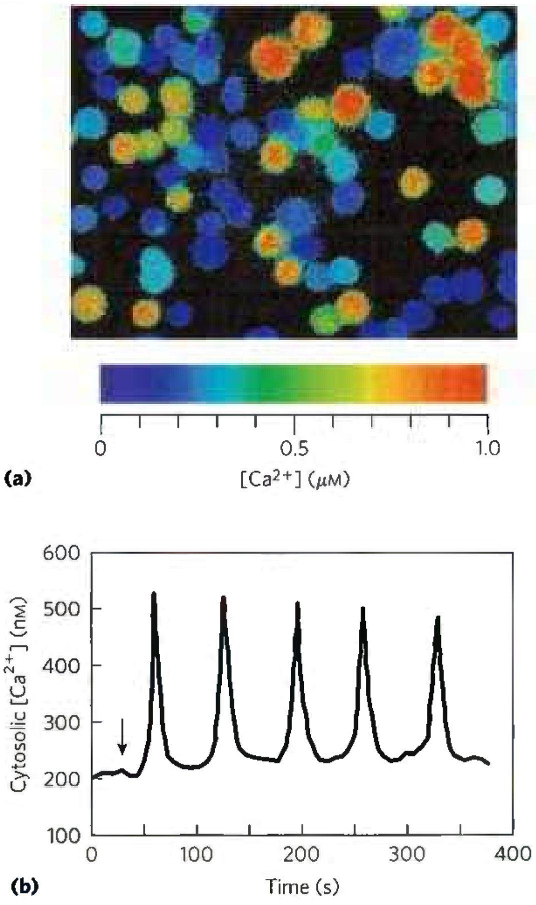


FIGURE 12-18 Triggering of oscillations in intracellular [Ca^{2+}] by extracellular signals. (a) The dye fura, which undergoes fluorescence changes when it binds Ca^{2+}, can be used with fluorescence microscopy to measure the instantaneous Ca^{2+} in cells. The color scale relates fluorescence intensity to [Ca^{2+}]. Here, thymus cells have been stimulated with extracellular ATP, which raises their internal [Ca^{2+}]. The cells are heterogeneous in their responses: some have high intracellular [Ca^{2+}] (red), others have much lower [Ca^{2+}] (blue). (b) When such a probe is used in a single hepatocyte, the agonist norepinephrine (added at the arrow) causes oscillations of [Ca^{2+}] from 200 to 500 nM. Similar oscillations are induced in other cell types by other extracellular signals. [(a) Courtesy Michael D. Cahalan, University of California, Irvine, Department of Physiology and Biophysics. (b) Data from T. A. Rooney et al., *J. Biol. Chem.* 264:17,131, 1989.]

SUMMARY 12.2 *G Protein–Coupled Receptors and Second Messengers*

- G protein–coupled receptors (GPCRs) have seven transmembrane helices and act through heterotrimeric G proteins. Ligand binding activates the G protein, which then stimulates or inhibits the activity of an effector enzyme, changing the local concentration of its second-messenger product cAMP.
- Epinephrine, acting through its GPCR and the G_s protein, stimulates adenylyl cyclase, which produces cAMP. Cyclic AMP activates protein kinase A (PKA), which then phosphorylates target proteins on a Ser or Thr residue, changing their biological activity.
- To end the response to epinephrine, a phosphodiesterase breaks down cAMP, and the G protein is inactivated by its own GTPase activity. Phosphoprotein phosphatases reverse the effects of PKA.
- When the epinephrine signal persists, β-adrenergic receptor–specific protein kinase phosphorylates the GPCR, creating a binding site for the protein β-arrestin, which prevents interaction between the GPCR and its G protein. Arrestin triggers desensitization by movement of the receptor into intracellular vesicles.
- GPCRs typically have their effects through the G protein-cAMP-PKA pathway; some act through G_s, raising [cAMP], others through G_i, lowering [cAMP]. Adaptor proteins such as AKAPs tether PKA and limit its area of action and its target proteins.
- Trimeric G proteins coupled to GPCRs are activated when their bound GDP is exchanged for GTP, and they remain active until their GTPase converts bound GTP to GDP. Their GTPase activity is modulated by GTPase activator proteins (GAPs) and regulators of G-protein signaling (RGSs).
- Monomeric (small) G proteins such as Ras, Rab, and Ran also serve as self-limiting switches. Defective G-protein signaling is common in individuals with some types of cancer.
- Some GPCRs are coupled to a G protein (G_q) that acts through a plasma membrane phospholipase C that cleaves PIP_2 to diacylglycerol and IP_3. By opening Ca^{2+} channels in the endoplasmic reticulum, IP_3 raises cytosolic

[Ca^{2+}]. Diacylglycerol and Ca^{2+} act together to activate protein kinase C, which phosphorylates and changes the activity of specific cellular proteins.

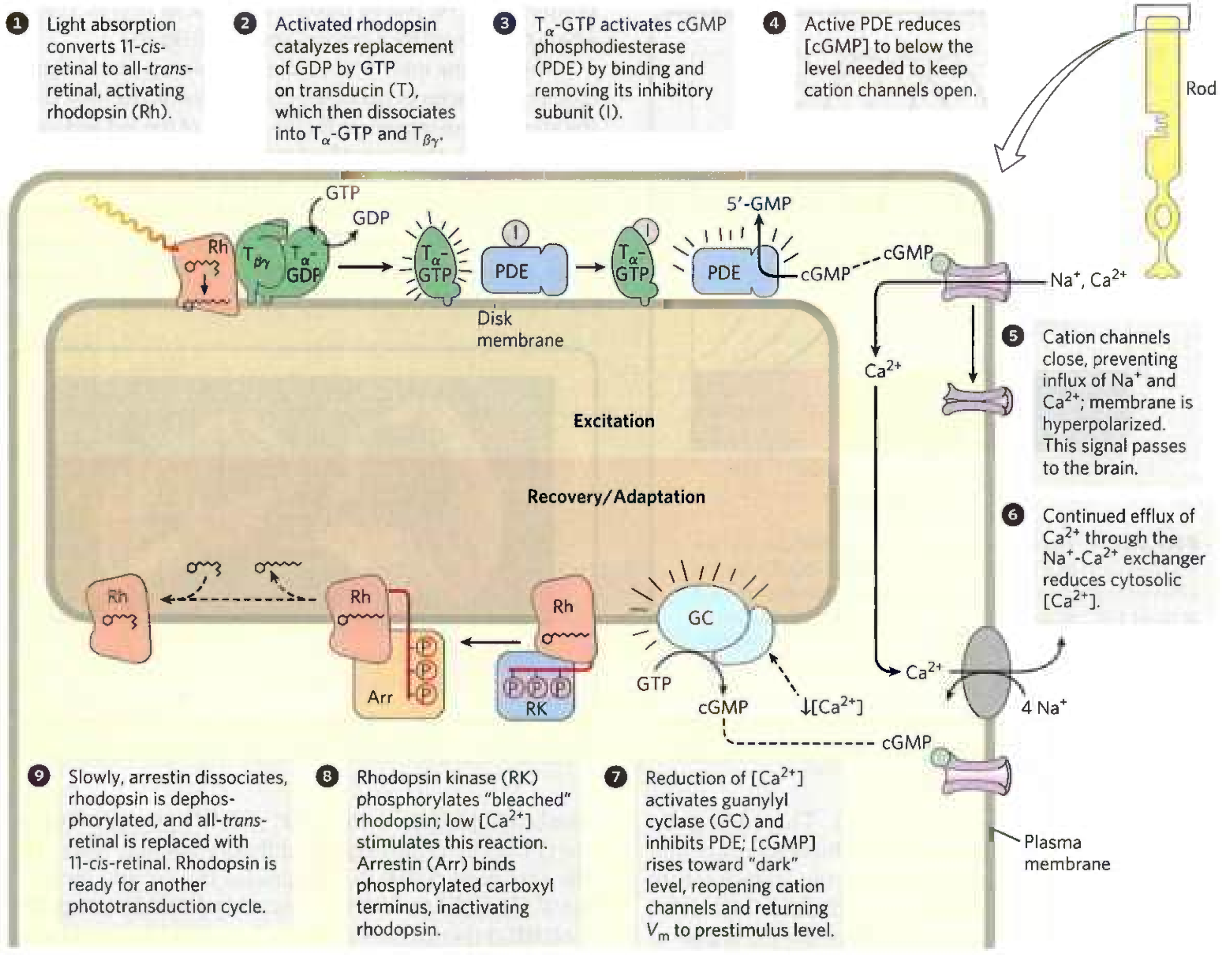
- Cellular [Ca^{2+}] also regulates (often through calmodulin) many other enzymes and proteins involved in secretion, cytoskeletal rearrangements, or contraction. Many of the target enzymes are in the family of Ca^{2+}-activated protein kinases (PKCs).

12.3 GPCRs in Vision, Olfaction, and Gustation

The detection of light, odors, and tastes (vision, olfaction, and gustation, respectively) in animals is accomplished by specialized sensory neurons that use signal-transduction mechanisms fundamentally similar to those that detect hormones, neurotransmitters, and growth factors. An initial sensory signal is greatly amplified by mechanisms that include gated ion channels and intracellular second messengers; the system adapts to continued stimulation by changing its sensitivity to the stimulus (desensitization); and sensory input from several receptors is integrated before the final signal goes to the brain.

The Vertebrate Eye Uses Classic GPCR Mechanisms

Visual transduction (**Fig. 12-19**) begins when light falls on **rhodopsin**, a GPCR in the disk membranes of rod cells of the vertebrate eye. (Rod cells do not detect colors; cone cells do, as we shall see in **Box 12-3**.) The light-absorbing pigment (chromophore) 11-*cis*-retinal is covalently attached to **opsin**, the protein component of rhodopsin, which lies near the middle of the disk membrane bilayer. When a photon is absorbed by the retinal component of rhodopsin (step ➊), the energy causes a photochemical change; 11-*cis*-retinal is converted



➊ Light absorption converts 11-*cis*-retinal to all-*trans*-retinal, activating rhodopsin (Rh).

➋ Activated rhodopsin catalyzes replacement of GDP by GTP on transducin (T), which then dissociates into T_α-GTP and $T_{\beta\gamma}$.

➌ T_α-GTP activates cGMP phosphodiesterase (PDE) by binding and removing its inhibitory subunit (I).

➍ Active PDE reduces [cGMP] to below the level needed to keep cation channels open.

➎ Cation channels close, preventing influx of Na^+ and Ca^{2+}; membrane is hyperpolarized. This signal passes to the brain.

➏ Continued efflux of Ca^{2+} through the Na^+-Ca^{2+} exchanger reduces cytosolic [Ca^{2+}].

➐ Reduction of [Ca^{2+}] activates guanylyl cyclase (GC) and inhibits PDE; [cGMP] rises toward "dark" level, reopening cation channels and returning V_m to prestimulus level.

➑ Rhodopsin kinase (RK) phosphorylates "bleached" rhodopsin; low [Ca^{2+}] stimulates this reaction. Arrestin (Arr) binds phosphorylated carboxyl terminus, inactivating rhodopsin.

➒ Slowly, arrestin dissociates, rhodopsin is dephosphorylated, and all-*trans*-retinal is replaced with 11-*cis*-retinal. Rhodopsin is ready for another phototransduction cycle.

FIGURE 12-19 Molecular consequences of photon absorption by rhodopsin in the rod outer segment. The top half of the figure (steps ➊ to ➎) describes excitation; the bottom shows post-illumination steps: recovery (steps ➏ and ➐) and adaptation (steps ➑ and ➒).

BOX 12-3 MEDICINE

Color Blindness: John Dalton's Experiment from the Grave

Color vision involves a path of sensory transduction in specialized cells in the retina. Three types of cone cells are specialized to detect light from different regions of the spectrum, using three related photoreceptor proteins (opsins). Each cone cell expresses only one kind of opsin, but each type is closely related to rhodopsin in size, amino acid sequence, and, presumably, three-dimensional structure. The differences among the opsins, however, are great enough to place the chromophore, 11-*cis*-retinal, in three slightly different environments, with the result that the three photoreceptors have different absorption spectra (Fig. 1). We discriminate colors and hues by integrating the output from the three types of cone cells, each containing one of the three types of photoreceptors.

Color blindness, such as the inability to distinguish red from green, is a fairly common, genetically inherited trait in humans. The various types of color blindness result from different opsin mutations. One form is due to loss of the red photoreceptor; affected individuals are **red$^-$ dichromats** (they see only two primary colors). Others lack the green pigment and are **green$^-$ dichromats.** In some cases, the red and green photoreceptors are present but have a changed amino acid sequence that causes a change in their absorption spectra, resulting in abnormal color vision. Depending on which pigment is altered, these individuals are **red-anomalous trichromats** or **green-anomalous trichromats.** Examination of the genes for the visual receptors has allowed the diagnosis of color blindness in the chemist John Dalton more than a century after his death.

Dalton (of atomic theory fame) was color-blind. He thought it probable that the vitreous humor of his eyes (the fluid that fills the eyeball behind the lens) was tinted blue, unlike the colorless fluid of normal eyes. He proposed that after his death, his eyes should be dissected and the color of the vitreous humor determined. His wish was honored. The day after Dalton's death in July 1844, Joseph Ransome dissected his eyes and found the vitreous humor to be perfectly colorless. Ransome, like many scientists, was reluctant to throw samples away. He placed Dalton's eyes in a jar of preservative, where they stayed for a century and a half (Fig. 2).

Then, in the mid-1990s, molecular biologists in England took small samples of Dalton's retinas and extracted DNA. Using the known gene sequences for the opsins of the red and green light receptors, they amplified the relevant sequences using PCR and determined that Dalton had the opsin gene for the red photopigment but lacked the opsin gene for the green photopigment. Dalton was a green$^-$ dichromat. So, 150 years after his death, the experiment Dalton started—by hypothesizing about the cause of his color blindness—was finally finished.

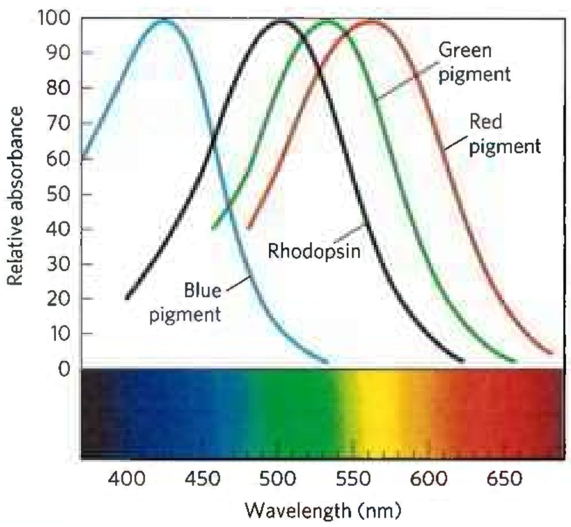


FIGURE 1 Absorption spectra of purified rhodopsin and the red, green, and blue receptors of cone cells. The receptor spectra, obtained from individual cone cells isolated from cadavers, peak at about 420, 530, and 560 nm, and the maximum absorption for rhodopsin is at about 500 nm. For reference, the visible spectrum for humans is about 380 to 750 nm. [Data from J. Nathans, *Sci. Am.* 260 (February):42, 1989.]

FIGURE 2 Dalton's eyes. [Professor J. D. Mollon, Cambridge University, Department of Experimental Psychology.]

to all-*trans*-retinal (see Fig. 10-20). This change in the structure of the chromophore forces conformational changes in the rhodopsin molecule, allowing it to interact with and thus activate its trimeric G protein, transducin. Rhodopsin now stimulates the exchange of bound GDP on transducin for GTP from the cytosol (Fig. 12-19, step ②), and activated transducin stimulates the membrane protein cyclic GMP (cGMP) phosphodiesterase (PDE) by removing an inhibitory subunit (step ③). The activated cGMP PDE degrades the second messenger 3′,5′-cGMP to 5′-GMP, lowering [cGMP] (step ④). A cGMP-dependent Na^+ or Ca^{2+} channel in the plasma membrane closes (step ⑤), while a Na^+-Ca^{2+} active antiporter continues to pump Ca^{2+} outward across the plasma membrane (step ⑥), making the transmembrane electrical potential more negative inside (that is, hyperpolarizing the rod cell). This electrical change passes through a series of specialized nerve cells to the visual cortex of the brain.

Several steps in the visual-transduction process result in a huge amplification of the signal. Each excited rhodopsin molecule activates at least 500 molecules of transducin, and each transducin molecule can activate a molecule of cGMP PDE. This phosphodiesterase has a remarkably high turnover number: each activated molecule hydrolyzes 4,200 molecules of cGMP per second. The binding of cGMP to cGMP-gated ion channels is cooperative, and a relatively small change in [cGMP] therefore registers as a large change in ion conductance. The result of these amplifications is exquisite sensitivity to light. Absorption of a single photon closes 1,000 or more ion channels for Na^+ and Ca^{2+}, hyperpolarizing the cell's membrane by about 1 mV.

As your eyes move across this line of type, the retinal images of the first words disappear rapidly—before you see the next series of words. In that short interval, a great deal of biochemistry has taken place. Very soon after illumination of the rod or cone cells stops, the photosensory system shuts off. The α subunit of transducin (T_α, with bound GTP) has GTPase activity. Within milliseconds after the decrease in light intensity, GTP is hydrolyzed and T_α reassociates with $T_{\beta\gamma}$. The inhibitory subunit of PDE, which had been bound to T_α-GTP, is released and reassociates with PDE, strongly inhibiting its activity and thus slowing cGMP breakdown.

At the same time, a second factor that helps to end the response to light is the reduction of intracellular [Ca^{2+}] that results from continued Ca^{2+} efflux through the Na^+-Ca^{2+} exchanger (Fig. 12-19, step ⑥). High [Ca^{2+}] inhibits the enzyme that makes cGMP (guanylyl cyclase; step ⑦), so cGMP production rises when [Ca^{2+}] falls, quickly reaching its prestimulus level.

In response to prolonged illumination, rhodopsin itself undergoes changes that limit the duration of its signaling activity. The conformational change induced in rhodopsin by light absorption exposes several Thr and Ser residues in its carboxyl-terminal domain, and these residues are phosphorylated by **rhodopsin kinase** (step ⑧), which is functionally and structurally homologous to the β-adrenergic kinase (βARK) that desensitizes the β-adrenergic receptor. The phosphorylated carboxyl-terminal domain of rhodopsin is bound by the protein **arrestin 1**, preventing further interaction between activated rhodopsin and transducin (see Fig. 12-10b). Arrestin 1 is a close homolog of arrestin 2 (βarr) of the β-adrenergic system. On a much longer time scale (step ⑨), the all-*trans*-retinal bound to light-bleached rhodopsin is removed and replaced with 11-*cis*-retinal, making rhodopsin ready to detect another photon.

Vertebrate Olfaction and Gustation Use Mechanisms Similar to the Visual System

The sensory cells that detect odors and tastes have much in common with the visual receptor system. Binding of an odorant molecule to one of its specific GPCRs (humans have about 800 different GPCRs; rodents have about 1,200) triggers a change in receptor conformation, activating a G protein, G_{olf}, analogous to transducin and to G_s of the β-adrenergic system. The activated G_{olf} activates adenylyl cyclase, raising the local [cAMP]. **P5** The cAMP-gated Na^+ and Ca^{2+} channels of the plasma membrane open, and the influx of Na^+ and Ca^{2+} produces a small depolarization called the **receptor potential**. If a sufficient number of odorant molecules encounter receptors, the receptor potential is strong enough to cause the neuron to fire an action potential. This signal is relayed to the brain in several stages and registers as a specific smell. All these events occur within 100 to 200 ms. When the olfactory stimulus is no longer present, the transducing machinery shuts itself off in several ways. A cAMP phosphodiesterase returns [cAMP] to the prestimulus level. G_{olf} hydrolyzes its bound GTP to GDP, thereby inactivating itself. Phosphorylation of the receptor by a specific kinase prevents its interaction with G_{olf}, by a mechanism analogous to that used to desensitize the β-adrenergic receptor and rhodopsin. Some odorants are detected by another mechanism we have seen in other signal transductions: activation of a phospholipase and production of IP_3, leading to a rise in intracellular [Ca^{2+}].

The sense of taste in vertebrates reflects the activity of gustatory neurons clustered in taste buds on the surface of the tongue. In taste sensory neurons, GPCRs are coupled to the heterotrimeric G protein **gustducin**. When the tastant molecule binds its receptor, gustducin is activated and stimulates cAMP production by adenylyl cyclase. The resulting elevation of [cAMP] activates PKA, which phosphorylates K^+ channels in the plasma membrane, causing them to close and sending an electrical signal to the brain. Other taste buds specialize in detecting bitter, sour, salty, or umami (savory) tastants, using various combinations of second messengers and ion channels in the transduction mechanisms.

All GPCR Systems Share Universal Features

We have now looked at several types of signaling systems (hormone signaling, vision, olfaction, and gustation) in which membrane receptors are coupled to second messenger–generating enzymes through G proteins. As we have intimated, signaling mechanisms must have arisen early in evolution; genomic studies have revealed hundreds of genes encoding GPCRs in vertebrates, arthropods (*Drosophila* and mosquito), and the roundworm *Caenorhabditis elegans*. Even the common budding yeast *Saccharomyces* uses GPCRs and G proteins to detect the opposite mating type. Overall patterns have been conserved, and the introduction of variety has given modern organisms the ability to respond to a wide range of stimuli (**Table 12-6**). Of the approximately 20,000 genes in the human genome, as many as 800 encode GPCRs, including hundreds for olfactory stimuli and many orphan receptors, for which the natural ligand is not yet known.

P2 All well-studied signal-transducing systems that act through heterotrimeric G proteins share some common features that reflect their evolutionary relatedness (**Fig. 12-20**). The receptors have seven transmembrane segments, a domain (generally the loop between transmembrane helices 6 and 7) that interacts with a G protein, and a carboxyl-terminal cytoplasmic domain that undergoes reversible phosphorylation on several Ser or Thr residues. The ligand-binding site (or, in the case of light reception, the light receptor) is buried deep in the membrane and includes residues from several of the transmembrane segments. Ligand binding (or light) induces a conformational change in the receptor, exposing a domain that can interact with a G protein. Heterotrimeric G proteins activate or inhibit effector enzymes (adenylyl cyclase, PDE, or PLC), which change the concentration of a second messenger (cAMP, cGMP, IP_3, or Ca^{2+}). In the hormone-detecting systems, the final output is an activated protein kinase that regulates some cellular process by phosphorylating a protein critical to that process. In sensory neurons, the output is a change in membrane potential and a consequent electrical signal that passes to another neuron in the pathway connecting the sensory cell to the brain.

All these systems self-inactivate. Bound GTP is converted to GDP by the GTPase activity of G proteins, often augmented by GTPase-activating proteins (GAPs) or regulators of G-protein signaling (RGS). In some cases, the effector enzymes that are the targets of modulation by G proteins also serve as GAPs. The desensitization mechanism involving phosphorylation of the carboxyl-terminal region followed by arrestin binding is widespread and may be universal.

TABLE 12-6 Some Signals That Act through GPCRs

Amines
- Acetylcholine (muscarinic)
- Dopamine
- Epinephrine
- Histamine
- Serotonin

Peptides
- Angiotensin
- Bombesin
- Bradykinin
- Chemokine
- Colecystokinin (CCK)
- Endothelin
- Gonadotropin-releasing hormone
- Interleukin-8
- Melanocortin
- Neuropeptide Y
- Neurotensin
- Orexin
- Somatostatin
- Tachykinin
- Thyrotropin-releasing hormone
- Vasopressin

Protein hormones
- Follicle-stimulating hormone
- Gonadotropin
- Lutropin-choriogonadotropic hormone
- Thyrotropin

Prostanoids
- Prostacyclin
- Prostaglandin
- Thromboxane

Others
- Cannabinoids
- Lysosphingolipids
- Melatonin
- Olfactory stimuli
- Opioids
- Rhodopsin

SUMMARY 12.3 *GPCRs in Vision, Olfaction, and Gustation*

- Vision, olfaction, and gustation in vertebrates employ GPCRs, which act through heterotrimeric G proteins to change the membrane potential (V_m) of a sensory neuron.

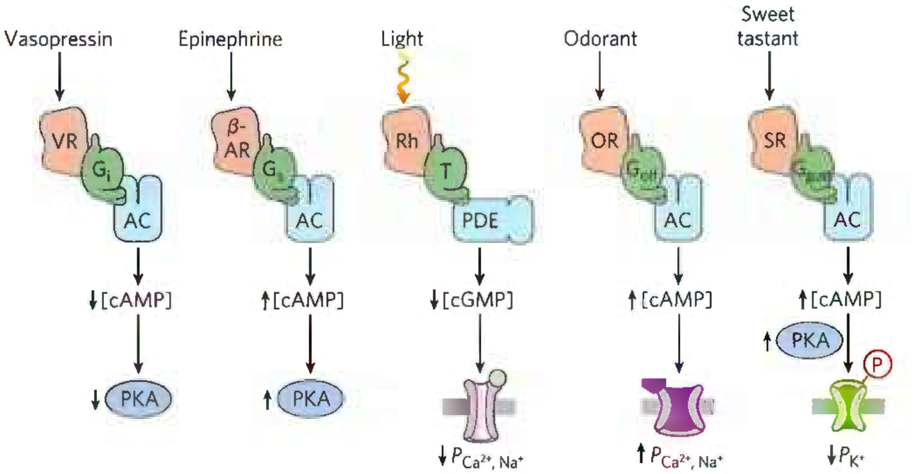


FIGURE 12-20 Common features of signaling systems that detect hormones, light, smells, and tastes. GPCRs provide signal specificity, and their interaction with G proteins provides signal amplification. Heterotrimeric G proteins activate or inhibit effector enzymes: adenylyl cyclase (AC) and phosphodiesterases (PDEs) that degrade cAMP or cGMP. Changes in concentration of the second messengers (cAMP, cGMP) result in alterations in enzymatic activities via phosphorylation or alterations in the permeability (P) of surface membranes to Ca^{2+}, Na^+, and K^+. The resulting depolarization or hyperpolarization of the sensory cell (the signal) passes through relay neurons to sensory centers in the brain. In the best-studied cases, desensitization includes phosphorylation of the receptor and binding of a protein (arrestin) that interrupts receptor–G protein interactions. (The path of odorant detection by production of IP_3 and increase in intracellular $[Ca^{2+}]$, mentioned in the text, is not shown here.) VR is the vasopressin receptor; β-AR, the β-adrenergic receptor; Rh, rhodopsin; OR, olfactory receptor; SR, sweet-taste receptor.

- Light activates the GPCR rhodopsin, which allosterically activates the trimeric G protein transducin. T_α activates a cGMP phosphodiesterase, lowering [cGMP] and closing cGMP-dependent ion channels. The resulting electrical impulse carries the signal to the brain.

- In olfactory neurons, olfactory stimuli, acting through GPCRs and G proteins, trigger an increase in [cAMP] (by activating adenylyl cyclase) or [Ca^{2+}] (by activating PLC). These second messengers affect ion channels and thus the V_m. Gustatory neurons have GPCRs that respond to tastants by altering levels of cAMP, which changes V_m by gating ion channels.

- There is a high degree of conservation of signaling proteins and transduction mechanisms across signaling systems and across species. GPCRs with seven transmembrane helices, G proteins with intrinsic GTPase activities, cyclic nucleotides, and protein kinases are central to signaling.

12.4 Receptor Tyrosine Kinases

P4 The **receptor tyrosine kinases (RTKs)**, a family of plasma membrane receptors with protein kinase activity, transduce extracellular signals by a mechanism fundamentally different from that of GPCRs. RTKs have a ligand-binding domain on the extracellular face of the plasma membrane and an enzyme active site on the cytoplasmic face, connected by a single transmembrane segment, as seen for insulin in **Figure 12-21a**. The cytoplasmic domain

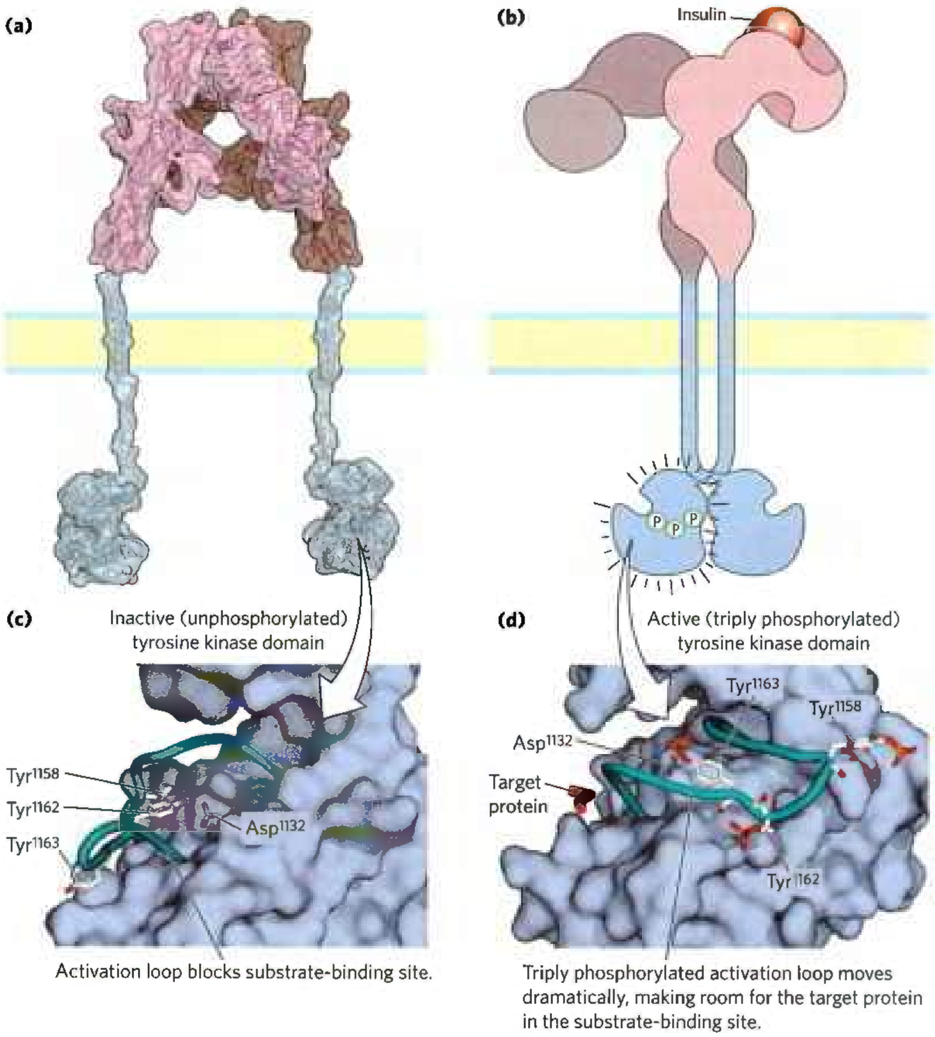

(a)
(b)
Insulin
P
P
P
(c)
Inactive (unphosphorylated) tyrosine kinase domain
Tyr1158
Tyr1162
Tyr1163
Asp1132
Activation loop blocks substrate-binding site.
(d)
Active (triply phosphorylated) tyrosine kinase domain
Tyr1163
Tyr1158
Asp1132
Target protein
Tyr1162
Triply phosphorylated activation loop moves dramatically, making room for the target protein in the substrate-binding site.

FIGURE 12-21 Activation of the insulin-receptor tyrosine kinase by autophosphorylation. (a) A structural model assembled from crystal structures of the extracellular and tyrosine kinase domains and NMR solution structure of the transmembrane domain. The insulin-binding region of the insulin receptor lies outside the cell and comprises two intertwined α subunits. (b) The binding of a single molecule of insulin is communicated through the single transmembrane helix of each β subunit to the paired Tyr kinase domains inside the cell, moving them toward each other and activating them to phosphorylate each other on three Tyr residues. (c) In the inactive form of the Tyr kinase domain, the activation loop (backbone shown in teal) sits in the active site, and none of the critical Tyr residues (stick structures) are phosphorylated. This conformation is stabilized by hydrogen bonding between Tyr^{1162} and Asp^{1132}. (d) Activation of the Tyr kinase allows each β subunit to phosphorylate three Tyr residues (Tyr^{1158}, Tyr^{1162}, Tyr^{1163}) on the other β subunit. (Phosphoryl groups are depicted in red and orange.) The introduction of three highly charged Ⓟ–Tyr residues forces a 30 Å change in the position of the activation loop, away from the substrate-binding site, which thus becomes available to bind and phosphorylate a target protein. [(a) Data and information from T. Gutmann et al., *J. Cell Biol.* 21:1643, 2018, Fig. 1. Data from (c) PDB ID 1IRK, S. R. Hubbard et al., *Nature* 372:746, 1994; (d) PDB ID 1IR3, S. R. Hubbard, *EMBO J.* 16:5572, 1997.]

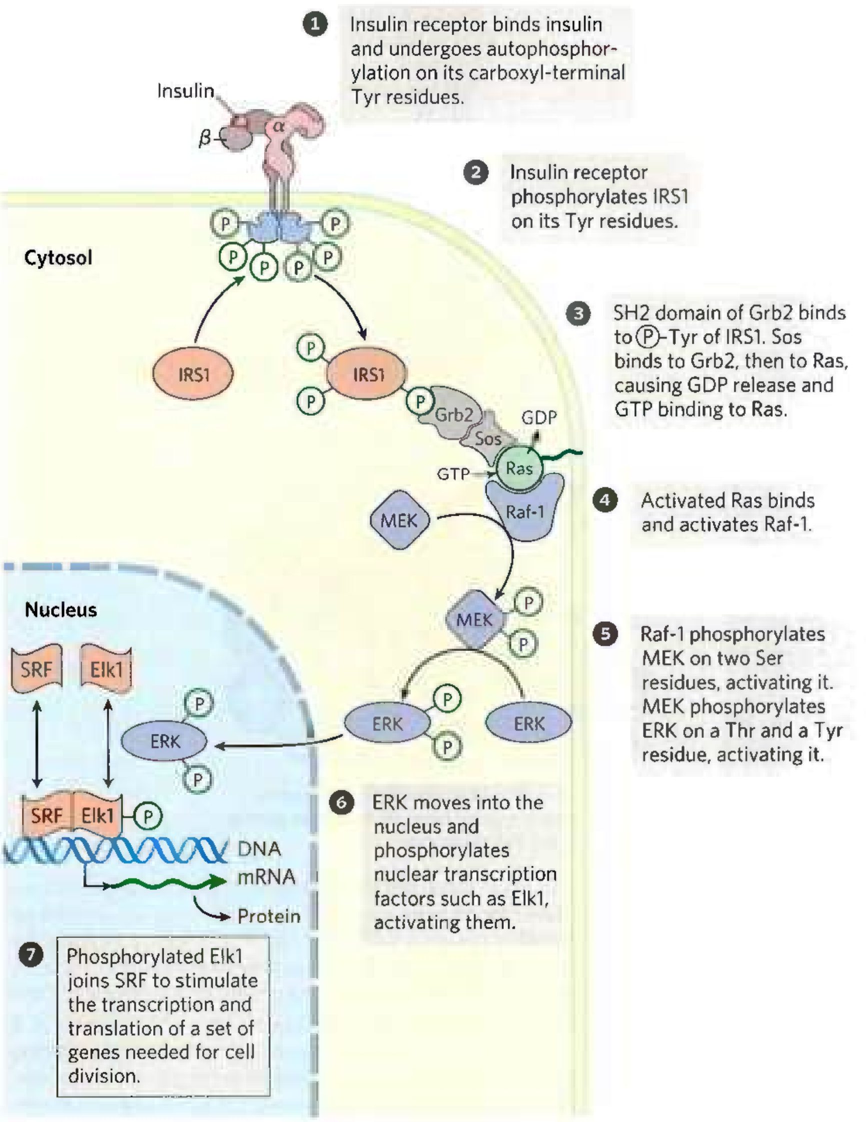
is a Tyr kinase, a protein kinase that phosphorylates specific Tyr residues in target proteins. The receptors for insulin and epidermal growth factor are prototypes for the 58 RTKs in humans (see Fig. 12-26).

Stimulation of the Insulin Receptor Initiates a Cascade of Protein Phosphorylation Reactions

Insulin regulates both metabolic enzymes and gene expression. It initiates a signal that travels divergent pathways from the plasma membrane receptor to insulin-sensitive enzymes in the cytosol, and to enzymes that act in the nucleus by stimulating the transcription of specific genes. The active insulin receptor protein (INSR) consists of two identical α subunits protruding from the outer face of the plasma membrane and two transmembrane β subunits with their carboxyl termini protruding into the cytosol—a dimer of $\alpha\beta$ monomers (Fig. 12-21). The α subunits contain the insulin-binding domain, and the intracellular domains of the β subunits contain the protein kinase activity that transfers a phosphoryl group from ATP to the hydroxyl group of Tyr residues in specific target proteins.

Signaling through INSR begins with the binding of one insulin molecule between the two subunits of the receptor on the extracellular side, causing movement of the Tyr kinase domains together and stimulating their activity. Each β subunit phosphorylates three essential Tyr residues near the carboxyl terminus of the other β subunit. This **autophosphorylation** opens the active site so that the enzyme can phosphorylate Tyr residues of other target proteins. The mechanism of activation of the INSR protein kinase is similar to that described for PKA and PKC: a region of the cytoplasmic domain called the activation loop that usually occludes the active site moves out of the active site after being phosphorylated, opening the site for the binding of target proteins (Fig. 12-21c, d).

When INSR is autophosphorylated (**Fig. 12-22**, step ①) and becomes an active Tyr kinase, one of its targets is the protein **insulin receptor substrate 1 (IRS1;**



① Insulin receptor binds insulin and undergoes autophosphorylation on its carboxyl-terminal Tyr residues.

② Insulin receptor phosphorylates IRS1 on its Tyr residues.

③ SH2 domain of Grb2 binds to Ⓟ-Tyr of IRS1. Sos binds to Grb2, then to Ras, causing GDP release and GTP binding to Ras.

④ Activated Ras binds and activates Raf-1.

⑤ Raf-1 phosphorylates MEK on two Ser residues, activating it. MEK phosphorylates ERK on a Thr and a Tyr residue, activating it.

⑥ ERK moves into the nucleus and phosphorylates nuclear transcription factors such as Elk1, activating them.

⑦ Phosphorylated Elk1 joins SRF to stimulate the transcription and translation of a set of genes needed for cell division.

FIGURE 12-22 Regulation of gene expression by insulin through a MAP kinase cascade. The signaling pathway by which insulin regulates the expression of specific genes consists of a cascade of protein kinases, each of which activates the next. The insulin receptor is a Tyr-specific kinase; the other kinases (all shown in blue) phosphorylate Ser or Thr residues. MEK is a dual-specificity kinase that phosphorylates both a Thr residue and a Tyr residue in ERK (*e*xtracellular *r*egulated *k*inase). MEK is *m*itogen-activated, *E*RK-activating *k*inase; SRF is *s*erum *r*esponse *f*actor.

step ②). Once phosphorylated on several of its Tyr residues, IRS1 becomes the point of nucleation for a complex of proteins (step ③) that carry the message from the insulin receptor to end targets in the cytosol and nucleus, through a long series of intermediate proteins. First, a Ⓟ–Tyr residue of IRS1 binds to the **SH2 domain** of the protein Grb2. Many signaling proteins contain SH2 (*S*rc *h*omology *2*) domains, all of which bind Ⓟ–Tyr residues in a protein partner. Grb2 (*g*rowth factor *r*eceptor-*b*ound protein *2*) is an adaptor protein, with no intrinsic enzymatic activity. Its function is to bring together two proteins (in this case, IRS1 and the protein Sos) that must interact to enable signal transduction. In addition to its SH2 (Ⓟ–Tyr-binding) domain, Grb2 contains a second protein-binding domain, SH3, that binds to a proline-rich region of Sos (*s*on *o*f *s*evenless), recruiting Sos to the growing receptor complex. When bound to Grb2, Sos acts as a guanosine nucleotide–exchange factor (GEF), catalyzing the replacement of bound GDP with GTP on the small G protein Ras.

In its GTP-bound active form, Ras can activate a protein kinase, Raf-1 (Fig. 12-22, step ④), the first of three protein kinases—Raf-1, MEK, and ERK—that form a cascade in which each kinase activates the next by phosphorylation (step ⑤). The protein kinases MEK and ERK are activated by phosphorylation of both a Thr residue and a Tyr residue. When activated, ERK mediates some of the biological effects of insulin by entering the nucleus and phosphorylating transcription factors, including Elk1 (step ⑥), that modulate the transcription of about 100 insulin-regulated genes (step ⑦), some of which encode proteins essential for cell division. Thus, insulin acts as a growth factor.

The proteins Raf-1, MEK, and ERK are members of three larger families, for which several nomenclatures are used. ERK is in the **MAPK** family (*m*itogen-*a*ctivated *p*rotein *k*inases; mitogens are extracellular signals that induce mitosis and cell division). Soon after discovery of the first MAPK enzyme, that enzyme was found to be activated by another protein kinase, which was named MAP kinase kinase (MEK belongs to this family), and when a third kinase that activated MAP kinase kinase was discovered, it was given the slightly ludicrous family name MAP kinase kinase kinase (Raf-1 is in this family). Somewhat less cumbersome are the abbreviations for these three families: MAPK, MAPKK, and MAPKKK. Kinases in the MAPK and MAPKKK families are specific for Ser residues or Thr residues, and MAPKKs (here, MEK) phosphorylate both a Ser residue and a Tyr residue in their substrate, a MAPK (here, ERK).

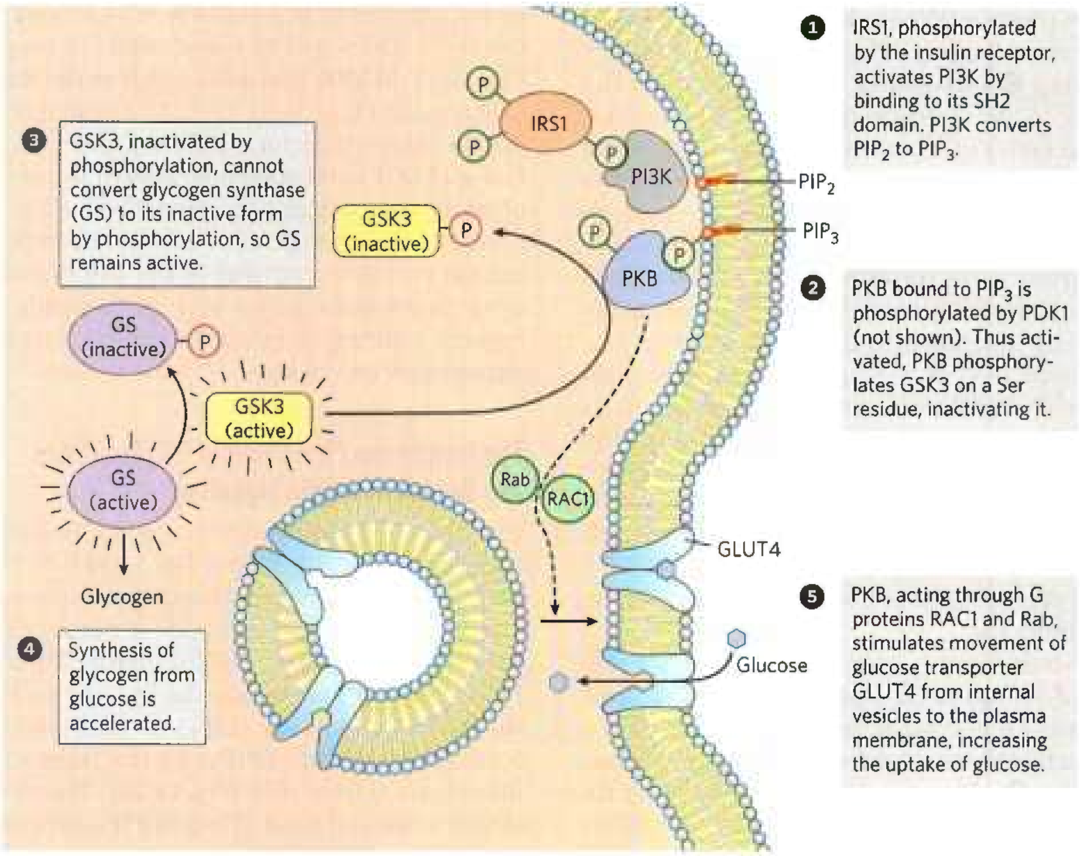
P4 This insulin pathway is but one instance of a more general scheme in which hormone signals, via pathways similar to that shown in Figure 12-22, result in a change in the phosphorylation of target enzymes by protein kinases or phosphoprotein phosphatases. The target of phosphorylation is often another protein kinase, which then phosphorylates a third protein kinase, and so on. The result is a cascade of reactions that amplifies the initial signal by many orders of magnitude (see Fig. 12-7). **MAPK cascades** (such as the Raf-MEK-ERK sequence in Fig. 12-22) mediate signaling initiated by a variety of growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), both of which are receptor tyrosine kinases like the insulin receptor. Another general scheme exemplified by the insulin receptor pathway is the use of nonenzymatic adaptor proteins, often with intrinsically disordered regions, to bring together the components of divergent signaling pathways, to which we now turn.

The Membrane Phospholipid PIP$_3$ Functions at a Branch in Insulin Signaling

The signaling pathway from insulin branches at IRS1 (step ② in Fig. 12-22 and **Fig. 12-23**). Grb2 is not the only protein that associates with phosphorylated IRS1. The enzyme phosphoinositide 3-kinase (PI3K) binds IRS1 through PI3K's SH2 domain. Thus activated, PI3K converts the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) by the transfer of a phosphoryl group from ATP (**Fig. 12-24**). The multiply (negatively) charged head group of PIP$_3$, protruding on the cytoplasmic side of the plasma membrane, is the starting point for a second signaling branch involving another cascade of protein kinases. When bound to PIP$_3$, protein kinase B (PKB; also called Akt) is phosphorylated and activated by yet another protein kinase, PDK1 (not shown in Fig. 12-23). The activated PKB then phosphorylates Ser or Thr residues in its target proteins, one of which is glycogen synthase kinase 3 (GSK3). In its active, nonphosphorylated form, GSK3 phosphorylates glycogen synthase, inactivating it and thereby contributing to the slowing of glycogen synthesis. (This mechanism is only part of the explanation for the effects of insulin on glycogen metabolism; see Fig. 15-16.) When phosphorylated by PKB, GSK3 is inactivated. By thus preventing inactivation of glycogen synthase in liver and muscle, the cascade of protein phosphorylations initiated by insulin ultimately stimulates glycogen synthesis (Fig. 12-23).

In a third signaling branch important in muscle and fat tissue, PKB, acting through two small G proteins (RAC1 and Rab) triggers the clathrin-aided movement of glucose transporters (GLUT4) from intracellular vesicles to the plasma membrane, stimulating glucose uptake from the blood (Fig. 12-23, step ⑤; see also Box 11-1). This increase in glucose uptake triggered by insulin has profound metabolic and medical consequences, as we shall see in Chapter 23.

How fast is the response to insulin? Phosphoproteomics uses high-resolution, high-throughput mass spectrometry to determine, for thousands of proteins in a cell type, which residues in which proteins are


IRS1
P
P
P
PI3K
PIP$_2$
PIP$_3$
GSK3 (inactive)
P
P
P
PKB
GS (inactive)
P
GSK3 (active)
GS (active)
Glycogen
Rab
RAC1
GLUT4
Glucose

① IRS1, phosphorylated by the insulin receptor, activates PI3K by binding to its SH2 domain. PI3K converts PIP$_2$ to PIP$_3$.

② PKB bound to PIP$_3$ is phosphorylated by PDK1 (not shown). Thus activated, PKB phosphorylates GSK3 on a Ser residue, inactivating it.

③ GSK3, inactivated by phosphorylation, cannot convert glycogen synthase (GS) to its inactive form by phosphorylation, so GS remains active.

④ Synthesis of glycogen from glucose is accelerated.

⑤ PKB, acting through G proteins RAC1 and Rab, stimulates movement of glucose transporter GLUT4 from internal vesicles to the plasma membrane, increasing the uptake of glucose.

FIGURE 12-23 Insulin action on glycogen synthesis and GLUT4 movement to the plasma membrane. The activation of PI3 kinase (PI3K) by phosphorylated IRS1 initiates (through protein kinase B, PKB) movement of the glucose transporter GLUT4 to the plasma membrane, and the activation of glycogen synthase.

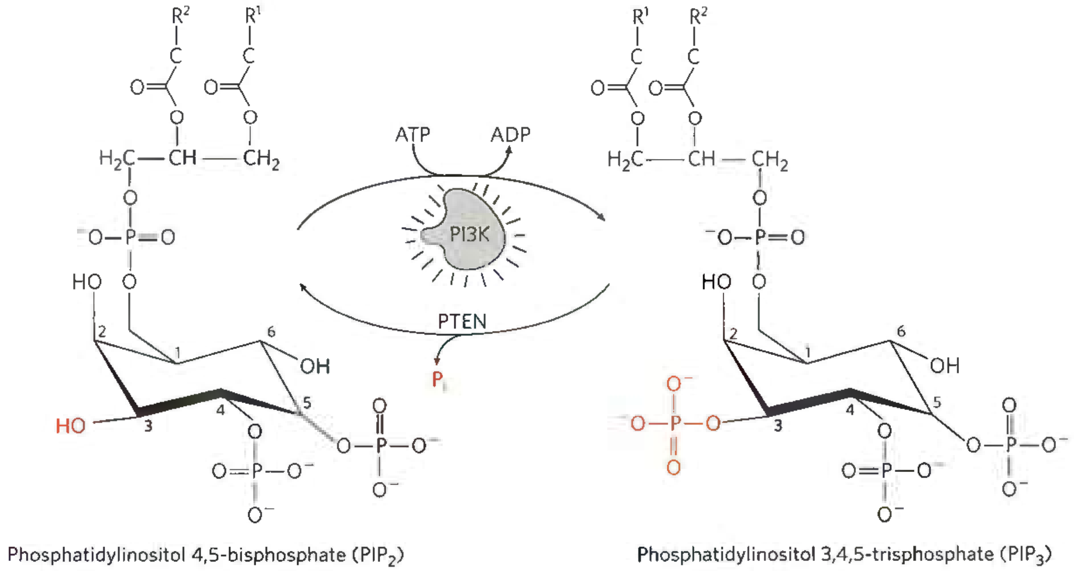

ATP
ADP
PI3K
PTEN
P$_i$
Phosphatidylinositol 4,5-bisphosphate (PIP$_2$)
Phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$)

FIGURE 12-24 Regulation of PIP$_3$ formation and breakdown. Phosphoinositide 3-kinase (PI3K) responds to the insulin signal by catalyzing the transfer of a phosphoryl group from ATP to C-3 of the inositol ring of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to form phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$), which serves as a nucleation point for proteins involved in the MAPK cascade. The enzyme PTEN (phosphatase and *tensin* homolog) ends the response by catalyzing removal of the same phosphoryl group, which is released as P$_i$.

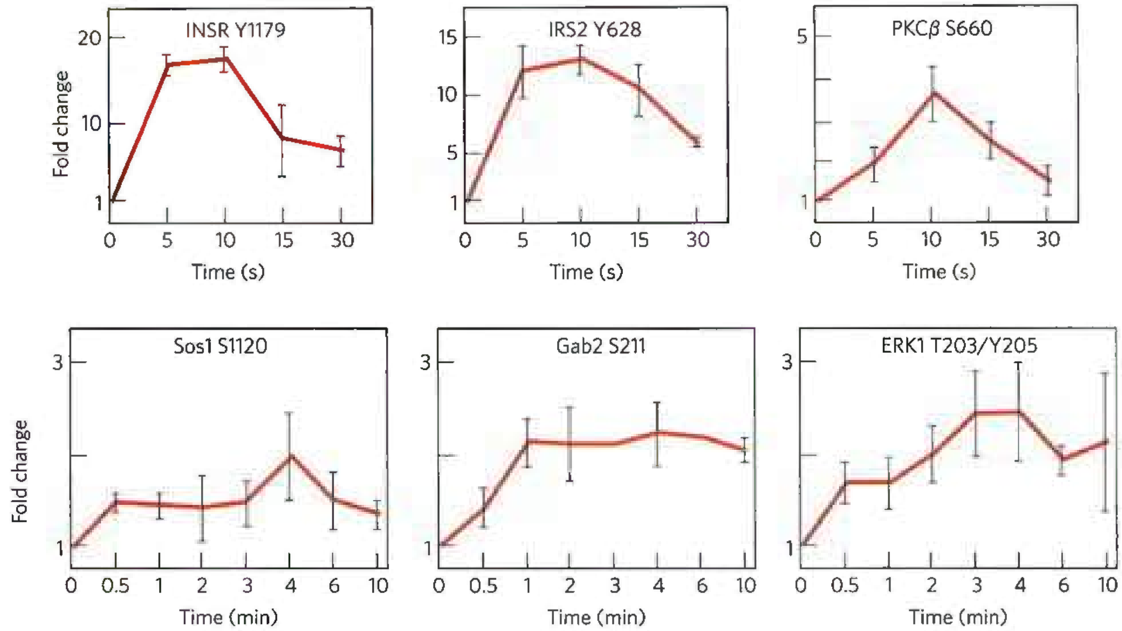


FIGURE 12-25 Time course of phosphorylations triggered by insulin. Mouse liver proteins were examined by mass spectrometry at intervals after injection of insulin to determine quantitatively for each protein which amino acid residues became phosphorylated and when phosphorylation occurred. Each graph represents the phosphorylation of a single residue in the protein, designated by its one-letter abbreviation and position in the primary sequence. Proteins are INSR, insulin receptor; IRS2, insulin receptor substrate 2; PKCβ, protein kinase C_β; Sos and ERK, as in Fig. 12-22. Gab2 is an adaptor protein involved in the MAPK and PI3K signaling pathways. [Data from S. J. Humphrey et al., *Nature Biotechnol.* 33:990, 2015, Fig. 4.]

phosphorylated in response to a stimulus such as insulin in the living animal (**Fig. 12-25**). Autophosphorylation of the insulin receptor and IRS1 occurs within a few seconds of insulin addition; protein kinase C_β is phosphorylated slightly later (within 15 s), and Sos and Gab are phosphorylated within 0.5 and 1 min. ERK1, the end target in this signaling pathway (Fig. 12-22), is maximally phosphorylated within 3 min. The movement of glucose transporter GLUT4 into the plasma membrane takes about 15 min; and the many changes in gene expression in response to insulin occur over several hours. A recently discovered action of insulin has a different mechanism and a slower time course. In some cases, insulin enters the cell and the nucleus, where, with the help of several other nuclear proteins, it regulates gene expression by binding to promoter regions on the DNA.

As in all signaling pathways, there is a mechanism for terminating the activity of the PI3K-PKB pathway. A PIP_3-specific phosphatase (in humans, PTEN) removes the phosphoryl group at the 3 position of PIP_3 to produce PIP_2 (see Fig. 12-24), which no longer serves as a binding site for PKB, and the signaling chain is broken. In several types of cancer, the *PTEN* gene has often undergone mutation, resulting in a defective regulatory circuit and abnormally high levels of PIP_3 activity. The result is a continuing signal for cell division and thus tumor growth. The unmutated *PTEN* gene is a tumor suppressor, the subject of Section 12.9. ■

P4 The insulin receptor is the prototype for several receptor enzymes with a similar structure and RTK activity (**Fig. 12-26**). The receptors for EGF and PDGF, for example, have structural and sequence similarities to INSR, and both have a protein Tyr kinase activity that phosphorylates IRS1. Many of these receptors dimerize after binding ligand and before autophosphorylation. INSR is the exception, as it is already an $(\alpha\beta)_2$ dimer before insulin binds. (The protomer of the insulin receptor is one $\alpha\beta$ unit.) The binding of adaptor proteins such as Grb2 to Ⓟ–Tyr residues is a common mechanism for promoting protein-protein interactions initiated by RTKs, a subject to which we return in Section 12.5.

P2 What spurred the evolution of such complicated regulatory machinery? This system allows one activated receptor to activate several IRS1 molecules, amplifying the insulin signal, and it provides for the integration of signals from different receptors such as EGFR and PDGFR, each of which can phosphorylate IRS1. Furthermore, because IRS1 can activate any of several proteins that contain SH2 domains, a single receptor acting through IRS1 can trigger two or more signaling pathways; insulin affects gene expression through the mitogenic Grb2-Sos-Ras-MAPK pathway and affects glycogen metabolism and glucose transport through the metabolic PI3K-PKB pathway. Finally, there are several closely related IRS proteins (IRS2, IRS3), each with its own characteristic tissue distribution and function, further enriching the signaling possibilities in pathways initiated by RTKs.

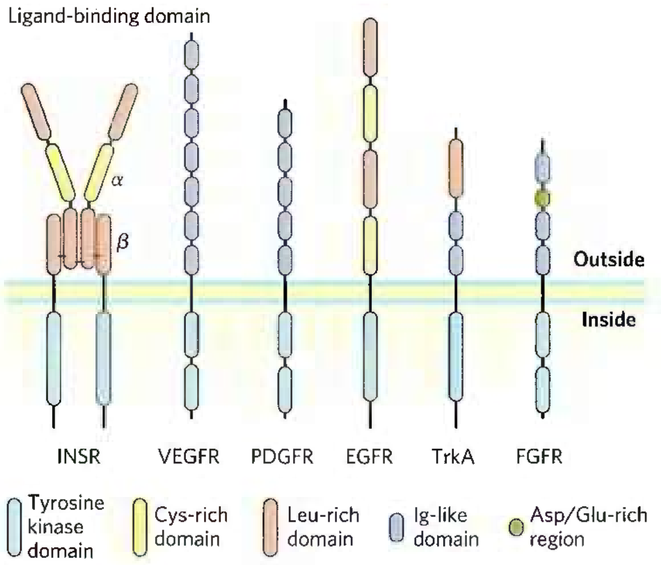


FIGURE 12-26 Receptor tyrosine kinases. Growth factor receptors that initiate signals through Tyr kinase activity include those for insulin (INSR), vascular endothelial growth factor (VEGFR), platelet-derived growth factor (PDGFR), epidermal growth factor (EGFR), high-affinity nerve growth factor (TrkA), and fibroblast growth factor (FGFR). All these receptors have a Tyr kinase domain (blue) on the cytoplasmic side of the plasma membrane. The extracellular domain is unique to each type of receptor, reflecting the different growth factor specificities. These extracellular domains are typically combinations of structural motifs such as Cys- or Leu-rich segments and segments containing one of several motifs common to immunoglobulins (Ig). Many other Tyr kinase receptors are encoded in the human genome, each with a different extracellular domain and ligand specificity. All of the receptors except INSR are monomeric and their Tyr kinase activity is silent until ligand binding triggers receptor dimerization and Tyr kinase activation. Only INSR is always dimeric, but its Tyr kinase is active only when insulin is bound.

Cross Talk among Signaling Systems Is Common and Complex

For simplicity, we have treated individual signaling pathways as separate sequences of events leading to separate metabolic consequences, but there is, in fact, extensive cross talk among signaling systems. The regulatory circuitry that governs metabolism is richly interwoven and multilayered. We have discussed the signaling pathways for insulin and epinephrine separately, but they do not operate independently. Insulin opposes the metabolic effects of epinephrine in most tissues, and activation of the insulin signaling pathway directly reduces signaling through the β-adrenergic signaling system. For example, the INSR kinase directly phosphorylates two Tyr residues in the cytoplasmic tail of a β_2-adrenergic receptor, and PKB, activated by insulin, phosphorylates two Ser residues in the same region (**Fig. 12-27**). Phosphorylation of these four residues triggers clathrin-aided internalization of the β_2-adrenergic receptor, lowering the cell's sensitivity to epinephrine.

A second type of cross talk between these receptors occurs when Ⓟ–Tyr residues on the β_2-adrenergic receptor, phosphorylated by INSR, serve as nucleation points for SH2 domain–containing proteins such as Grb2 (Fig. 12-27, left side). Activation of the MAPK ERK by insulin is 5- to 10-fold greater in the presence of the β_2-adrenergic receptor, presumably because of this cross talk. Signaling systems that use cAMP and Ca^{2+} also show extensive interaction; each second messenger affects the generation and concentration of the other. Yet another factor that further complicates the signaling picture is that certain metabolic intermediates, such as fatty acids and ceramides, amino acids, and bile acids, can influence insulin signaling. One of the major challenges of systems biology is to sort out the effects of such interactions on the overall metabolic patterns in each tissue—a daunting task.

SUMMARY 12.4 *Receptor Tyrosine Kinases*

- The insulin receptor, INSR, is the prototype of receptor enzymes with Tyr kinase activity. When insulin binds, the receptor Tyr kinase is activated, and phosphorylates Tyr residues on other proteins, such as IRS. Phosphotyrosine

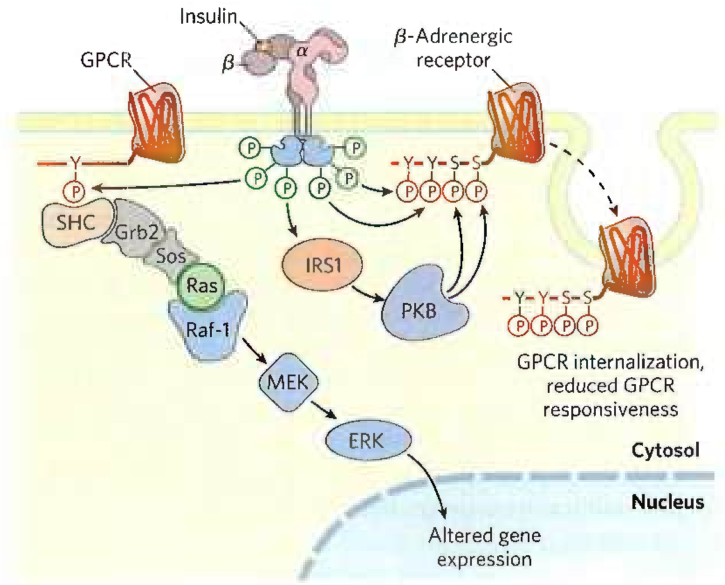


FIGURE 12-27 Cross talk between the insulin receptor and the β-adrenergic receptor (or other GPCR). When INSR is activated by insulin binding, its Tyr kinase directly phosphorylates the β-adrenergic receptor (right side) on two Tyr residues (Tyr^{350} and Tyr^{364}) near its carboxyl terminus, and indirectly causes phosphorylation of two Ser residues in the same region (through activation of protein kinase B (PKB). The effect of these phosphorylations is internalization of the adrenergic receptor, reducing the response to the adrenergic stimulus. Alternatively (left side), INSR-catalyzed phosphorylation of a GPCR (an adrenergic or other receptor) on a carboxyl-terminal Tyr creates the point of nucleation for activating the MAPK cascade (see Fig. 12-22), with Grb2 serving as the adaptor protein. In this case, INSR has used the GPCR to enhance its own signaling.

residues of IRS1 serve as binding sites for proteins with SH2 domains. These multivalent proteins can serve as adaptors that bring two proteins into proximity. Sos bound to Grb2 activates Ras, which in turn activates a MAPK cascade that ends with the phosphorylation of target proteins in the cytosol and nucleus. The result is specific metabolic changes and altered gene expression.

- The enzyme PI3K, activated by interaction with IRS1, phosphorylates the membrane lipid PIP_2 to PIP_3, which becomes the point of nucleation for proteins in a second and third branch of insulin signaling.
- Extensive interconnections among signaling pathways allow integration and fine-tuning of multiple hormonal effects.

12.5 Multivalent Adaptor Proteins and Membrane Rafts

Two generalizations have emerged from studies of signaling systems such as those we have discussed so far. First, protein kinases that phosphorylate Tyr, Ser, and Thr residues — and phosphatases that dephosphorylate them — are central to signaling, *directly* affecting the activities of a large number of protein substrates. **P4** Second, protein-protein interactions brought about by the reversible phosphorylation of Tyr, Ser, and Thr residues in signaling proteins create *docking sites* for other proteins that bring about *indirect* effects on proteins downstream in the signaling pathway. In fact, many signaling proteins are *multivalent*: they can interact with several different proteins simultaneously to form multiprotein signaling complexes. In this section we present a few examples to illustrate the general principles of phosphorylation-dependent protein interactions in signaling pathways.

Many of the proteins involved in signaling have intrinsically disordered regions (IDRs) that are flexible enough to allow specific interaction with more than one protein — and perhaps with many other proteins. Protein kinases, for example, have a well-conserved structure containing the substrate-binding and catalytic sites, but through evolution they have acquired additional sequences, typically 20–30 residues long, that are partially disordered and can fold to fit into various multienzyme regulatory networks. The activation loop found in most protein kinases is an IDR and is the universal regulator of kinase activity; its position changes when one or several residues become phosphorylated, switching on the kinase activity. Protein kinases of the PKA, PKB, and PKC families have a disordered carboxyl-terminal tail containing critical residues whose phosphorylation state switches between active and inactive kinase structures. In the MAPK cascade, an amino-terminal IDR plays a similar role as a docking site in the multienzyme complex. Given the ~1,000 protein kinase genes in humans, the many noncatalytic scaffold proteins, and the multiple interactions possible for IDRs in many of these proteins, the number of possible permutations and combinations available for switching and regulating metabolic processes by protein phosphorylation is impressively large.

Protein Modules Bind Phosphorylated Tyr, Ser, or Thr Residues in Partner Proteins

The protein Grb2 in the insulin signaling pathway (Figs. 12-22 and 12-27) binds through its SH2 domain to other proteins that have exposed Ⓟ–Tyr residues. The human genome encodes at least 87 SH2-containing proteins, many known to participate in signaling. The Ⓟ–Tyr residue is bound in a deep pocket in an SH2 domain, with each of its phosphate oxygens participating in hydrogen bonding or electrostatic interactions; the positive charges on two Arg residues figure prominently in the binding. Subtle differences in the structure of SH2 domains account for the specificities of the interactions of SH2-containing proteins with various Ⓟ–Tyr-containing proteins. The SH2 domain typically interacts with a Ⓟ–Tyr (which is assigned the index position 0) and the next three residues toward the carboxyl terminus (designated +1, +2, +3). Some proteins with SH2 domains (Src, Fyn, Hck, Nck) favor negatively charged residues in the +1 and +2 positions; others (PLCγ1, SHP2) have a long hydrophobic groove that binds to aliphatic residues in positions +1 to +5. These differences define subclasses of SH2 domains that have different partner specificities.

Phosphotyrosine-binding domains, or **PTB domains**, are another binding partner for Ⓟ–Tyr proteins (**Fig. 12-28**), but their critical sequences and three-dimensional

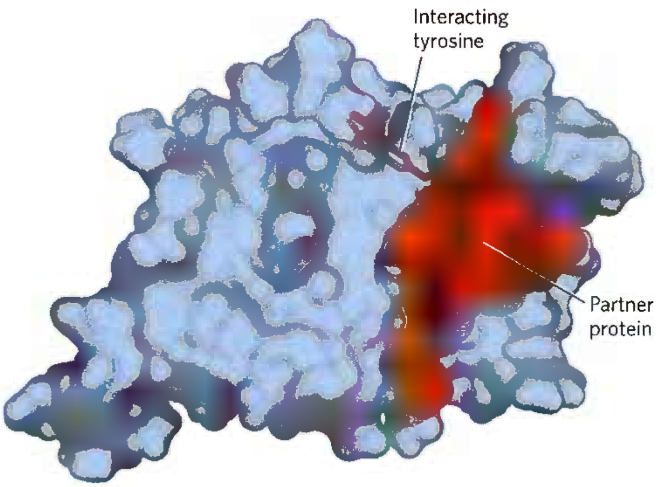


FIGURE 12-28 Interaction of a PTB domain with Ⓟ–Tyr residue in a partner protein. The PTB domain is represented as a blue surface contour. The partner protein is held to the kinase by multiple noncovalent interactions, which confer specificity on the interaction and position the Ⓟ–Tyr residue in a binding pocket at the enzyme's active site. [Data from PDB ID 1SHC, M. M. Zhou et al., *Nature* 378:584, 1995.]

structure distinguish them from SH2 domains. The human genome encodes at least 24 proteins that contain PTB domains, including IRS1, which we have already encountered in its role as an adaptor protein in insulin-signal transduction (Fig. 12-23). The Ⓟ–Tyr-binding sites for SH2 and PTB domains on partner proteins are created by Tyr kinases and eliminated by protein tyrosine phosphatases (PTPs).

As we have seen, other signaling protein kinases, including PKA, PKC, PKG, and members of the MAPK cascade, phosphorylate Ser or Thr residues in their target proteins. In some cases, these proteins acquire the ability to interact with partner proteins through the phosphorylated residue, triggering a downstream process. An alphabet soup of domains that bind Ⓟ–Ser or Ⓟ–Thr residues has been identified, and more are sure to be found. Each domain favors a certain sequence around the phosphorylated residue, so proteins with that domain bind to and interact with a specific subset of phosphorylated proteins.

In some cases, the region on a protein that binds Ⓟ–Tyr of a substrate protein is masked by the region's interaction with a Ⓟ–Tyr in the same protein. For example, the soluble protein Tyr kinase Src, when phosphorylated on a specific Tyr residue, is rendered inactive; an SH2 domain needed to bind the substrate protein instead binds the internal Ⓟ–Tyr. Removal of this Ⓟ–Tyr residue by a phosphoprotein phosphatase turns on the Tyr kinase activity of Src (**Fig. 12-29a**). Similarly, glycogen synthase kinase 3 (GSK3) is inactive when phosphorylated on a Ser residue in its autoinhibitory domain (Fig. 12-29b). Dephosphorylation of that domain frees the enzyme to bind (and then phosphorylate) its target proteins.

In addition to the three commonly phosphorylated residues in proteins (Tyr, Ser, Thr), there is a fourth phosphorylated structure that nucleates the formation of supramolecular complexes of signaling proteins: the phosphorylated head group of the membrane phosphatidylinositols. Many signaling proteins contain domains such as SH3 and PH (pleckstrin homology) that bind tightly to PIP_3 protruding on the cytoplasmic side of the plasma membrane. Wherever the enzyme PI3K creates this head group (as it does in response to the insulin signal), proteins that bind PIP_3 will cluster at the membrane surface.

Most of the proteins involved in signaling at the plasma membrane have one or more phosphoprotein- or phospholipid-binding domain; many have three or more, and thus are multivalent in their interactions with other signaling proteins. **Figure 12-30** shows just a few of the multivalent proteins known to participate in signaling. Many of the complexes include components with membrane-binding domains. Given the location of so many signaling processes at the inner surface of the plasma membrane, the molecules that must collide to produce the signaling response are effectively confined to two-dimensional space—the membrane surface; collisions are far more likely here than in the three-dimensional space of the cytosol.

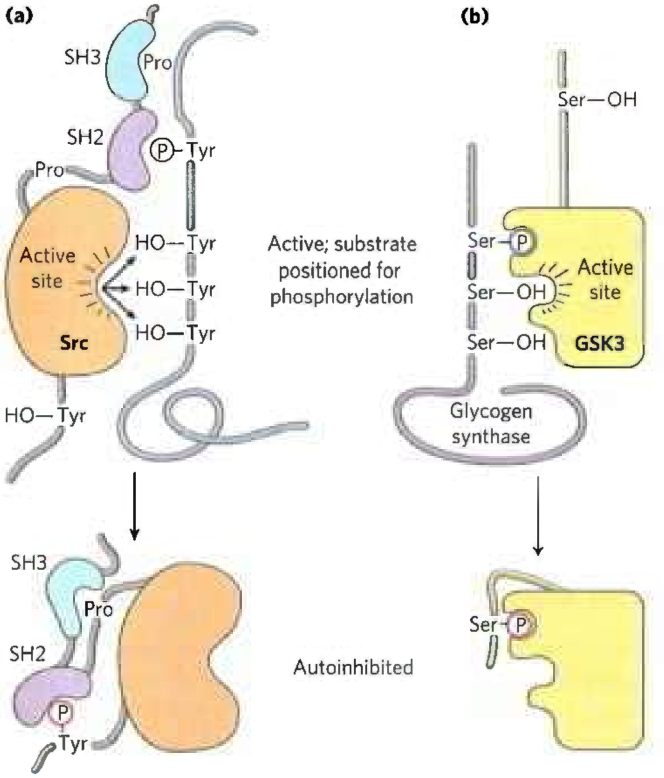


FIGURE 12-29 Mechanism of autoinhibition of Src and GSK3. (a) In the active form of the Tyr kinase Src, an SH2 domain binds a Ⓟ–Tyr in the protein substrate, and an SH3 domain binds a proline-rich region of the substrate, thus lining up the active site of the kinase with several target Tyr residues in the substrate (top). When Src is phosphorylated on a specific Tyr residue (bottom), the SH2 domain binds the internal Ⓟ–Tyr instead of the Ⓟ–Tyr of the substrate, and the SH3 domain binds an internal proline-rich region, preventing productive enzyme-substrate binding; the enzyme is thus autoinhibited. (b) In the active form of glycogen synthase kinase 3 (GSK3), an internal Ⓟ–Ser-binding domain is available to bind Ⓟ–Ser in its substrate (glycogen synthase) and to position the kinase to phosphorylate neighboring Ser residues (top). Phosphorylation of an internal Ser residue allows this internal kinase segment to occupy the Ⓟ–Ser-binding site, blocking substrate binding (bottom).

In summary, a remarkable picture of signaling pathways has emerged from studies of many signaling proteins and their multiple binding domains. P5 An initial signal results in phosphorylation of the receptor or a target protein, triggering the assembly of large multiprotein complexes, held together on scaffolds with multivalent binding capacities. Some of these complexes contain several protein kinases that activate each other in turn, producing a cascade of phosphorylation and a great amplification of the initial signal. The interactions between cascade kinases are not left to the vagaries of random collisions in three-dimensional space. In the MAPK cascade, for example, a scaffold protein, KSR, binds all three kinases (MAPK, MAPKK, and MAPKKK), ensuring their proximity and correct orientation and even conferring allosteric properties on the interactions among the kinases, which makes their serial phosphorylation sensitive to very small stimuli (**Fig. 12-31**).

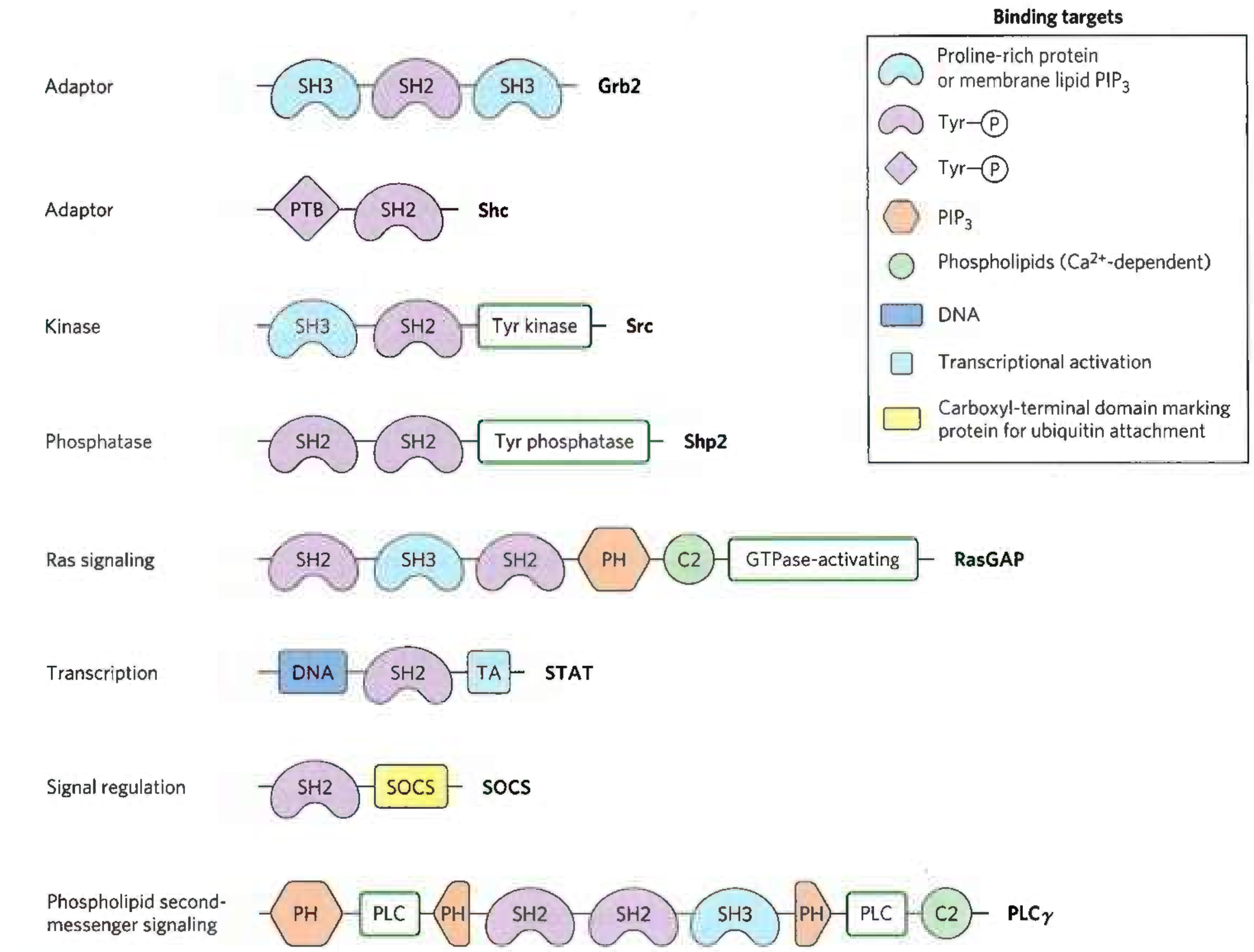

Binding targets
Proline-rich protein or membrane lipid PIP$_3$
Tyr–Ⓟ
Tyr–Ⓟ
PIP$_3$
Phospholipids (Ca^{2+}-dependent)
DNA
Transcriptional activation
Carboxyl-terminal domain marking protein for ubiquitin attachment
Adaptor
SH3 SH2 SH3
Grb2
Adaptor
PTB SH2
Shc
Kinase
SH3 SH2 Tyr kinase
Src
Phosphatase
SH2 SH2 Tyr phosphatase
Shp2
Ras signaling
SH2 SH3 SH2 PH C2 GTPase-activating
RasGAP
Transcription
DNA SH2 TA
STAT
Signal regulation
SH2 SOCS
SOCS
Phospholipid second-messenger signaling
PH PLC PH SH2 SH2 SH3 PH PLC C2
PLCγ

FIGURE 12-30 Some binding modules of signaling proteins. These signaling proteins interact with phosphorylated proteins or phospholipids in many permutations and combinations to form integrated signaling complexes. Each protein is represented by a line (with the amino terminus to the left); symbols indicate the location of conserved binding domains (with specificities as listed in the key; abbreviations are explained in the text); green boxes indicate catalytic activities. The name of each protein is given at its carboxyl-terminal end. [Information from T. Pawson et al., *Trends Cell Biol.* 11:504, 2001, Fig. 5.]

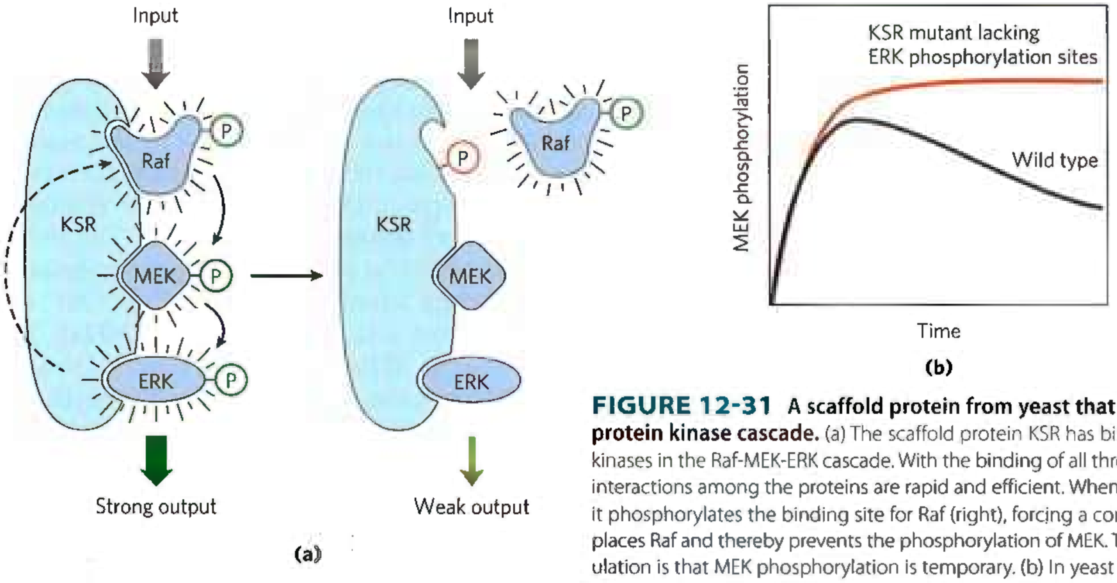

Input
Raf
P
KSR
MEK
P
ERK
P
Strong output
Input
P
Raf
P
KSR
MEK
ERK
Weak output
(a)
KSR mutant lacking ERK phosphorylation sites
Wild type
MEK phosphorylation
Time
(b)

FIGURE 12-31 A scaffold protein from yeast that organizes and regulates a protein kinase cascade. (a) The scaffold protein KSR has binding sites for all three of the kinases in the Raf-MEK-ERK cascade. With the binding of all three in appropriate orientations, interactions among the proteins are rapid and efficient. When ERK has been activated (left), it phosphorylates the binding site for Raf (right), forcing a conformational change that displaces Raf and thereby prevents the phosphorylation of MEK. The result of this feedback regulation is that MEK phosphorylation is temporary. (b) In yeast cells with mutant KSR lacking the phosphorylation sites (red curve), no feedback occurs, producing a different time course of signaling. [Information from M. C. Good et al., *Science* 332:680, 2011, Fig. 2E.]

Phosphotyrosine phosphatases remove the phosphate from Ⓟ–Tyr residues, reversing the effect of phosphorylation. At least 37 genes in the human genome encode protein tyrosine phosphatases (PTPs). About half of these are receptorlike integral proteins with a single transmembrane domain; they are presumably controlled by extracellular factors not yet identified. Other PTPs are soluble and contain SH2 domains that determine their molecular partners and intracellular location. In addition, animal cells have protein Ⓟ–Ser and Ⓟ–Thr phosphatases such as PP1 that reverse the effects of Ser- and Thr-specific protein kinases.

We can see, then, that signaling occurs in protein circuits, which are effectively hardwired from signal receptor to response effector and can be switched off instantly by the hydrolysis of a single upstream phosphate ester bond. In these circuits, protein kinases are the writers, domains such as SH2 are the readers, and PTPs and other phosphatases are the erasers. The multivalency of signaling proteins allows the assembly of many different combinations of Lego-like signaling modules, each combination suited to particular signals, cell types, and metabolic circumstances, yielding diverse signaling circuits of extraordinary complexity.

Membrane Rafts and Caveolae Segregate Signaling Proteins

Membrane rafts (Chapter 11) are regions of the membrane bilayer enriched in sphingolipids, sterols, and certain proteins, including many proteins attached to the bilayer by GPI (*g*lycosylated derivatives of *p*hosphatidyl*i*nositol) anchors. The β-adrenergic receptor is segregated in rafts that also contain G proteins, adenylyl cyclase, PKA, and the protein phosphatase PP2, which together provide a highly integrated signaling unit. By segregating in a small region of the plasma membrane all of the elements required for responding to and ending the signal, the cell is able to produce a highly localized and brief "puff" of second messenger.

Some RTKs (EGFR and PDGFR) are also localized in rafts, and this sequestration very likely has functional significance. In isolated fibroblasts, EGFR is usually concentrated in specialized rafts called caveolae (see Fig. 11-23). When the cells are treated with EGF, the receptor leaves the raft, separating it from the other components of the EGF signaling pathway. This migration depends on the receptor's protein kinase activity; mutant receptors lacking this activity remain in the raft during treatment with EGF. Such experiments suggest that spatial segregation of signaling proteins in rafts is yet another dimension of the already complex processes initiated by extracellular signals.

SUMMARY 12.5 *Multivalent Adaptor Proteins and Membrane Rafts*

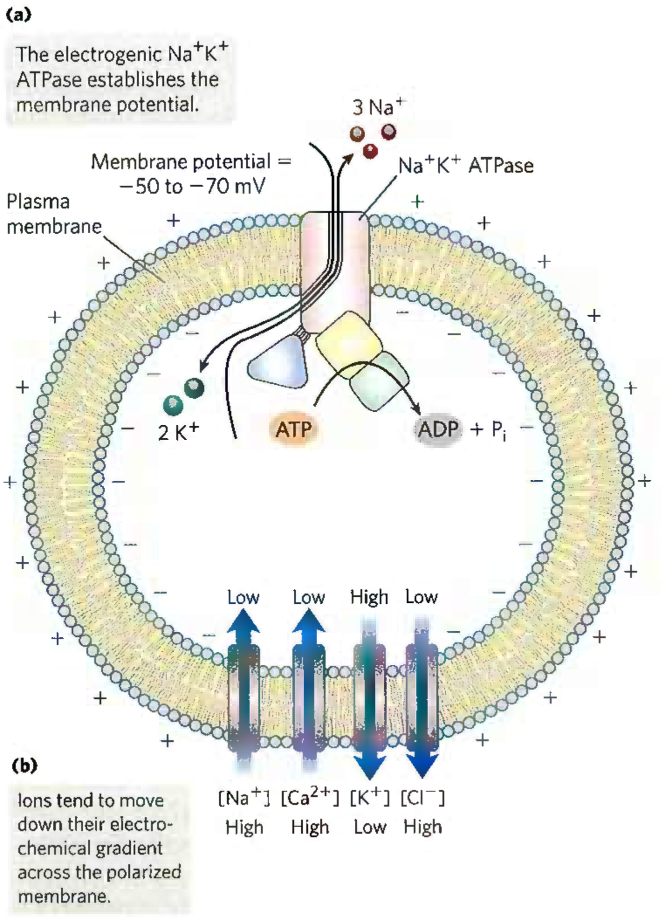
- Many signaling proteins have domains that bind phosphorylated Tyr, Ser, or Thr residues in other proteins; the binding specificity for each domain is determined by sequences that adjoin the phosphorylated residue in the substrate. SH2 and PTB domains bind to proteins containing Ⓟ–Tyr residues; other domains bind Ⓟ–Ser and Ⓟ–Thr residues in various contexts. SH3 and PH domains bind the membrane phospholipid PIP_3.
- Many signaling proteins are multivalent, with several different binding modules. By combining the substrate specificities of various protein kinases with the specificities of domains that bind phosphorylated Tyr, Ser, or Thr residues, and with phosphatases that can rapidly inactivate a signaling pathway, cells create a large number of multiprotein signaling complexes.
- Membrane rafts and caveolae sequester groups of signaling proteins in small regions of the plasma membrane, effectively raising their local concentrations and making signaling more efficient.

12.6 Gated Ion Channels

Certain cells in multicellular organisms are "excitable": they can detect an external signal, convert it into an electrical signal (specifically, a change in plasma membrane potential), and pass it on. Changes in membrane potential are effected by gated ion channels. Excitable cells play central roles in nerve conduction, muscle contraction, hormone secretion, sensory processes, and learning and memory.

Ion Channels Underlie Rapid Electrical Signaling in Excitable Cells

P6 The excitability of sensory cells, neurons, and myocytes depends on ion channels, signal transducers that provide a regulated path for the movement of inorganic ions such as Na^+, K^+, Ca^{2+}, and Cl^- across the plasma membrane in response to various stimuli. Recall from Chapter 11 that these ion channels are gated: they may be open or closed, depending on whether the associated receptor has been activated by the binding of its specific ligand (a neurotransmitter, for example) or by a change in the transmembrane electrical potential (**voltage-gated ion channels**). The Na^+K^+ ATPase is electrogenic; it creates a charge imbalance across the plasma membrane by carrying 3 Na^+ out of the cell for every 2 K^+ carried in (**Fig. 12-32a**). The action of the Na^+K^+ ATPase makes the inside of the cell negative relative to the outside. Inside the cell, $[K^+]$ is much higher and $[Na^+]$ is much lower than outside the cell (Fig. 12-32b). The direction of spontaneous ion flow across a polarized membrane is dictated by the electrochemical potential of that ion across the membrane, which has two components: the difference in concentration of the ion on the two sides of the membrane, and the difference in electrical potential (V_m),



The electrogenic Na$^+$K$^+$ ATPase establishes the membrane potential.

Ions tend to move down their electrochemical gradient across the polarized membrane.

FIGURE 12-32 Transmembrane electrical potential. (a) The electrogenic Na$^+$K$^+$ ATPase produces a transmembrane electrical potential of about –60 mV (inside negative). (b) Blue arrows show the direction in which ions tend to move spontaneously across the plasma membrane in an animal cell, driven by the combination of chemical and electrical gradients. The chemical gradient drives Na$^+$ and Ca^{2+} inward (producing depolarization) and K$^+$ outward, against its electrical gradient (producing hyperpolarization). The electrical gradient drives Cl$^-$ outward, against its concentration gradient (producing depolarization).

typically expressed in millivolts (see Eqn 11-4, p. 392). Given the ion concentration differences and a V_m of about –60 mV (inside negative), opening of a Na$^+$ or Ca^{2+} channel will result in a spontaneous inward flow of Na$^+$ or Ca^{2+} (and depolarization), whereas opening of a K$^+$ channel will result in a spontaneous outward flow of K$^+$ (and hyperpolarization) (Fig. 12-32b). In this case, K$^+$ moves out of the cell against the electrical gradient, because the large concentration difference exerts a stronger effect than the V_m. For Cl$^-$, the membrane potential predominates, so when a Cl$^-$ channel opens, Cl$^-$ flows outward.

The number of ions that must flow to produce a physiologically significant change in the membrane potential is negligible relative to the concentrations of Na$^+$, K$^+$, and Cl$^-$ in cells and extracellular fluid, so the ion fluxes that occur during signaling in excitable cells have essentially no effect on the concentrations of these ions. With Ca^{2+}, the situation is different; because the intracellular [Ca^{2+}] is generally very low (~10^{-7} M), inward flow of Ca^{2+} can significantly alter the cytosolic [Ca^{2+}], allowing it to serve as a second messenger.

The membrane potential of a cell at a given time is the result of the types and numbers of ion channels open at that instant. P6 The precisely timed opening and closing of ion channels and the resulting transient changes in membrane potential underlie the electrical signaling by which the nervous system stimulates the skeletal muscles to contract, the heart to beat, or secretory cells to release their contents. Moreover, many hormones exert their effects by altering the membrane potential of their target cells. These mechanisms are not limited to animals; ion channels play important roles in the responses of bacteria, protists, and plants to environmental signals.

To illustrate the action of ion channels in cell-to-cell signaling, we describe the mechanisms by which a neuron passes a signal along its length and across a synapse to the next neuron (or to a myocyte) in a cellular circuit, using acetylcholine as the neurotransmitter.

Voltage-Gated Ion Channels Produce Neuronal Action Potentials

Signaling in the nervous system is accomplished by networks of neurons, specialized cells that carry an electrical impulse (action potential) from one end of the cell (the cell body) through an elongated cytoplasmic extension (the axon), to the synapse with the next neuron. The electrical signal triggers release of the neurotransmitter acetylcholine into the synaptic cleft, carrying the signal to the next cell (neuron) in the circuit. Initially, the plasma membrane of the presynaptic neuron is polarized (inside negative) through the action of the electrogenic Na$^+$K$^+$ ATPase, which pumps out three Na$^+$ for every two K$^+$ pumped in (Fig. 12-32). A rapid sequence of opening and closing of several types of ion channels (**Fig. 12-33**) produces a wave of depolarization (an **action potential**) that sweeps the neuron from the cell body to the end of an axon. First, opening of a voltage-gated Na$^+$ channel allows Na$^+$ entry, and the resulting local depolarization causes the adjacent Na$^+$ channel to open, and so on (Fig. 12-33, step ①). The directionality of movement of the action potential is ensured by the brief refractory period that follows the opening of each voltage-gated Na$^+$ channel. A split second after the action potential passes a point in the axon, voltage-gated K$^+$ channels open (step ②), allowing K$^+$ exit, which brings about repolarization of the membrane to make it ready for the next action potential (step ③).

When the wave of depolarization reaches the axon tip, voltage-gated Ca^{2+} channels open, allowing Ca^{2+} entry (step ④). The resulting increase in internal [Ca^{2+}] triggers exocytotic release of the neurotransmitter acetylcholine into the synaptic cleft (step ⑤). Acetylcholine binds

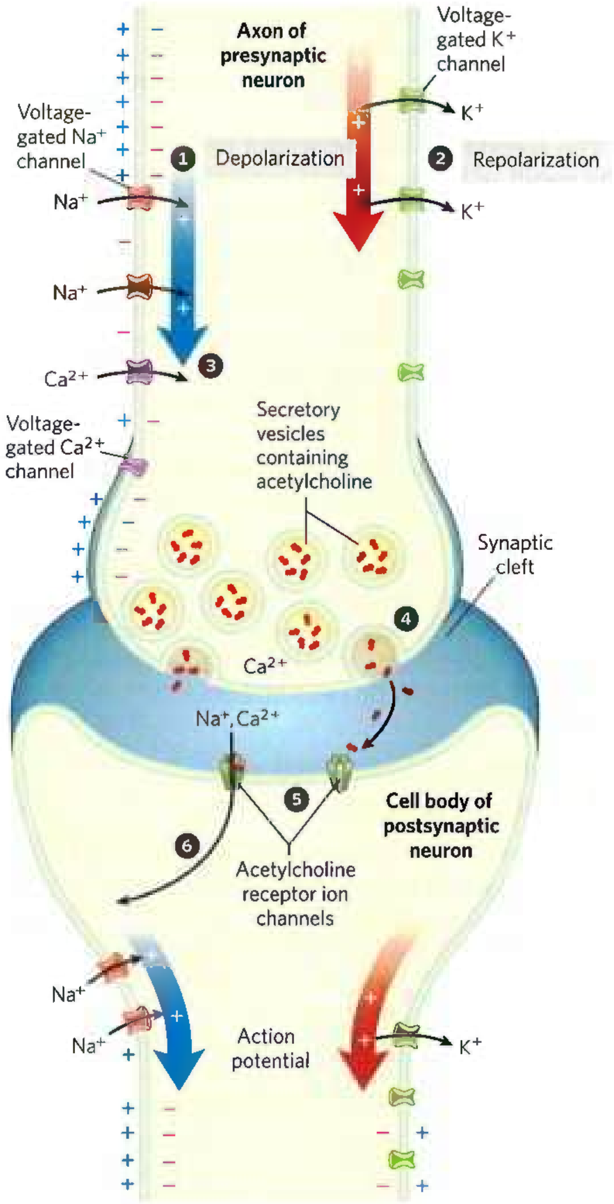

Axon of presynaptic neuron
Voltage-gated K⁺ channel
K⁺
Voltage-gated Na⁺ channel
1 Depolarization
2 Repolarization
Na⁺
K⁺
Na⁺
Ca²⁺
3
Voltage-gated Ca²⁺ channel
Secretory vesicles containing acetylcholine
Synaptic cleft
4
Ca²⁺
Na⁺, Ca²⁺
5
Cell body of postsynaptic neuron
6
Acetylcholine receptor ion channels
Na⁺
Na⁺
Action potential
K⁺
+
−

FIGURE 12-33 Role of voltage-gated and ligand-gated ion channels in neural transmission. The steps are described in the text. Stimulation of the presynaptic neuron (top) triggers a wave of depolarization (blue arrow) followed by repolarization (red arrow), producing an action potential that sweeps along the axon. At the synapse, depolarization opens Ca^{2+} channels, and Ca^{2+} entry triggers acetylcholine release into the synaptic cleft. Acetylcholine diffuses across the cleft, opening receptor channels, depolarizing the postsynaptic cell, and starting an action potential in the postsynaptic cell. Note that, for clarity, Na^+ channels and K^+ channels are drawn on opposite sides of the axon, but both types are uniformly distributed in the axonal membrane. Also, positive and negative charges are shown only on the left, but as the wave of potential sweeps the axon, the membrane potential is the same at any given point along the axon.

to a receptor on the postsynaptic neuron or myocyte, causing its ligand-gated ion channel to open (step ❻). Extracellular Na^+ and Ca^{2+} enter through this channel, depolarizing the postsynaptic cell. The electrical signal has thus passed to the cell body of the postsynaptic neuron and will move along its axon to a third neuron (or a myocyte) by this same sequence of events. We see, then, that gated ion channels convey signals in either of two ways: by changing the cytoplasmic concentration of an ion (such as Ca^{2+}), which then serves as an intracellular second messenger, or by changing V_m and affecting other membrane proteins that are sensitive to V_m. The passage of an electrical signal through one neuron and on to the next illustrates both types of mechanism.

Neurons Have Receptor Channels That Respond to Different Neurotransmitters

Animal cells, especially those of the nervous system, contain a variety of ion channels gated by ligands, voltage, or both. Receptors that are themselves ion channels are classified as **ionotropic**, to distinguish them from receptors that generate a second messenger (**metabotropic** receptors). Acetylcholine acts on an ionotropic receptor in the postsynaptic cell. The acetylcholine receptor is a cation channel. When occupied by acetylcholine, the receptor opens to the passage of cations (Na^+, K^+, and Ca^{2+}), triggering depolarization of the cell. The neurotransmitters serotonin, glutamate, and glycine all can act through ionotropic receptors that are structurally related to the acetylcholine receptor. Serotonin and glutamate trigger the opening of cation (Na^+, K^+, Ca^{2+}) channels, whereas glycine opens Cl^--specific channels.

P6 Depending on which ion passes through a channel, binding of the ligand (neurotransmitter) for that channel results in either depolarization or hyperpolarization of the target cell. A single neuron normally receives input from many other neurons, each releasing its own characteristic neurotransmitter with its characteristic depolarizing or hyperpolarizing effect. The target cell's V_m therefore reflects the *integrated* input (see Fig. 12-1f) from multiple neurons. The cell responds with an action potential only if the integrated input adds up to a net depolarization of sufficient magnitude.

The receptor channels for acetylcholine, glycine, glutamate, and γ-aminobutyric acid (GABA) are gated by *extracellular* ligands. *Intracellular* second messengers — such as cAMP, cGMP, IP_3, Ca^{2+}, and ATP — regulate ion channels of the type we saw in the sensory transductions of vision, olfaction, and gustation.

Toxins Target Ion Channels

Many of the most potent toxins found in nature act on ion channels. For example, dendrotoxin (from the black mamba snake) blocks the action of voltage-gated K^+ channels, tetrodotoxin (produced by puffer fish) acts on voltage-gated Na^+ channels, and cobrotoxin (from cobras) disables acetylcholine receptor ion channels. Why, in the course of evolution, have ion channels become the preferred target of toxins, rather than some critical metabolic target such as an enzyme essential in energy metabolism?

Ion channels are extraordinary amplifiers; opening of a single channel can allow the flow of 10 million ions per second. Consequently, relatively few molecules of an ion-channel protein are needed per neuron for signaling functions. This means that a relatively small number of toxin molecules with high affinity for ion channels, acting from outside the cell, can have a pronounced effect on neurosignaling throughout the body. A comparable effect by way of a metabolic enzyme, typically present in cells at much higher concentrations than ion channels, would require far greater numbers of the toxin molecule.

SUMMARY 12.6 *Gated Ion Channels*

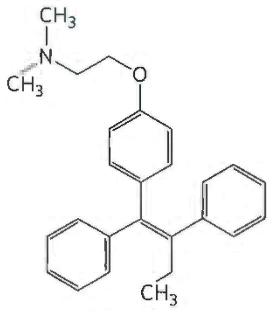
- Ion channels gated by membrane potential or ligands are central to signaling in neurons and other cells.
- The voltage-gated Na^+ and K^+ channels of neuronal membranes carry the action potential along the axon as a wave of depolarization (Na^+ influx) followed by repolarization (K^+ efflux). Arrival of an action potential at the distal end of a presynaptic neuron triggers neurotransmitter release.
- The neurotransmitter (acetylcholine, for example) diffuses to the postsynaptic neuron (or the myocyte, at a neuromuscular junction), binds to specific receptors in the plasma membrane, and triggers a change in V_m. The cell body of the neuron has receptors for a variety of neurotransmitters or extracellular signals. The neuron's V_m is the sum of the effects of all ion-channel contributions.
- Neurotoxins, produced by many organisms, attack neuronal ion channels and are therefore fast-acting and deadly.

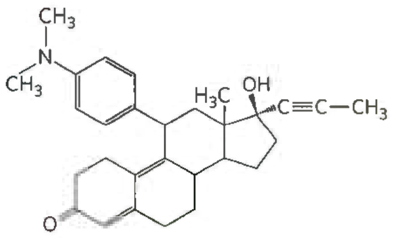
12.7 Regulation of Transcription by Nuclear Hormone Receptors

P7 The steroid, retinoic acid (retinoid), and thyroid hormones form a large group of receptor ligands that exert at least part of their effects by a mechanism fundamentally different from that of other hormones: they act directly in the nucleus to alter gene expression. We discuss their mode of action in detail in Chapter 28, along with other mechanisms for regulating gene expression. Here we give a brief overview.

Steroid hormones (estrogen, progesterone, vitamin D, and cortisol, for example), too hydrophobic to dissolve readily in the blood, are transported on specific carrier proteins from their point of release to their target tissues. In target cells, these hormones pass through the plasma membrane and nuclear membrane by simple diffusion and bind to specific receptor proteins in the nucleus (**Fig. 12-34**). Hormone binding triggers changes in the conformation of a receptor protein so that it becomes capable of interacting with specific regulatory sequences in DNA called **hormone response elements (HREs)**, thus altering gene expression (see Fig. 28-34). The bound receptor-hormone complex enhances the expression of specific genes adjacent to HREs, with the help of several other proteins essential for transcription. Hours or days are required for these regulators to have their full effect—the time required for the changes in RNA synthesis and subsequent protein synthesis to become evident in altered metabolism.

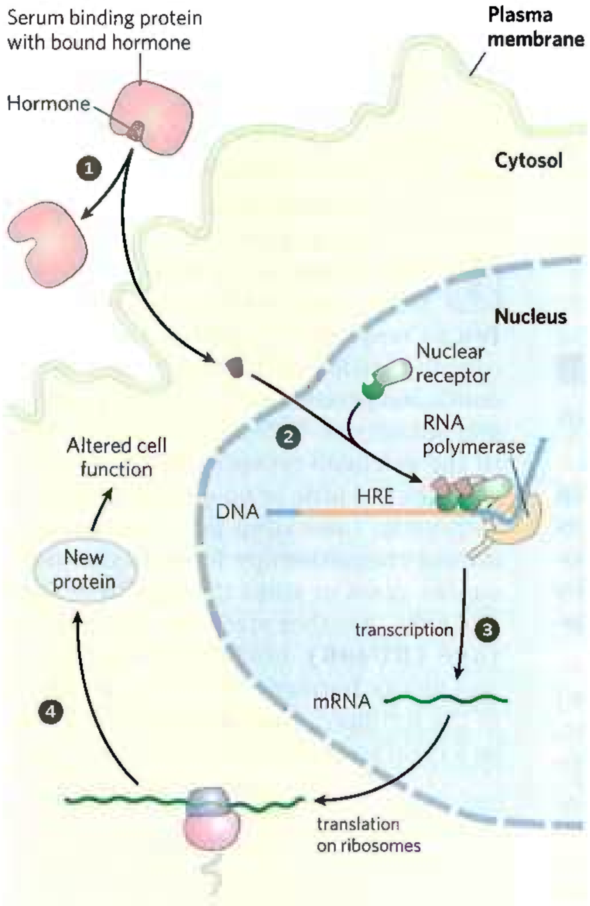
The specificity of the steroid-receptor interaction is exploited in the use of the drug **tamoxifen** to treat breast cancer. In some types of breast cancer, division of the cancerous cells depends on the continued presence of estrogen. Tamoxifen is an estrogen antagonist; it competes with estrogen for binding to the estrogen receptor, but the tamoxifen-receptor complex has little or no effect on gene expression. Consequently, tamoxifen administered after surgery or during chemotherapy for hormone-dependent breast cancer slows or stops the growth of remaining cancerous cells. Another steroid analog, the drug **mifepristone (RU486)**, binds to the progesterone receptor and blocks hormone actions essential to implantation of the fertilized ovum in the uterus, and thus functions as a contraceptive.


Tamoxifen


Mifepristone
(RU486)

SUMMARY 12.7 *Regulation of Transcription by Nuclear Hormone Receptors*

- Steroid hormones enter cells by simple diffusion and bind to specific receptor proteins, inducing a structural change that exposes a specific binding site on the DNA.
- The hormone-receptor complex binds specific regions of DNA at the hormone response elements, and interacts with other proteins to regulate the expression of nearby genes.



1. Hormone, carried to the target tissue on serum binding proteins, diffuses across the plasma membrane and binds to its specific receptor protein in the nucleus.

2. Hormone binding changes the conformation of the receptor; it forms homo- or heterodimers with other hormone-receptor complexes and binds to specific regulatory regions called hormone response elements (HREs) in the DNA adjacent to specific genes.

3. Receptor attracts coactivator or corepressor protein(s) and, with them, regulates transcription of the adjacent gene(s), increasing or decreasing the rate of mRNA formation.

4. Altered levels of the hormone-regulated gene product produce the cellular response to the hormone.

FIGURE 12-34 General mechanism by which steroid and thyroid hormones, retinoids, and vitamin D regulate gene expression. The details of transcription and protein synthesis are discussed in Chapters 26 and 27. Some steroids also act through plasma membrane receptors by a completely different mechanism.

12.8 Regulation of the Cell Cycle by Protein Kinases

One of the most dramatic manifestations of signaling pathways is the regulation of the eukaryotic cell cycle. During embryonic growth and later development, cell division occurs in virtually every tissue. In the adult organism, cells of most tissues stop dividing, becoming quiescent. A cell's "decision" to divide or not is of crucial importance to the organism. **P9** When the regulatory mechanisms that limit cell division are defective and cells undergo unregulated division, the result is catastrophic—cancer. Proper cell division requires a precisely ordered sequence of biochemical events that assures every daughter cell a full complement of the molecules required for life. Investigations into the control of cell division in diverse eukaryotic cells have revealed universal regulatory mechanisms. Signaling mechanisms much like those discussed above are central in determining whether and when a cell undergoes cell division, and they also ensure orderly passage through the stages of the cell cycle.

The Cell Cycle Has Four Stages

Cell division accompanying mitosis in eukaryotes occurs in four well-defined stages (**Fig. 12-35**). In the S (synthesis) phase, the DNA is replicated to produce copies for both daughter cells. In the G2 phase (*G* indicates the gap between divisions), new proteins are synthesized and the cell approximately doubles in size. In the M phase (mitosis), the maternal nuclear envelope breaks down, paired chromosomes are pulled to opposite poles of the cell, each set of daughter chromosomes is surrounded by a newly formed nuclear envelope, and cytokinesis pinches the cell in half, producing two daughter cells (see Fig. 24-22). In embryonic or rapidly proliferating tissue, each daughter cell divides again, but only after a waiting period (G1). In animal cells in the laboratory, the entire process takes about 24 hours.

After passing through mitosis and into G1, a cell either continues through another division or ceases to divide, entering a quiescent phase (G0) that may last hours, days, or the lifetime of the cell. When a cell in G0 begins to divide again, it reenters the division cycle

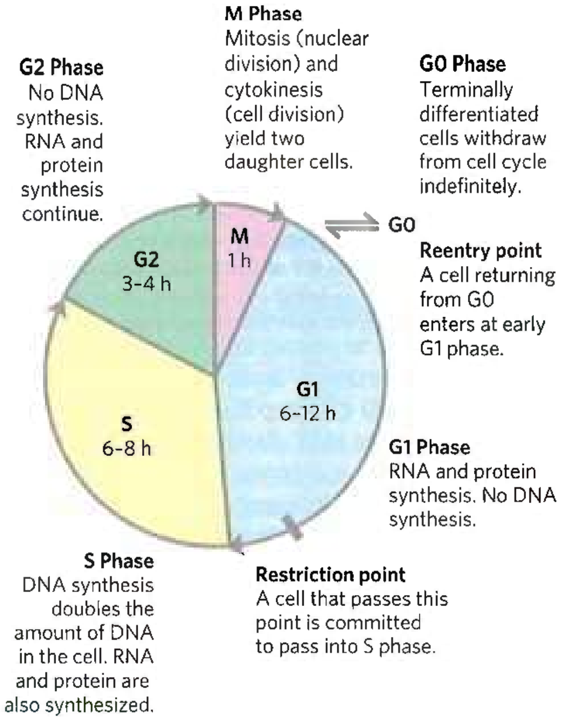

M Phase
Mitosis (nuclear division) and cytokinesis (cell division) yield two daughter cells.
G2 Phase
No DNA synthesis. RNA and protein synthesis continue.
G0 Phase
Terminally differentiated cells withdraw from cell cycle indefinitely.
M
1 h
G0
G2
3-4 h
Reentry point
A cell returning from G0 enters at early G1 phase.
G1
6-12 h
S
6-8 h
G1 Phase
RNA and protein synthesis. No DNA synthesis.
S Phase
DNA synthesis doubies the amount of DNA in the cell. RNA and protein are also synthesized.
Restriction point
A cell that passes this point is committed to pass into S phase.

FIGURE 12-35 The eukaryotic cell cycle. The durations (in hours) of the four stages vary, but those shown are typical.

through the G1 phase. Differentiated cells such as hepatocytes or adipocytes have acquired their specialized function and form; they remain in the G0 phase. Stem cells retain their potential to divide and to differentiate into any of a number of cell types.

Levels of Cyclin-Dependent Protein Kinases Oscillate

P8 The timing of the cell cycle is controlled by a family of protein kinases with activities that change in response to cellular signals. By phosphorylating specific proteins at precisely timed intervals, these protein kinases orchestrate the metabolic activities of the cell to produce orderly cell division. The kinases are heterodimers with a regulatory subunit, a **cyclin**, and a catalytic subunit, a **cyclin-dependent protein kinase (CDK)**. In the absence of the cyclin, the catalytic subunit is virtually inactive. When the cyclin binds, the catalytic site opens up, a residue essential to catalysis becomes accessible, and the protein kinase activity of the catalytic subunit increases 10,000-fold. Animal cells have at least 10 different cyclins (designated A, B, and so forth) and at least 8 CDKs (CDK1 through CDK8), which act in various combinations at specific points in the cell cycle.

In a population of animal cells undergoing synchronous division, some CDK activities show striking oscillations (**Fig. 12-36**). These oscillations are the result of four mechanisms for regulating CDK activity: phosphorylation or dephosphorylation of the CDK, controlled degradation of the cyclin subunit, periodic synthesis of CDKs and cyclins, and the action of specific CDK-inhibiting proteins. The precisely timed activation and inactivation of a series of CDKs produces signals serving as a master clock that orchestrates the events in normal cell division and ensures that one stage is completed before the next begins.

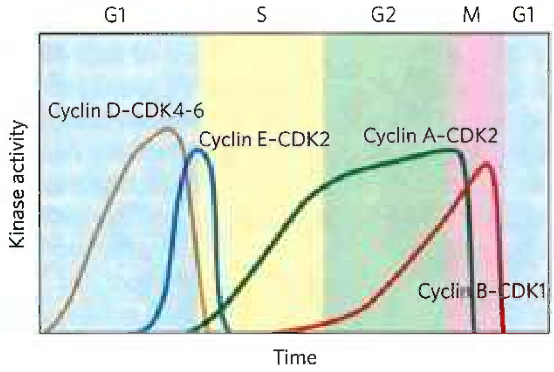


FIGURE 12-36 Variations in the activities of specific CDKs during the cell cycle in animals. Early in G1, the activity of cyclin D–CDK4-6 rises slowly, then drops sharply as G1 ends. Near the end of G1, cyclin E–CDK2 activity rises and peaks near the G1 phase–S phase boundary, when the active enzyme triggers synthesis of enzymes required for DNA synthesis (see Fig. 12-40). Cyclin A–CDK2 activity rises during the S and G2 phases, then drops sharply in the M phase, as cyclin B–CDK1 peaks. [Data from P. Icard et al., *Trends Biochem. Sci.* 44:490, 2019, Fig. 3.]

CDKs Are Regulated by Phosphorylation, Cyclin Degradation, Growth Factors, and Specific Inhibitors

The activity of a CDK is strikingly affected by phosphorylation and dephosphorylation of two specific residues in the protein (**Fig. 12-37**). Phosphorylation of Thr^{160} of CDK2 stabilizes a conformation in which an autoinhibitory "T loop" is moved away from the substrate-binding cleft in the kinase, opening it to bind protein substrates. Dephosphorylation of Ⓟ-Tyr^{15} of CDK2 removes a negative charge that blocks ATP from approaching its binding site. This mechanism for activating a CDK is self-reinforcing; the enzyme (PTP) that dephosphorylates Ⓟ-Tyr^{15} is itself a substrate for the CDK and is activated by phosphorylation. The combination of these factors activates the CDK manyfold, allowing it to phosphorylate downstream protein targets required for progression of the cell cycle (**Fig. 12-38a**).

The presence of a single-strand break in DNA signals arrest of the cell cycle in G2 by activating two proteins (ATM and ATR; see Fig. 12-40). These proteins trigger a cascade of responses that include inactivation of the PTP that dephosphorylates Tyr^{15} of the CDK. With the CDK inactivated, the cell is arrested in G2, unable to divide until the DNA is repaired and the effects of the cascade are reversed.

Highly specific and precisely timed proteolytic breakdown of mitotic cyclins regulates CDK activity throughout the cell cycle (Fig. 12-38b). How is the timing of cyclin breakdown controlled? A feedback loop occurs in

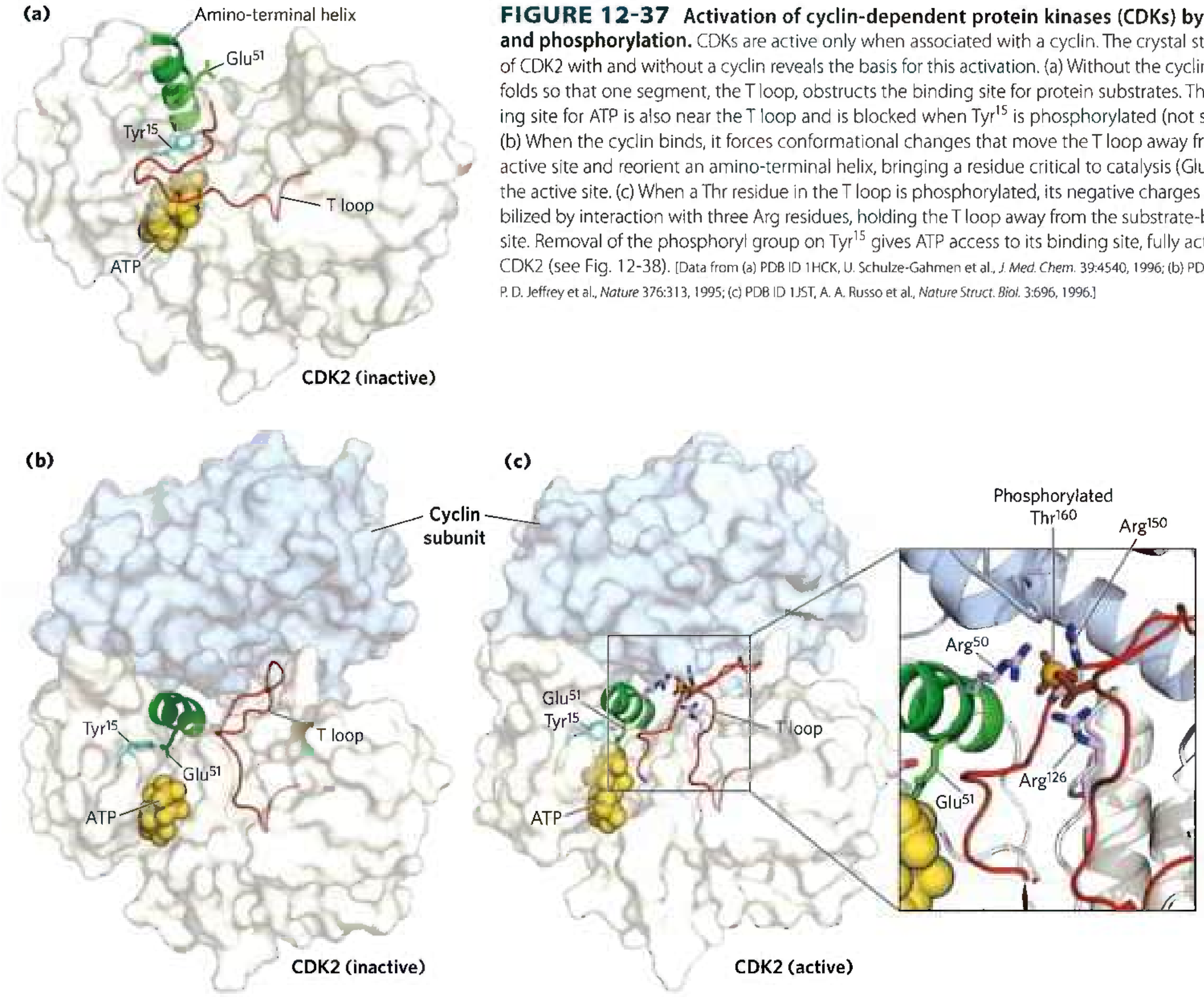


FIGURE 12-37 Activation of cyclin-dependent protein kinases (CDKs) by cyclin and phosphorylation. CDKs are active only when associated with a cyclin. The crystal structure of CDK2 with and without a cyclin reveals the basis for this activation. (a) Without the cyclin, CDK2 folds so that one segment, the T loop, obstructs the binding site for protein substrates. The binding site for ATP is also near the T loop and is blocked when Tyr^{15} is phosphorylated (not shown). (b) When the cyclin binds, it forces conformational changes that move the T loop away from the active site and reorient an amino-terminal helix, bringing a residue critical to catalysis (Glu^{51}) into the active site. (c) When a Thr residue in the T loop is phosphorylated, its negative charges are stabilized by interaction with three Arg residues, holding the T loop away from the substrate-binding site. Removal of the phosphoryl group on Tyr^{15} gives ATP access to its binding site, fully activating CDK2 (see Fig. 12-38). [Data from (a) PDB ID 1HCK, U. Schulze-Gahmen et al., *J. Med. Chem.* 39:4540, 1996; (b) PDB ID 1FIN, P. D. Jeffrey et al., *Nature* 376:313, 1995; (c) PDB ID 1JST, A. A. Russo et al., *Nature Struct. Biol.* 3:696, 1996.]

the overall process shown in Figure 12-38. As a cell enters mitosis, the M-phase CDK is inactive (step ❶). As cyclin is synthesized (step ❷), the cyclin-CDK complex forms (step ❸). The T loop lies in the substrate-binding site of CDK, and Ⓟ-Tyr^{15} blocks its ATP-binding site, keeping the complex inactive. When Thr^{160} in the T loop is phosphorylated, the loop moves out of the substrate-binding site, and when Tyr^{15} is dephosphorylated, ATP can bind. These two changes make the cyclin-CDK complex many times more active (step ❹). Further activation is achieved as CDK also phosphorylates and activates the enzyme that dephosphorylates Ⓟ-Tyr^{15} (step ❺). The active cyclin-CDK complex triggers its own inactivation by phosphorylation of DBRP (destruction box recognizing protein; step ❻). DBRP and ubiquitin ligase then attach several molecules of **ubiquitin** (U) to the cyclin (step ❼), targeting it for destruction by proteolytic enzyme complexes called **proteasomes** (step ❽). The role of ubiquitin and proteasomes is not limited to the regulation of cyclins; as we shall see in Chapter 27, both also take part in the turnover of cellular proteins, a process fundamental to cellular housekeeping.

The third mechanism for changing CDK activity is regulation of the rate of synthesis of the cyclin or CDK or both. Extracellular signals such as **growth factors** and cytokines (developmental signals that trigger cell division) activate, by phosphorylation, the nuclear transcription factors Jun and Fos, which promote the synthesis of many gene products, including cyclins, CDKs, and the transcription factor E2F. In turn, E2F stimulates production of several enzymes essential for the synthesis of deoxynucleotides and DNA, and the CDK and cyclin allow the cell to enter the S phase (**Fig. 12-39**).

Finally, specific protein inhibitors bind to and inactivate specific CDKs. One such protein is p21, which we discuss below.

These four control mechanisms modulate the activity of specific CDKs that, in turn, control whether a cell will divide, differentiate, become permanently quiescent, or begin a new cycle of division after a period of quiescence.

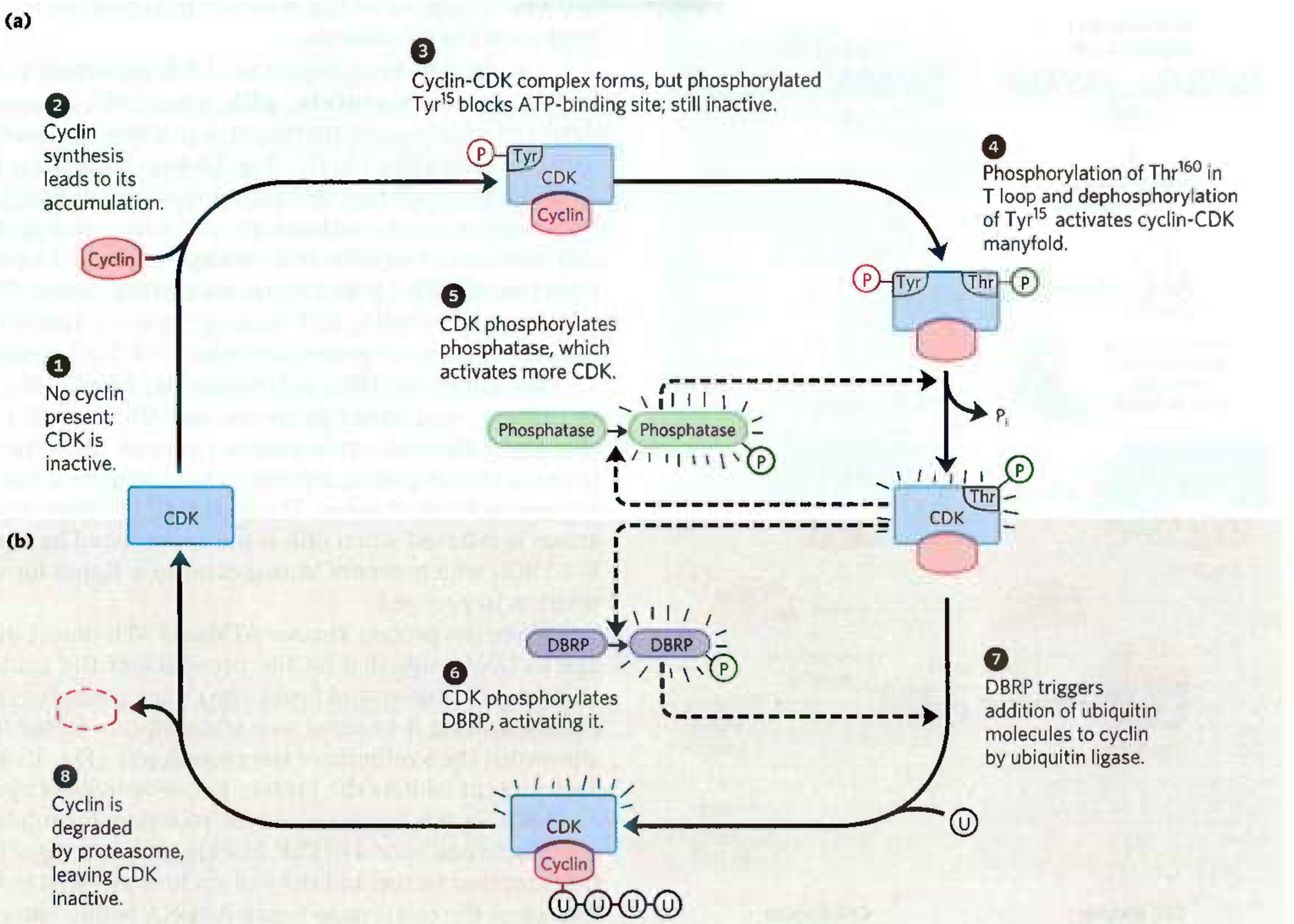

(a)
③ Cyclin-CDK complex forms, but phosphorylated Tyr15 blocks ATP-binding site; still inactive.
② Cyclin synthesis leads to its accumulation.
④ Phosphorylation of Thr160 in T loop and dephosphorylation of Tyr15 activates cyclin-CDK manyfold.
⑤ CDK phosphorylates phosphatase, which activates more CDK.
① No cyclin present; CDK is inactive.
Cyclin
CDK
Tyr
Thr
P
Pi
Phosphatase
DBRP
(b)
⑥ CDK phosphorylates DBRP, activating it.
⑦ DBRP triggers addition of ubiquitin molecules to cyclin by ubiquitin ligase.
⑧ Cyclin is degraded by proteasome, leaving CDK inactive.
U

FIGURE 12-38 Regulation of CDK by phosphorylation and proteolysis. (a) The series of events that leads to activation of a cyclin-dependent protein kinase. (b) The periodic proteolytic degradation of cyclin, inactivating the cyclin-dependent protein kinase. The steps are described in the text.

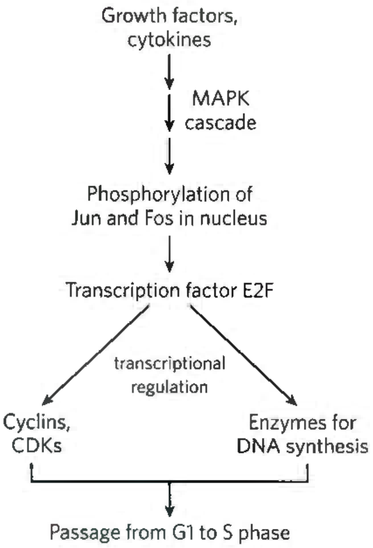

Growth factors, cytokines
MAPK cascade
Phosphorylation of Jun and Fos in nucleus
Transcription factor E2F
transcriptional regulation
Cyclins, CDKs
Enzymes for DNA synthesis
Passage from G1 to S phase

FIGURE 12-39 Regulation of cell division by growth factors.

The details of cell cycle regulation, such as the number of different cyclins and kinases and the combinations in which they act, differ from species to species, but the basic mechanism has been conserved in the evolution of all eukaryotic cells.

CDKs Regulate Cell Division by Phosphorylating Critical Proteins

We have examined how cells maintain close control of CDK activity, but how does the activity of CDKs control the cell cycle? There are scores of known CDK targets, and much remains to be learned. But we can see a general pattern behind CDK regulation by inspecting the effect of CDKs on the structures of lamin and myosin and on the activity of retinoblastoma protein.

The structure of the nuclear envelope is maintained in part by highly organized meshworks of intermediate filaments composed of the protein **lamin**. Breakdown

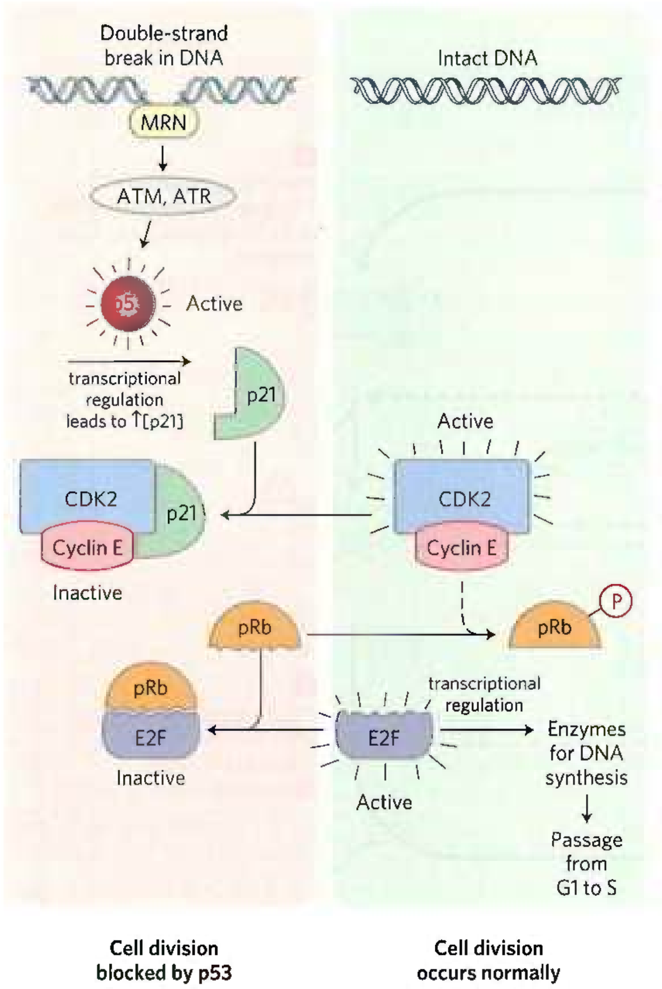

Double-strand break in DNA
Intact DNA
MRN
ATM, ATR
p53
Active
transcriptional regulation leads to ↑[p21]
p21
Active
CDK2
p21
Cyclin E
Inactive
CDK2
Cyclin E
pRb
pRb
P
pRb
E2F
Inactive
transcriptional regulation
E2F
Active
Enzymes for DNA synthesis
Passage from G1 to S
Cell division blocked by p53
Cell division occurs normally

FIGURE 12-40 Regulation of passage from G1 to S by phosphorylation of pRb. Transcription factor E2F promotes transcription of genes for certain enzymes essential to DNA synthesis. The retinoblastoma protein, pRb, can bind E2F (lower left), inactivating it and preventing transcription of these genes. Phosphorylation of pRb by CDK2 prevents it from binding and inactivating E2F, and the genes are transcribed, allowing cell division. Damage to the cell's DNA (upper left) triggers a series of events that inactivate CDK2, blocking cell division. When the protein MRN detects damage to the DNA, it activates two protein kinases, ATM and ATR, and they phosphorylate and activate the transcription factor p53. Active p53 promotes the synthesis of another protein, p21, an inhibitor of CDK2. Inhibition of CDK2 stops the phosphorylation of pRb, which therefore continues to bind and inhibit E2F. With E2F inactivated, genes essential to cell division are not transcribed and cell division is blocked. When DNA has been repaired, this inhibition is released, and the cell divides.

of the nuclear envelope before segregation of the sister chromatids in mitosis is partly due to the phosphorylation of lamin by a CDK, which causes lamin filaments to depolymerize.

A second kinase target is the ATP-driven contractile machinery (actin and myosin) that pinches a dividing cell into two equal parts during cytokinesis. After the division, a CDK phosphorylates a small regulatory subunit of myosin, causing dissociation of myosin from actin filaments and inactivating the contractile machinery. Subsequent dephosphorylation allows reassembly of the contractile apparatus for the next round of cytokinesis.

A third and very important CDK substrate is the **retinoblastoma protein, pRb**; when DNA damage is detected, this protein participates in a mechanism that arrests cell division in G1 (**Fig. 12-40**). Named for the retinal tumor cell line in which it was discovered, pRb functions in most, perhaps all, cell types to regulate cell division in response to a variety of stimuli. Unphosphorylated pRb binds the transcription factor E2F; while bound to pRb, E2F cannot promote transcription of a group of genes necessary for DNA synthesis (the genes for DNA polymerase α, ribonucleotide reductase, and other proteins; see Chapter 25). In this state, the cell cycle cannot proceed from the G1 phase to the S phase, the step that commits a cell to mitosis and cell division. The pRb-E2F blocking mechanism is relieved when pRb is phosphorylated by cyclin E–CDK2, which occurs in response to a signal for cell division to proceed.

When the protein kinases ATM and ATR detect damage to DNA (signaled by the presence of the protein MRN at a double-strand break site), they phosphorylate p53, activating it to serve as a transcription factor that stimulates the synthesis of the protein p21 (Fig. 12-40). This protein inhibits the protein kinase activity of cyclin E–CDK2. In the presence of p21, pRb remains unphosphorylated and bound to E2F, blocking the activity of this transcription factor, and the cell cycle is arrested in G1. This gives the cell time to repair its DNA before entering the S phase, thereby avoiding the potentially disastrous transfer of a defective genome to one or both daughter cells. When the damage is too severe to allow effective repair, this same machinery triggers apoptosis (described below), a process that leads to the death of the cell, preventing the possible development of a cancer.

SUMMARY 12.8 *Regulation of the Cell Cycle by Protein Kinases*

- Progression through the cell cycle is regulated by the cyclin-dependent protein kinases (CDKs), which act at specific points in the cycle, phosphorylating key proteins and modulating their activities. The catalytic subunit of CDKs is inactive unless associated with the regulatory cyclin subunit.
- The activity of a cyclin-CDK complex changes during the cell cycle through differential synthesis of CDKs, specific degradation of the cyclin, phosphorylation and dephosphorylation of critical residues in CDKs, and binding of inhibitory proteins to specific cyclin-CDKs.
- A cyclin sequence (the destruction box) marks cyclin for tagging with ubiquitin and degradation in proteasomes. The rise of cyclin concentration by its synthesis ultimately triggers its degradation, yielding oscillations in cyclin level keyed to the cell cycle.

■ Cells receive extracellular signals that determine the timing of their division. Scores of proteins are known targets of CDKs, many with unknown functions. Among the targets phosphorylated by cyclin-CDKs are proteins of the nuclear envelope and proteins required for cytokinesis and DNA repair.

12.9 Oncogenes, Tumor Suppressor Genes, and Programmed Cell Death

Tumors and cancer are the result of uncontrolled cell division. Normally, cell division is regulated by a family of extracellular growth factors, proteins that cause resting cells to divide and, in some cases, differentiate. The result is a precise balance between the formation of new cells and cell destruction. Regulation of cell division ensures that skin cells are replaced every few weeks and white blood cells are replaced every few days. This is homeostasis at the organismal level. P9 When this balance is disturbed by defects in regulatory proteins, the result is sometimes the formation of a clone of cells that divide repeatedly and without regulation (a tumor) until their presence interferes with the function of normal tissues—cancer. The direct cause is almost always a genetic defect in one or more of the proteins that regulate cell division. In some cases, a defective gene is inherited from one parent; in other cases, the mutation occurs when a toxic compound from the environment (a mutagen or a carcinogen) or high-energy radiation interacts with the DNA of a single cell to damage it and introduce a mutation. In most cases there is both an inherited contribution and an environmental contribution, and in most cases, more than one mutation is required in order to cause completely unregulated division and full-blown cancer.

Oncogenes Are Mutant Forms of the Genes for Proteins That Regulate the Cell Cycle

P9 **Oncogenes** are mutated versions of genes encoding signaling proteins involved in cell cycle regulation. Oncogenes were originally discovered in tumor-causing viruses, then later found to be derived from genes in animal host cells, **proto-oncogenes**, which encode growth-regulating proteins. During a viral infection, the host DNA sequence of a proto-oncogene is sometimes copied into the viral genome, where it proliferates with the virus. In subsequent viral infection cycles, the proto-oncogenes can become defective by truncation or mutation. Viruses, unlike animal cells, do not have effective mechanisms for correcting mistakes during DNA replication, so they accumulate mutations rapidly. When a virus carrying an oncogene infects a new host cell, the viral DNA (and oncogene) can be incorporated into the host cell's DNA, where it can now interfere with the regulation of cell division in the host cell. In an alternative, nonviral mechanism, a single cell in a tissue exposed to carcinogens may suffer DNA damage that renders one of its regulatory proteins defective, with the same effect as the viral oncogenic mechanism: failed regulation of cell division.

The mutations that produce oncogenes are genetically dominant; if either of a pair of chromosomes contains a defective gene, that gene product sends the signal "divide," and a tumor may result. The oncogenic defect can be in any of the proteins involved in communicating the "divide" signal. Oncogenes discovered thus far include those that encode secreted proteins that act as signaling molecules, growth factors, transmembrane proteins (receptors), cytoplasmic proteins (G proteins and protein kinases), and the nuclear transcription factors that control the expression of genes essential for cell division (Jun, Fos).

Some oncogenes encode growth factor receptors with unregulated Tyr kinase activity; they signal continued cell division even when the growth factor is absent, leading to tumor formation. Tumor-producing mutations have been found in many of the signaling protein kinases we have discussed here, all of which use ATP as their substrate for phosphoryl transfer to another element in the signaling cascade. The development of drugs that inhibit the protein kinase activity is an obvious approach to treating cancers that result from unregulated kinase activity. However, most known protein kinase inhibitors act by blocking the binding site for ATP, which is similar in all protein kinases. Inhibitors that are effective against one protein kinase are likely to have intolerable side effects due to their inhibition of other, essential kinases. Nonetheless, the prominent role played by protein kinases in signaling processes related to normal and abnormal cell division has made these enzymes a prime target in the development of drugs for the treatment of cancer (**Box 12-4**). ■

Defects in Certain Genes Remove Normal Restraints on Cell Division

Tumor suppressor genes encode proteins that normally restrain cell division. Mutation in one or more of these genes can lead to tumor formation. Unregulated growth due to defective tumor suppressor genes, unlike that due to oncogenes, is genetically recessive; tumors form only if *both* chromosomes contain a defective gene. This is because the function of these genes is to prevent cell division, and if either copy of the gene is normal, it will produce a normal protein and normal inhibition of division. In a person who inherits one correct copy and one defective copy, every cell begins with one defective copy of the gene. If any one of the individual's 10^{12} somatic cells undergoes mutation in the one good copy, a tumor may grow from that doubly mutant cell. Mutations in both copies of the genes for pRb, p53, or p21 yield cells in which the normal restraint on cell division is lost and a tumor forms.

BOX 12-4 MEDICINE

Development of Protein Kinase Inhibitors for Cancer Treatment

When a single cell divides without any regulatory limitation, it eventually gives rise to a clone of cells so large that it interferes with normal physiological functions (Fig. 1). This is cancer, a leading cause of death in the developed world, and increasingly so in the developing world. In all types of cancer, the normal regulation of cell division has become dysfunctional due to defects in one or more genes. For example, genes encoding proteins that normally send intermittent signals for cell division become oncogenes, producing constitutively active signaling proteins, or genes encoding proteins that normally restrain cell division (tumor suppressor genes) mutate to produce proteins that lack this braking function. In many tumors, both kinds of mutation have occurred.

Many oncogenes and tumor suppressor genes encode protein kinases or proteins that act in pathways upstream from protein kinases. It is therefore reasonable to hope that specific inhibitors of protein kinases could prove valuable in the treatment of cancer. For example, a mutant form of the EGF (epidermal growth factor) receptor is a constantly active receptor Tyr kinase (RTK), signaling cell division whether EGF is present or not. In about 30% of all women with invasive breast cancer, the gene for the receptor ErbB2 (also called HER2/neu) is overexpressed, sometimes by as much as 100-fold. Another RTK, **vascular endothelial growth factor receptor (VEGFR)**, must be activated for the formation of new blood vessels (angiogenesis) to provide a solid tumor with its own blood supply, and inhibition of VEGFR might starve a tumor of essential nutrients. Nonreceptor Tyr kinases can also mutate, resulting in constant signaling and unregulated cell division. For example, the oncogene *Abl* (from the *Ab*elson *l*eukemia virus) is associated with acute myeloid leukemia, a relatively rare blood disease (~5,000 cases a year in the United States). Another group of oncogenes encode unregulated cyclin-dependent protein kinases. In each of these cases, specific protein kinase inhibitors might be valuable chemotherapeutic agents in the treatment of disease. Not surprisingly, huge efforts are under way to develop such inhibitors. How should one approach this challenge?

Protein kinases of all types show striking conservation of structure at the active site. All share with the prototypical PKA structure the features shown in Figure 2: two lobes that enclose the active site, with a P loop that helps to align and bind the phosphoryl groups of ATP, an activation loop that moves to open the active site to the protein substrate, and a C helix that changes position as the enzyme is activated, bringing the residues in the substrate-binding cleft into their binding positions. Detailed knowledge of the structure around the ATP-binding site makes it possible to design drugs that inhibit a *specific* protein kinase by (1) blocking the critical ATP-binding site, while (2) interacting with residues around that site that are *unique* to that particular protein kinase.

The simplest protein kinase inhibitors are ATP analogs that occupy the ATP-binding site but cannot serve as phosphoryl group donors. Many such compounds are known, but their clinical usefulness is limited by their lack of selectivity—they inhibit

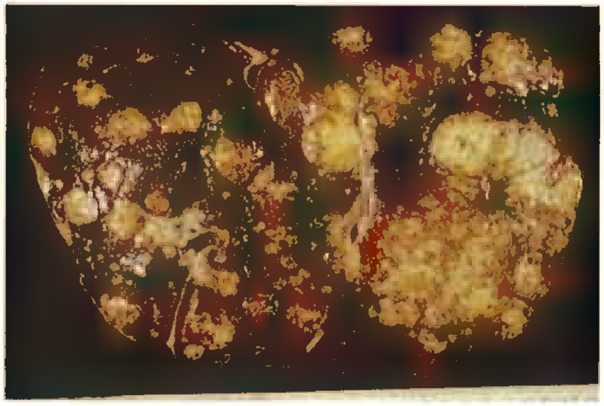


FIGURE 1 Unregulated division of a single cell in the colon led to a primary cancer that metastasized to the liver. Secondary cancers are seen as white patches in this liver obtained at autopsy. [CNRI/Science Source.]

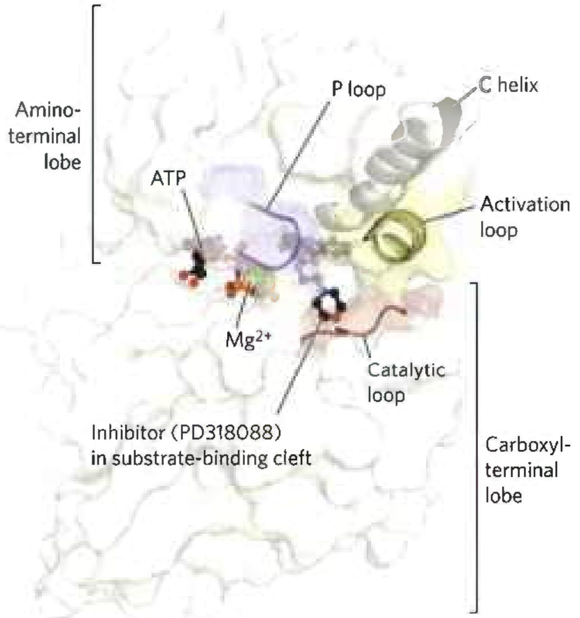
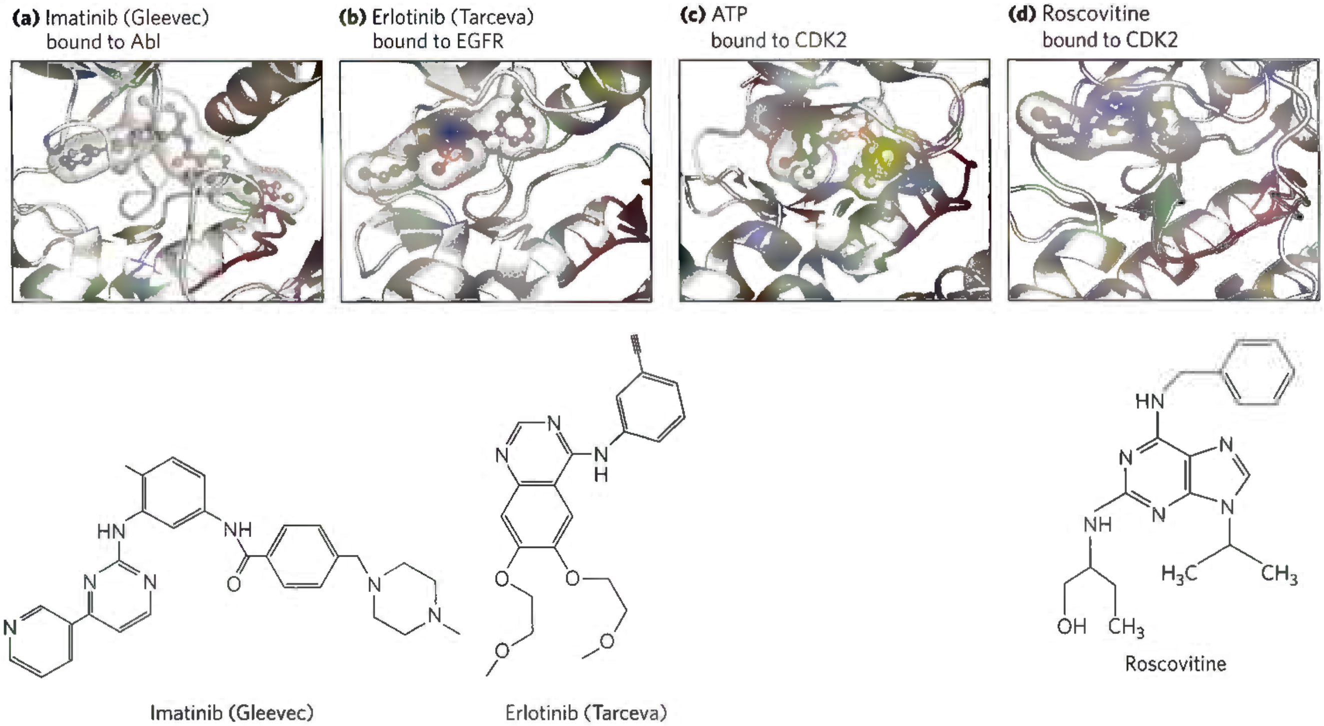


FIGURE 2 Conserved features of the active site of protein kinases. The amino-terminal and carboxyl-terminal lobes surround the active site of the enzyme, near the catalytic loop and the site where ATP binds. The activation loop of this and many other kinases undergoes phosphorylation, then moves away from the active site to expose the substrate-binding cleft, which in this image is occupied by a specific inhibitor of this enzyme, PD318088. The P loop is essential in the binding of ATP, and the C helix must also be correctly aligned for ATP binding and kinase activity. [Data from PDB ID 1S9I, J. F. Ohren et al., *Nature Struct. Mol. Biol.* 11:1192, 2004.]



(a) Imatinib (Gleevec) bound to Abl

(b) Erlotinib (Tarceva) bound to EGFR

(c) ATP bound to CDK2

(d) Roscovitine bound to CDK2

Imatinib (Gleevec)

Erlotinib (Tarceva)

Roscovitine

FIGURE 3 Some protein kinase inhibitors now in clinical trials or clinical use, showing their binding to the target protein. (a) Imatinib binds to the Abl kinase (an oncogene product) active site; it occupies both the ATP-binding site and a region adjacent to that site. (b) Erlotinib binds to the active site of EGFR. (c), (d) Roscovitine is an inhibitor of the cyclin-dependent kinases CDK2, CDK7, and CDK9; shown here are normal Mg^{2+}-ATP binding at the active site (c) and roscovitine binding (d), which prevents the binding of ATP. [Data from (a) PDB ID 1IEP, B. Nagar et al., *Cancer Res.* 62:4236, 2002; (b) PDB ID 1M17, J. Stamos et al., *J. Biol. Chem.* 277:46,265, 2002; (c) PDB ID 1S9I, J. F. Ohren et al., *Nature Struct. Mol. Biol.* 11:1192, 2004; (d) PDB ID 2A4L, W. F. De Azevedo et al., *Eur. J. Biochem.* 243:518, 1997.]

virtually all protein kinases and would produce unacceptable side effects. More selectivity is seen with compounds that fill part of the ATP-binding site but also interact outside this site with parts of the protein unique to the target protein kinase. A third possible strategy is based on the fact that although the active conformations of all protein kinases are similar, their inactive conformations are not. Drugs that target the inactive conformation of a specific protein kinase and prevent its conversion to the active form may have a higher specificity of action. A fourth approach employs the great specificity of antibodies. For example, monoclonal antibodies (p. 167) that bind the extracellular portions of specific RTKs could eliminate the receptors' kinase activity by preventing dimerization or by causing their removal from the cell surface. In some cases, an antibody selectively binding to the surface of cancer cells could cause the immune system to attack those cells.

The search for drugs active against specific protein kinases has yielded encouraging results. For example, imatinib mesylate (Gleevec; Fig. 3a), a small-molecule inhibitor, has proved nearly 100% effective in bringing about remission in patients with early-stage chronic myeloid leukemia. Erlotinib (Tarceva; Fig. 3b), which targets EGFR, is effective against advanced non-small-cell lung cancer (NSCLC). Because many cell-division signaling systems involve more than one protein kinase, inhibitors that act on several protein kinases may be useful in the treatment of cancer. Sunitinib (Sutent) and sorafenib (Nexavar) target several protein kinases, including VEGFR and PDGFR. These two drugs are in clinical use for patients with gastrointestinal stromal tumors and advanced renal cell carcinoma, respectively. Trastuzumab (Herceptin), cetuximab (Erbitux), and bevacizumab (Avastin) are monoclonal antibodies that target ErbB2/HER2/neu, EGFR, and VEGFR, respectively; all three drugs are in clinical use for certain types of cancer.

At least a hundred more compounds are in preclinical trials. Among the drugs being evaluated are some obtained from natural sources and some produced by synthetic chemistry. Indirubin is a component of a Chinese herbal preparation traditionally used to treat certain leukemias; it inhibits CDK2 and CDK5. Roscovitine (Fig. 3d), a substituted adenine, has a benzyl ring that makes it highly specific as an inhibitor of CDK2. With several hundred potential anticancer drugs heading toward clinical testing, it is realistic to hope that some will prove more effective or more target-specific than those now in use.

Retinoblastoma occurs in children and causes blindness if not surgically treated. The cells of a retinoblastoma have two defective versions of the *Rb* gene (two defective alleles). Very young children who develop retinoblastoma commonly have multiple tumors in both eyes. These children have inherited one defective copy of the *Rb* gene, which is present in every cell; each tumor is derived from a single retinal cell that has undergone a mutation in its remaining good copy of the *Rb* gene. (A fetus with two mutant alleles in every cell is nonviable.) People with retinoblastoma who survive childhood also have a high incidence of cancers of the lung, prostate, and breast later in life.

A far less likely event is that a person born with two good copies of the *Rb* gene will have independent mutations in both copies in the *same* cell. Some individuals do develop retinoblastomas later in childhood, usually with only one tumor in one eye. These individuals, presumably, were born with two good copies (alleles) of *Rb* in every cell, but both *Rb* alleles in a single retinal cell have undergone mutation, leading to a tumor. After the child reaches about age 3, retinal cells stop dividing, and retinoblastomas at later ages are quite rare.

Stability genes (also called caretaker genes) encode proteins that function in the repair of major genetic defects that result from aberrant DNA replication, ionizing radiation, or environmental carcinogens. Mutations in these genes lead to a high frequency of unrepaired damage (mutations) in other genes, including proto-oncogenes and tumor suppressor genes, and thus to cancer. Among the stability genes are *ATM* (see Fig. 12-40); the *XP* gene family, in which mutations lead to xeroderma pigmentosum; and the *BRCA1* genes associated with some types of breast cancer (see Box 25-1). Mutations in the gene for p53 also cause tumors; in more than 90% of human cutaneous squamous cell carcinomas (skin cancers) and in about 50% of all other human cancers, *p53* is defective. Those very rare individuals who *inherit* one defective copy of *p53* commonly have the Li-Fraumeni cancer syndrome, with multiple cancers (of the breast, brain, bone, blood, lung, and skin) occurring at high frequency and at an early age. The explanation for multiple tumors in this case is the same as that for *Rb* mutations: an individual born with one defective copy of *p53* in every somatic cell is likely to suffer a second *p53* mutation in more than one cell during his or her lifetime.

P9 In summary, then, three classes of defects can contribute to the development of cancer: (1) oncogenes, in which the defect is the equivalent of a car's accelerator pedal being stuck down, with the engine racing; (2) mutated tumor suppressor genes, in which the defect leads to the equivalent of brake failure; and (3) mutated stability genes, with the defect leading to unrepaired damage to the cell's replication machinery—the equivalent of an unskilled car mechanic.

Mutations in oncogenes and tumor suppressor genes do not have an all-or-none effect. In some cancers, perhaps in all, the progression from a normal cell to a malignant tumor requires an accumulation of mutations (sometimes over several decades), none of which, alone, is responsible for the end effect. For example, the development of colorectal cancer has several recognizable stages, each associated with a mutation (**Fig. 12-41**). If an epithelial cell in the colon undergoes mutation of both copies of the tumor

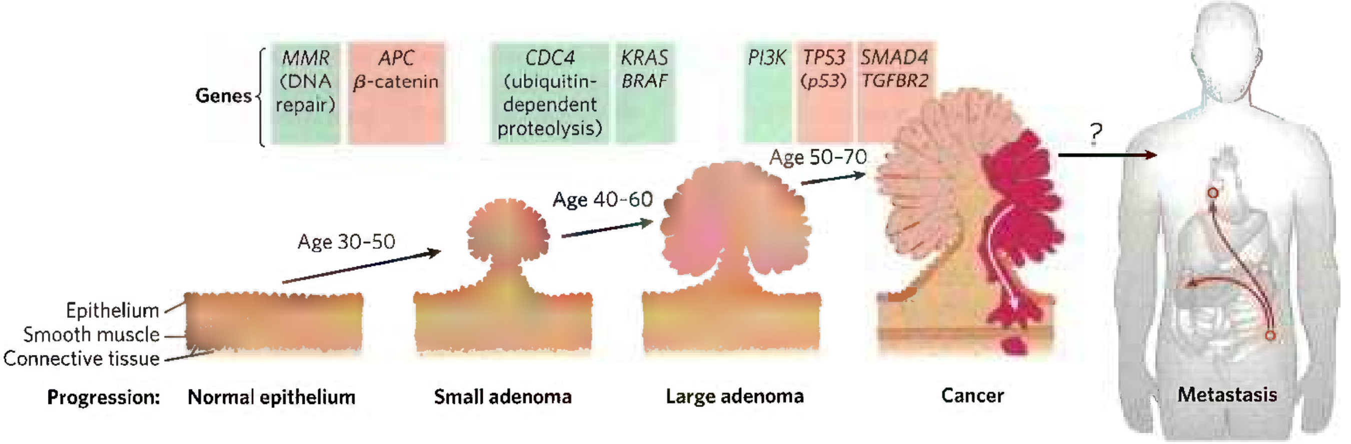


FIGURE 12-41 Multistep transition from normal epithelial cell to colorectal cancer. Serial mutations in oncogenes (green) or tumor suppressor genes (red) lead to progressively less control of cell division, until finally an active tumor forms, which can sometimes metastasize (spread from the initial site to other regions of the body). Mutation of the *MMR* gene leads to defective DNA repair and consequently to a higher rate of mutation. Mutations in both copies of the tumor suppressor gene *APC* lead to benign clusters of epithelial cells that multiply too rapidly (early adenoma). The *CDC4* oncogene results in defective ubiquitination, which is essential to the regulation of cyclin-dependent kinases (see Fig. 12-38). The oncogenes *KRAS* and *BRAF* encode Ras and Raf proteins (see Fig. 12-22), and this further disruption of signaling leads to the formation of a large adenoma, which may be detected by colonoscopy as a benign polyp. Oncogenic mutations in the *PI3K* gene, which encodes the enzyme phosphoinositide-3 kinase, or in *PTEN*, which regulates the synthesis of this enzyme, lead to a further strengthening of the signal: divide now. When a cell in one of the polyps undergoes further mutations, such as in the tumor suppressor genes *DCC* and *p53* (see Fig. 12-40), increasingly aggressive tumors form. Finally, mutations in other tumor suppressor genes such as *SMAD4* lead to a malignant tumor and sometimes to a metastatic tumor that can spread to other tissues. [Information from S. D. Markowitz and M. M. Bertagnolli, *N. Engl. J. Med.* 361:2449, 2009, Fig. 2.]

suppressor gene *APC* (adenomatous polyposis coli), it begins to divide faster than normal and produces a clone of itself, a benign polyp (early adenoma). For reasons not yet known, the *APC* mutation results in chromosomal instability, and whole regions of a chromosome are lost or rearranged during cell division. This instability can lead to another mutation, commonly in *ras*, that converts the clone into an intermediate (precancerous) adenoma.

A third mutation (often in the tumor suppressor gene *DCC*) leads to a late adenoma. Only when both copies of *p53* become defective does this cell mass become a carcinoma—a malignant, life-threatening tumor. The full sequence therefore requires at least seven genetic "hits": two on each of three tumor suppressor genes (*APC*, *DCC*, and *p53*) and one on the proto-oncogene *ras*. There are probably several other routes to colorectal cancer as well, but the principle that full malignancy results only from multiple mutations is likely to hold true for all of them. Because mutations accumulate over time, the chances of developing full-blown metastatic cancer rise with age.

When a polyp is detected in the early adenoma stage and the cells containing the first mutations are removed surgically, late adenomas and carcinomas will not develop; hence the importance of early detection. Cells and organisms, too, have their early detection systems. For example, the ATM and ATR proteins can detect DNA damage too extensive to be repaired effectively. They then trigger, through a pathway that includes p53, the process of apoptosis, in which a cell that has become dangerous to the organism kills itself.

The development of fast and inexpensive sequencing methods has opened a new window on the process by which cancer develops. In a typical study of cancers in humans, the sequences of all 20,000 genes were determined in about 3,300 different tumors, and then compared with the gene sequences in noncancerous tissue from the same patient. Almost 300,000 mutations were detected in all. Only a small fraction of these mutations, the **driver mutations**, were the *cause* of unregulated cell division; the vast majority (>99.9%) were "passenger mutations," which occurred randomly and did not confer a selective growth advantage on the tissue in which they occurred. Among the driver mutations were those in about 75 tumor suppressor genes and about 65 oncogenes. These 140 driver mutations fell in three general categories: those that affect cell survival (in genes encoding Ras, PI3K, MAPK, for example), those that affect cells' ability to maintain an intact genome (ATM, ATR), and those that affect cell fate, causing cells to divide, differentiate, or become quiescent (APC is one example). A relatively small number of mutations were very common in multiple types of cancer, in the genes for Ras, p53, and pRb, for example. ■

Apoptosis Is Programmed Cell Suicide

Many cells can precisely control the time of their own death by the process of **programmed cell death**, or **apoptosis** (pronounced app′-a-toe′-sis; from the Greek for "dropping off," as in leaves dropping in the fall). One trigger for apoptosis is irreparable damage to DNA. Programmed cell death also occurs during the normal development of an embryo, when some cells must die to give a tissue or an organ its final shape. Carving fingers from stubby limb buds requires the precisely timed death of cells between developing finger bones. During development of the nematode *C. elegans* from a fertilized egg, exactly 131 cells (of a total of 1,090 somatic cells in the embryo) must undergo programmed death in order to construct the adult body.

Apoptosis also has roles in processes other than development. If a developing antibody-producing cell generates antibodies against a protein or glycoprotein that is normally present in the body, that cell undergoes programmed death in the thymus gland—an essential mechanism for eliminating anti-self antibodies (the cause of many autoimmune diseases). The monthly sloughing of cells of the uterine wall (menstruation) is another case of apoptosis mediating normal cell death. The dropping of leaves in the fall is the result of apoptosis in specific cells of the stem of a plant. Sometimes cell suicide is not programmed but occurs in response to biological circumstances that threaten the rest of the organism. For example, a virus-infected cell that dies before completion of the infection cycle prevents spread of the virus to nearby cells. Severe stresses such as heat, hyperosmolarity, UV light, and gamma irradiation also trigger cell suicide; presumably the organism is better off with any aberrant, potentially mutated cells dead.

P8 The regulatory mechanisms that trigger apoptosis involve some of the same proteins that regulate the cell cycle. The signal for suicide often comes from outside, through a surface receptor. Tumor necrosis factor (TNF), produced by cells of the immune system, interacts with cells through specific TNF receptors. These receptors have TNF-binding sites on the outer face of the plasma membrane and a "death domain" (~80 amino acid residues) that carries the self-destruct signal through the membrane to cytosolic proteins such as TRADD (TNF receptor–associated death domain) (**Fig. 12-42**).

When caspase-8, an "initiator" caspase, is activated by an apoptotic signal carried through TRADD, it further self-activates by cleaving its own proenzyme form. Mitochondria are one target of active caspase-8. The protease causes the release of certain proteins contained between the inner and outer mitochondrial membranes: cytochrome *c* and several "effector" caspases (see Fig. 19-39). Cytochrome *c* binds to the proenzyme form of the effector enzyme caspase-9 and stimulates its proteolytic activation. The activated caspase-9, in turn, catalyzes wholesale destruction of cellular proteins—a major cause of apoptotic cell death. One specific target of caspase action is a caspase-activated deoxyribonuclease.

In apoptosis, the monomeric products of protein and DNA degradation (amino acids and nucleotides) are

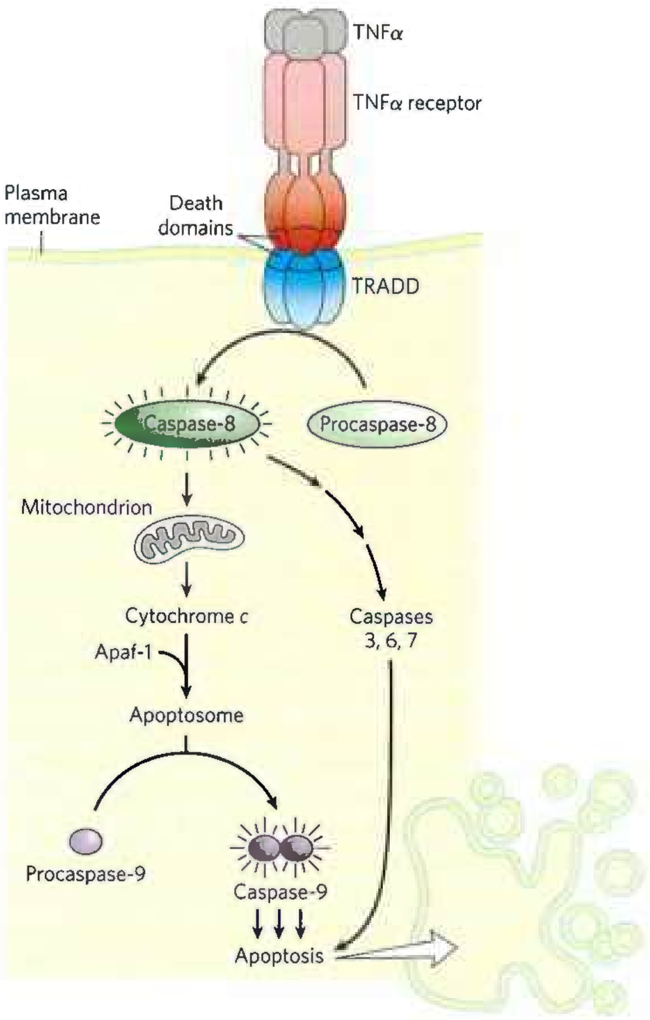


FIGURE 12-42 **Initial events of apoptosis.** An apoptosis-triggering signal from outside the cell (TNFα) binds to its specific receptor in the plasma membrane. The occupied receptor interacts with the cytosolic protein TRADD through "death domains" (80-residue domains on both TNFα receptor and TRADD), activating TRADD. Activated TRADD initiates a proteolytic cascade that leads to apoptosis: TRADD activates caspase-8, which acts to release cytochrome c from mitochondria, which, in concert with protein Apaf-1, activates caspase-9, triggering apoptosis.

released in a controlled process that allows them to be taken up and reused by neighboring cells. Apoptosis thus allows the organism to eliminate a cell that is unneeded or potentially dangerous without wasting its components.

SUMMARY 12.9 *Oncogenes, Tumor Suppressor Genes, and Programmed Cell Death*

- Oncogenes encode defective signaling proteins. By continually giving the signal for cell division, they lead to tumor formation. Oncogenes are genetically dominant and may encode defective growth factors, receptors, G proteins, protein kinases, or nuclear regulators of transcription.
- Tumor suppressor genes encode regulatory proteins that normally inhibit cell division; mutations in these genes are genetically recessive but can lead to tumor formation. Cancer is generally the result of an accumulation of mutations in oncogenes and tumor suppressor genes.
- When stability genes, which encode proteins necessary for the repair of genetic damage, are mutated, other mutations go unrepaired, including mutations in proto-oncogenes and tumor suppressor genes that can lead to cancer.
- Apoptosis is programmed and controlled cell death that functions during normal development and adulthood to destroy and recycle unnecessary, damaged, or infected cells. Apoptosis can be triggered by extracellular signals such as TNF, acting through plasma membrane receptors.

KEY TERMS

Terms in bold are defined in the glossary.

signal transduction	408
specificity	409
sensitivity	409
amplification	409
enzyme cascade	409
modularity	409
scaffold proteins	409
desensitization	409
integration	409
divergence	409
response localization	409
G protein–coupled receptors (GPCRs)	412
guanosine nucleotide–binding proteins	412
G proteins	412
second messenger	412
agonist	412
antagonist	412
β-adrenergic receptors	413
seven-transmembrane (7tm) receptors	413
guanosine nucleotide–exchange factor (GEF)	413
stimulatory G protein (G_s)	413
adenylyl cyclase	413
cAMP-dependent protein kinase (protein kinase A; PKA)	413
consensus sequence	414
green fluorescent protein (GFP)	416
fluorescence resonance energy transfer (FRET)	416
β-adrenergic receptor kinase (βARK)	418
β-arrestin (βarr)	418
G protein–coupled receptor kinases (GRKs)	419
cAMP response element binding protein (CREB)	420
inhibitory G protein (G_i)	420
adaptor proteins	420
AKAPs (A kinase anchoring proteins)	420
Ras	421
guanosine 3′,5′-cyclic monophosphate (cyclic GMP; cGMP)	422
cGMP-dependent protein kinase (protein kinase G; PKG)	422
GTPase activator protein (GAP)	422
regulator of G-protein signaling (RGS)	422
NO synthase	423
phospholipase C (PLC)	425
inositol 1,4,5-trisphosphate (IP_3)	425
IP_3-gated Ca^{2+} channel	425

protein kinase C (PKC)	425
calmodulin (CaM)	426
Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases)	426
rhodopsin	429
rhodopsin kinase	431
receptor potential	431
receptor Tyr kinase (RTK)	433
autophosphorylation	434
SH2 domain	435
MAPKs	435
voltage-gated ion channels	442
ionotropic	444
metabotropic	444
hormone response element (HRE)	445
cyclin	447
cyclin-dependent protein kinase (CDK)	447
ubiquitin	448
proteasome	448
growth factors	448
retinoblastoma protein (pRb)	450
oncogene	451
proto-oncogene	451
tumor suppressor gene	451
programmed cell death	455
apoptosis	455

PROBLEMS

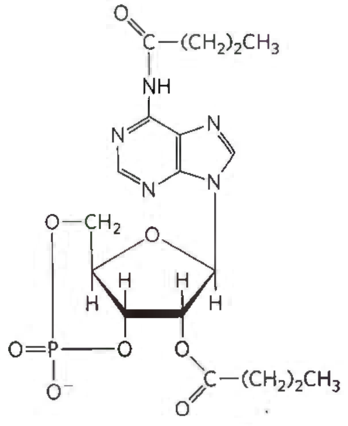
1. Hormone Experiments in Cell-Free Systems In the 1950s, Earl W. Sutherland, Jr., and his colleagues carried out pioneering experiments to elucidate the mechanism of action of epinephrine and glucagon. Given what you have learned in this chapter about hormone action, interpret each of the experiments described below. Identify substance X and indicate the significance of the results.

(a) Addition of epinephrine to a homogenate of normal liver resulted in an increase in the activity of glycogen phosphorylase. However, when the homogenate was first centrifuged at a high speed and epinephrine or glucagon was added to the clear supernatant fraction that contains phosphorylase, no increase in the phosphorylase activity occurred.

(b) When the particulate fraction from the centrifugation in (a) was treated with epinephrine, substance X was produced. The substance was isolated and purified. Unlike epinephrine, substance X activated glycogen phosphorylase when added to the clear supernatant fraction of the centrifuged homogenate.

(c) Substance X was heat-stable; that is, heat treatment did not affect its capacity to activate phosphorylase. (Hint: Would this be the case if substance X were a protein?) Substance X was nearly identical to a compound obtained when pure ATP was treated with barium hydroxide. (Fig. 8-6 will be helpful.)

2. Effect of Dibutyryl cAMP versus cAMP on Intact Cells In principle, the physiological effects of epinephrine should be mimicked by addition of cAMP to the target cells. In practice, addition of cAMP to intact target cells elicits only a minimal physiological response. Why? When the structurally related derivative dibutyryl cAMP (shown) is added to intact cells, the expected physiological response is readily apparent. Explain the basis for the difference in cellular response to these two substances. Dibutyryl cAMP is widely used in studies of cAMP function.



Dibutyryl cAMP
(N^6,$O^{2'}$-Dibutyryl adenosine 3′,5′-cyclic monophosphate)

3. Effect of Cholera Toxin on Adenylyl Cyclase The gram-negative bacterium *Vibrio cholerae* produces a protein, cholera toxin (M_r 90,000), that is responsible for the characteristic symptoms of cholera: extensive loss of body water and Na^+ through continuous, debilitating diarrhea. If body fluids and Na^+ are not replaced, severe dehydration results; untreated, the disease is often fatal. When the cholera toxin gains access to the human intestinal tract, it binds tightly to specific sites in the plasma membrane of the epithelial cells lining the small intestine, causing adenylyl cyclase to undergo prolonged activation (hours or days).

(a) What is the expected effect of cholera toxin on [cAMP] in the intestinal cells?

(b) Based on the information above, suggest how cAMP normally functions in intestinal epithelial cells.

(c) Suggest a possible treatment for cholera.

4. Mutations in PKA Explain how mutations in the R or C subunit of cAMP-dependent protein kinase (PKA) might lead to **(a)** a constantly active PKA or **(b)** a constantly inactive PKA.

5. Therapeutic Effects of Albuterol The respiratory symptoms of asthma result from constriction of the bronchi and bronchioles of the lungs, caused by contraction of the smooth muscle of their walls. Raising [cAMP] in the smooth muscle reverses the constriction of the bronchi and bronchioles. Explain the therapeutic effects of albuterol, an inhaled β-adrenergic agonist, in treating asthma. Would you expect this drug to have any side effects? If so, what design change could you make to the drug to minimize side effects?

6. Termination of Hormonal Signals Signals carried by hormones must eventually be terminated. Describe several mechanisms for signal termination.

7. Using FRET to Explore Protein-Protein Interactions In Vivo Figure 12-9 shows the interaction between β-arrestin and the β-adrenergic receptor. How would you use FRET (see Box 12-1) to demonstrate this interaction in living cells? Which proteins would you fuse? Which wavelengths would you use to illuminate the cells, and which wavelengths would you monitor? What would you expect to observe if the interaction occurred? If it did not occur? How might you explain the failure of this approach to demonstrate this interaction?

8. EGTA Injection EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid) is a chelating agent with high affinity and specificity for Ca^{2+}. By microinjecting a cell with an appropriate Ca^{2+}-EGTA solution, an experimenter can prevent cytosolic $[Ca^{2+}]$ from rising above 10^{-7} M. How would EGTA microinjection affect a cell's response to vasopressin (see Table 12-4)? To glucagon?

9. Amplification and Termination of Hormonal Signals In the β-adrenergic system, which of these contributes to the amplification of the signal (epinephrine) and which to the termination of the signal? Do any contribute to both amplification and termination of the signal?

(a) One G_α activates many adenylyl cyclase molecules.

(b) One protein kinase A (PKA) phosphorylates many target proteins.

(c) The intrinsic GTPase of G protein converts bound GTP to GDP.

(d) A phosphodiesterase acts on many molecules of cAMP.

(e) One epinephrine molecule activates many adrenergic receptors.

(f) One protein kinase phosphorylates many molecules of another protein kinase.

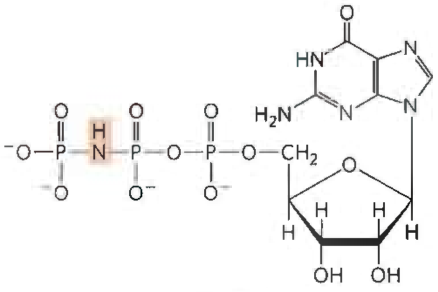
10. The Insulin Signaling System Place these components of the insulin receptor system in the order in which they occur in the sequence of events triggered by insulin: MEK, Ras, ERK, GRK, Raf, Sos, IRS1, PKA, Grb2. Some of these may not participate in that path.

11. Mutations in *ras* How would a mutation in *ras* that leads to formation of a Ras protein with no GTPase activity affect a cell's response to insulin?

12. Differences among G Proteins Compare the G protein G_s, which acts in transducing the signal from β-adrenergic receptors, and the G protein Ras. What properties do they share? How do they differ? What is the functional difference between G_s and G_i?

13. Mechanisms for Regulating Protein Kinases Identify eight general types of protein kinases found in eukaryotic cells, and explain what factor is *directly* responsible for activating each type.

14. Nonhydrolyzable GTP Analogs Many enzymes can hydrolyze GTP between the β and γ phosphates. The GTP analog β,γ-imidoguanosine 5′-triphosphate (Gpp(NH)p), shown here, cannot be hydrolyzed between the β and γ phosphates.



Gpp(NH)p
(β,γ-imidoguanosine 5′-triphosphate)

Predict the effect of microinjection of Gpp(NH)p into a myocyte on the cell's response to β-adrenergic stimulation.

15. Visual Desensitization Oguchi disease is an inherited form of night blindness. Affected individuals are slow to recover vision after a flash of bright light against a dark background, such as the headlights of a car on the freeway. Suggest what the molecular defect(s) might be in Oguchi disease. Explain in molecular terms how this defect would account for night blindness.

16. Effect of a Permeant cGMP Analog on Rod Cells An analog of cGMP, 8-Br-cGMP, will permeate cellular membranes, is only slowly degraded by a rod cell's PDE activity, and is as effective as cGMP in opening the gated channel in the cell's outer segment. If you suspended rod cells in a buffer containing a relatively high [8-Br-cGMP], then illuminated the cells while measuring their membrane potential, what would you expect to see?

17. Effect of Insulin on Glycogen Synthesis Protein kinase B (PKB) inactivates glycogen synthase kinase (GSK3), and GSK3 inactivates glycogen synthase. Predict the effect of insulin on glycogen synthesis.

18. Role of Intrinsically Disordered Regions of Signaling Proteins Signaling proteins, including protein kinases, often have intrinsically disordered regions (IDRs) that are important in signaling. Describe a case in which IDRs and their interactions with other proteins are important in signaling.

19. The Action Potential Place these events in the order in which they occur after a presynaptic neuron releases acetylcholine into the synaptic cleft.

(a) Vesicles containing a neurotransmitter fuse with the cell membrane.

(b) Ligand-gated Na^+ channels open, causing an influx of Na^+ ions.

(c) Voltage-gated Na^+ channels open in the axon.

(d) Membrane depolarization triggers voltage-gated Ca^{2+} channels to open.

(e) Local membrane depolarization in the axon triggers an efflux of K^+.

20. Hot and Cool Taste Sensations The sensations of heat and cold are transduced by a group of temperature-gated cation channels. For example, TRPV1, TRPV3, and TRPM8 are usually closed, but they open at different temperatures. TRPV1 opens at ≥43 °C, TRPV3 opens at ≥33 °C, and TRPM8 opens at <25 °C. These channel proteins are expressed in sensory neurons known to be responsible for temperature sensation.

(a) Propose a reasonable model to explain how exposing a sensory neuron containing TRPV1 to high temperature leads to a sensation of heat.

(b) Capsaicin, one of the active ingredients in "hot" peppers, is an agonist of TRPV1. Capsaicin shows 50% activation of the TRPV1 response at a concentration of 32 nM—a property known as EC_{50}. Explain why even a very few drops of hot pepper sauce can taste very "hot" without actually burning you.

(c) Menthol, one of the active ingredients in mint, is an agonist of TRPM8 ($EC_{50} = 30\ \mu M$) and TRPV3 ($EC_{50} = 20$ mM). What sensation would you expect from contact with low levels of menthol? With high levels?

21. Oncogenes, Tumor Suppressor Genes, and Tumors For each of the situations listed, provide a plausible explanation for how it could lead to unrestricted cell division.

(a) Colon cancer cells often contain mutations in the gene encoding the prostaglandin E_2 receptor. PGE_2 is a growth factor required for the division of cells in the gastrointestinal tract.
(b) Kaposi sarcoma, a common tumor in people with untreated AIDS, is caused by a virus carrying a gene for a protein similar to the chemokine receptors CXCR1 and CXCR2. Chemokines are cell-specific growth factors.
(c) Adenovirus, a tumor virus, carries a gene for the protein E1A, which binds to the retinoblastoma protein, pRb. (Hint: See Fig. 12-40.)
(d) An important feature of many oncogenes and tumor suppressor genes is their cell-type specificity. For example, mutations in the PGE_2 receptor are not typically found in lung tumors. Explain this observation. (Note that PGE_2 acts through a GPCR in the plasma membrane.)

22. Mutations in Tumor Suppressor Genes and Oncogenes Explain why mutations in tumor suppressor genes are recessive (both copies of the gene must be defective for the regulation of cell division to be defective), whereas mutations in oncogenes are dominant.

23. Retinoblastoma in Children Explain why some children with retinoblastoma develop multiple tumors of the retina in both eyes, whereas others have a single tumor in only one eye.

24. Specificity of a Signal for a Single Cell Type Discuss the validity of the proposition that a signaling molecule (hormone, growth factor, or neurotransmitter) elicits identical responses in different types of target cells if those cells contain identical receptors.

DATA ANALYSIS PROBLEM

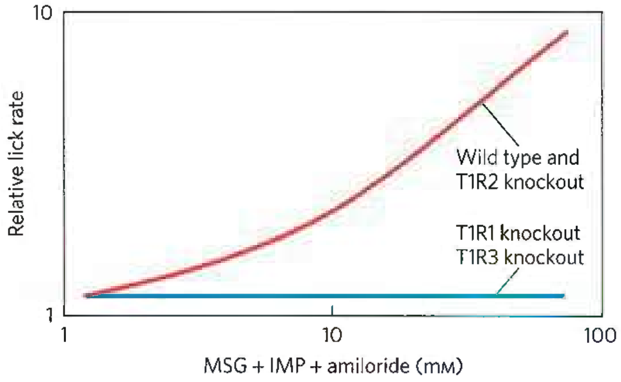
25. Exploring Taste Sensation in Mice Pleasing tastes are an evolutionary adaptation to encourage animals to consume nutritious foods. Zhao and coauthors (2003) examined the two major pleasurable taste sensations: sweet and umami. Umami is a "distinct savory taste" triggered by amino acids, especially aspartate and glutamate, and it probably encourages animals to consume protein-rich foods. Monosodium glutamate (MSG) is a flavor-enhancer that exploits this sensitivity.

At the time the article was published, three taste receptor proteins for sweet and umami had been tentatively characterized: T1R1, T1R2, and T1R3. These proteins function as heterodimeric receptor complexes: T1R1-T1R3 was tentatively identified as the umami receptor, and T1R2-T1R3 as the sweet receptor. It was not clear how taste sensation was encoded and sent to the brain, and two possible models had been suggested. In the cell-based model, individual taste-sensing cells express only one kind of receptor; that is, there are "sweet cells," "bitter cells," "umami cells," and so on, and each type of cell sends its information to the brain via a different nerve. The brain "knows" which taste is detected by the identity of the nerve fiber that transmits the message. In the receptor-based model, individual taste-sensing cells have several kinds of receptors and send different messages along the same nerve fiber to the brain, the message depending on which receptor is activated. Also unclear at the time was whether there was any interaction between the different taste sensations, or whether parts of one taste-sensing system were required for other taste sensations.

(a) Previous work had shown that different taste receptor proteins are expressed in nonoverlapping sets of taste receptor cells. Which model does this support? Explain your reasoning.

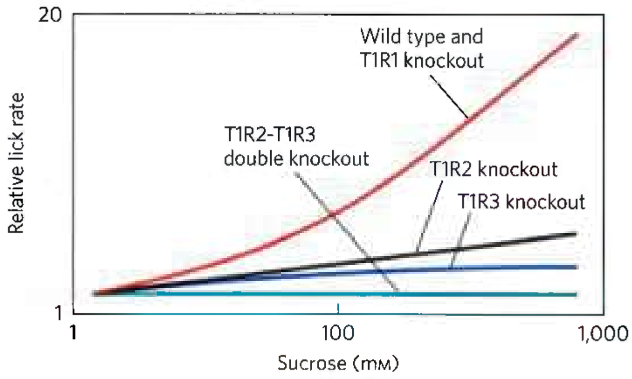
Zhao and colleagues constructed a set of "knockout mice"—mice homozygous for loss-of-function alleles for one of the three receptor proteins, T1R1, T1R2, or T1R3—and double-knockout mice with nonfunctioning T1R2 and T1R3. The researchers measured the taste perception of these mice by measuring their "lick rate" of solutions containing different taste molecules. Mice will lick the spout of a feeding bottle with a pleasant-tasting solution more often than one with an unpleasant-tasting solution. The researchers measured relative lick rates: how often the mice licked a sample solution compared with water. A relative lick rate of 1 indicated no preference; <1, an aversion; and >1, a preference.
(b) All four types of knockout strains had the same responses to salt and bitter tastes as did wild-type mice. Which of the above issues did this experiment address? What do you conclude from these results?

The researchers then studied umami taste reception by measuring the relative lick rates of the different mouse strains with different quantities of MSG in the feeding solution. Note that the solutions also contained inosine monophosphate (IMP), a strong potentiator of umami taste reception (and a common ingredient in ramen soups, along with MSG), and amiloride, which suppresses the pleasant salty taste imparted by the sodium of MSG. The results are shown in the graph.



(c) Are these data consistent with the umami taste receptor consisting of a heterodimer of T1R1 and T1R3? Why or why not?
(d) Which model(s) of taste encoding does this result support? Explain your reasoning.

Zhao and coworkers then performed a series of similar experiments using sucrose as a sweet taste. These results are shown below.



(e) Are these data consistent with the sweet taste receptor consisting of a heterodimer of T1R2 and T1R3? Why or why not?

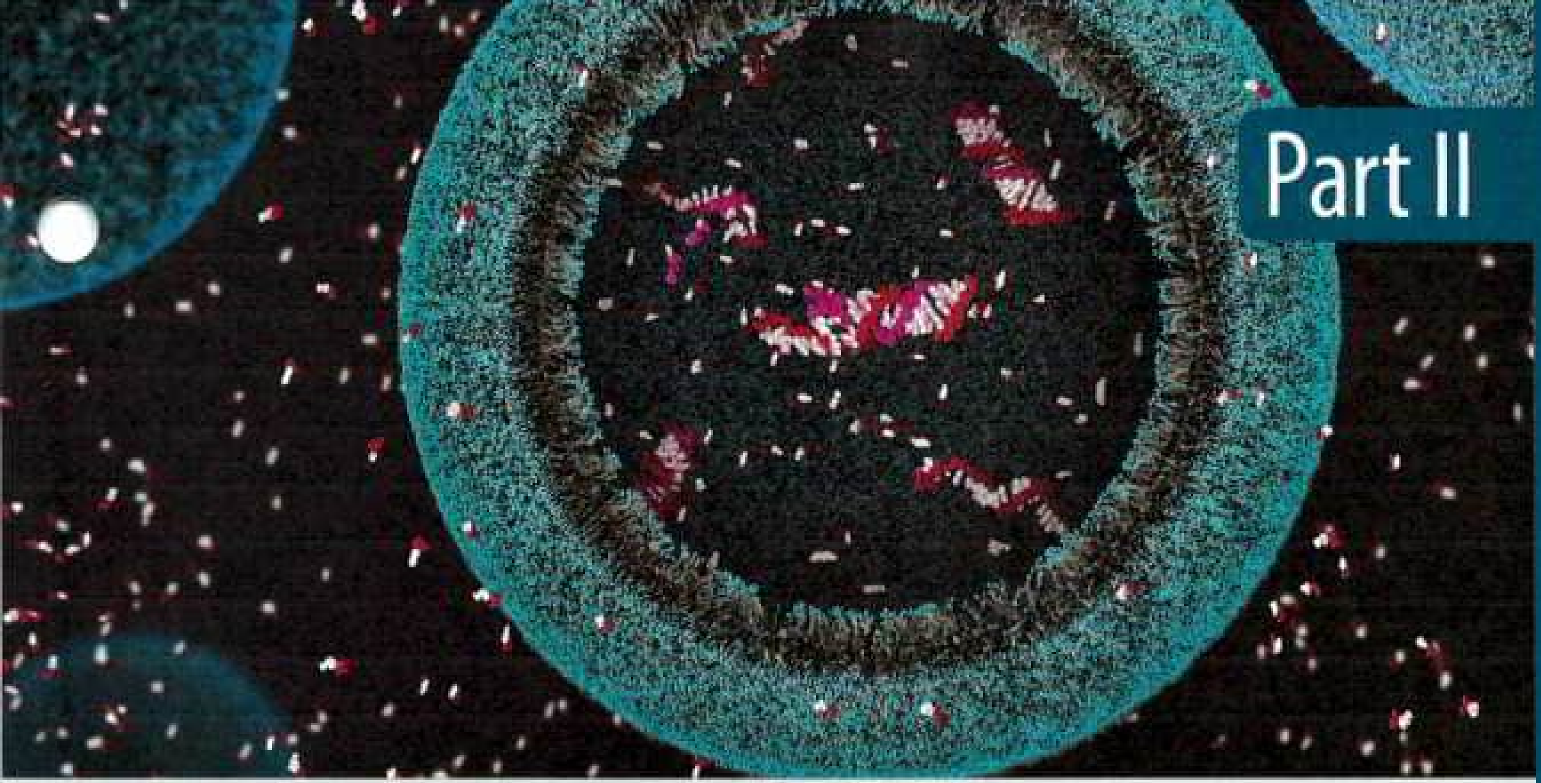
(f) There were some unexpected responses at very high sucrose concentrations. How do these complicate the idea of a heterodimeric system as presented above?

In addition to sugars, humans also taste other compounds (e.g., saccharin and the peptides monellin and aspartame) as sweet; mice do not taste these as sweet. Zhao and coworkers inserted into TIR2-knockout mice a copy of the human *T1R2* gene under the control of the mouse T1R2 promoter. These modified mice now tasted monellin and saccharin as sweet. The researchers then went further, adding to T1R1-knockout mice the RASSL protein—a G protein–linked receptor for the synthetic opiate spiradoline; the *RASSL* gene was under the control of a promoter that could be induced by feeding the mice tetracycline. These mice did not prefer spiradoline in the absence of tetracycline; in the presence of tetracycline, they showed a strong preference for nanomolar concentrations of spiradoline.

(g) Do these results strengthen your conclusions about the mechanism of taste sensation?

Reference

Zhao, G.Q., Y. Zhang, M.A. Hoon, J. Chandrashekar, I. Erlenbach, N.J.P. Ryba, and C. Zuker. 2003. The receptors for mammalian sweet and umami taste. *Cell* 115:255–266.



Part II

BIOENERGETICS AND METABOLISM

PART OUTLINE

13 Introduction to Metabolism 465

14 Glycolysis, Gluconeogenesis, and the Pentose Phosphate Pathway 510

15 The Metabolism of Glycogen in Animals 556

16 The Citric Acid Cycle 574

17 Fatty Acid Catabolism 601

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19 Oxidative Phosphorylation 659

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21 Lipid Biosynthesis 744

22 Biosynthesis of Amino Acids, Nucleotides, and Related Molecules 794

23 Hormonal Regulation and Integration of Mammalian Metabolism 841

Every enzyme-catalyzed reaction and reaction sequence serves an important role in an organism's physiology: to (1) obtain chemical energy by capturing solar energy or degrading energy-rich nutrients from the environment; (2) convert nutrient molecules into the cell's own characteristic molecules, including precursors of macromolecules; (3) polymerize monomeric precursors into macromolecules: proteins, nucleic acids, and polysaccharides; and (4) synthesize and degrade biomolecules required for specialized cellular functions, such as membrane lipids, intracellular messengers, and pigments.

Although metabolism embraces many thousands of different enzyme-catalyzed reactions, our major concern in Part II is the central metabolic pathways, which are few in number and remarkably similar in all forms of life. Living organisms can be divided into two large groups according to the chemical form in which they obtain carbon from the environment. **Autotrophs** (such as photosynthetic bacteria, green algae, and vascular plants) can use carbon dioxide from the atmosphere as their sole source of carbon, from which they construct all their carbon-containing biomolecules (see Fig. 1). **Heterotrophs** cannot use atmospheric carbon dioxide and must obtain carbon from their environment in

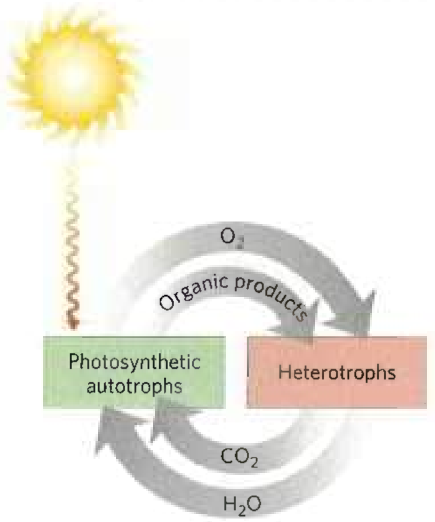


FIGURE 1 Cycling of carbon dioxide and oxygen between the autotrophic (photosynthetic) and heterotrophic domains in the biosphere. The flow of mass through this cycle is enormous; about 4×10^{11} metric tons of carbon are turned over in the biosphere annually.

the form of relatively complex organic molecules such as glucose. Multicellular animals and most microorganisms are heterotrophic. Autotrophic cells and organisms are relatively self-sufficient, whereas heterotrophic cells and organisms, with their requirements for carbon in more complex forms, must subsist on the products of other organisms.

Many autotrophic organisms are photosynthetic and obtain their energy from sunlight, whereas heterotrophic organisms obtain their energy from the degradation of organic nutrients produced by autotrophs. In our biosphere, autotrophs and heterotrophs live together in a vast, interdependent cycle in which autotrophic organisms use atmospheric carbon dioxide to build their organic biomolecules, some of them generating oxygen from water in the process. Heterotrophs, in turn, use the organic products of autotrophs as nutrients, which they oxidize, releasing to the atmosphere the CO_2 produced by oxidation. Thus carbon, oxygen, and water are constantly cycled between the heterotrophic and autotrophic worlds, with solar energy as the driving force for this global process. A similarly complex global web connects molecular nitrogen (N_2) with nitrogen in its other oxidation states, including NH_3 (the form in which nitrogen enters metabolism). The cycling of carbon, oxygen, and nitrogen, which ultimately involves all species, depends on a proper balance between the activities of the producers (autotrophs) and consumers (heterotrophs) in our biosphere. Global warming by the greenhouse effect (the result of rising CO_2 concentrations in our atmosphere) is a biochemical phenomenon, occurring on a very large scale.

These cycles of matter are driven by an enormous flow of energy into and through the biosphere, beginning with the capture of solar energy by photosynthetic organisms and use of this energy to generate energy-rich carbohydrates and other organic nutrients; these nutrients are then used as energy sources by heterotrophic organisms.

Precursors are converted into products through a series of intermediates called **metabolites**. The term **intermediary metabolism** is often applied to the combined activities of all the metabolic pathways that interconvert precursors, metabolites, and products of low molecular weight (generally, $M_r < 1{,}000$). **Catabolism** is the degradative phase of metabolism in which organic nutrient molecules (carbohydrates, fats, and proteins) are converted into smaller, simpler end products (such as lactic acid, CO_2, and NH_3). Catabolic pathways release energy, some of which is conserved in the formation of ATP and reduced electron carriers (NADH or NADPH); the rest is lost as heat. In **anabolism**, also called biosynthesis, small, simple precursors are built up into larger and more complex molecules, including lipids, polysaccharides, proteins, and nucleic acids. Anabolic reactions require an input of energy, generally in the form of the phosphoryl group transfer potential of ATP and the reducing power of NADH and NADPH (Fig. 2).

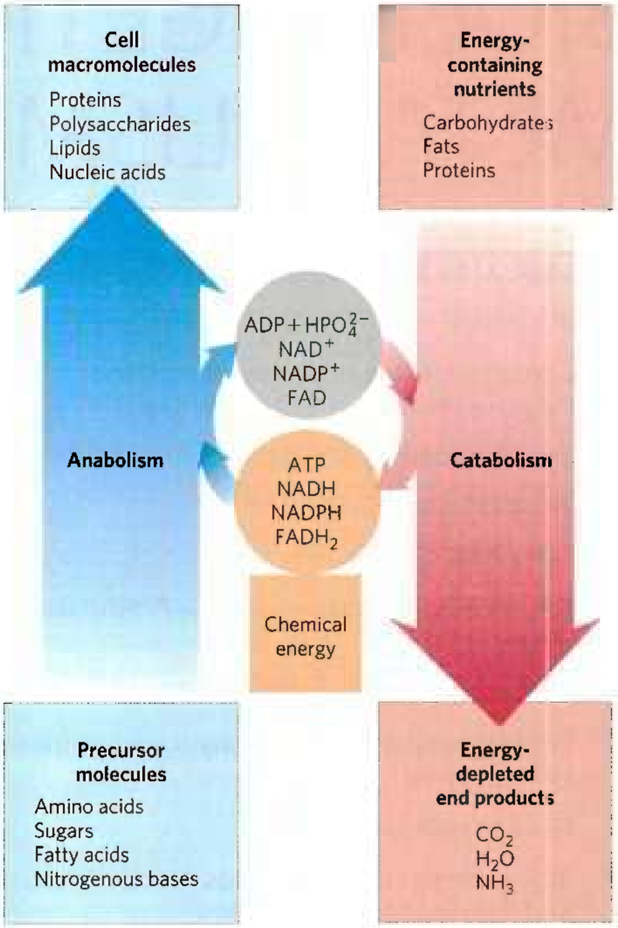


FIGURE 2 The big picture: energy relationships between catabolic and anabolic pathways. Catabolic pathways deliver chemical energy in the form of ATP, NADH, NADPH, and $FADH_2$. These energy carriers are used in anabolic pathways to convert small precursor molecules into cellular macromolecules.

Some metabolic pathways are linear, and some are branched, yielding multiple useful end products from a single precursor or converting several starting materials into a single product. In general, catabolic pathways are *convergent* and anabolic pathways are *divergent* (Fig. 3). Some pathways are cyclic: one starting component of the pathway is regenerated in a series of reactions that converts another starting component into a product. We shall see examples of each type of pathway in the following chapters.

Most cells have the enzymes to carry out both the degradation and the synthesis of the important categories of biomolecules—fatty acids, for example. The simultaneous synthesis and degradation of fatty acids would be wasteful, however. This is prevented by reciprocally regulating the anabolic and catabolic reaction sequences: when one sequence is active, the other is suppressed. Such regulation could not occur if anabolic and catabolic pathways were catalyzed by exactly the same set of enzymes, operating in one direction for anabolism, the opposite direction for catabolism: inhibition of an enzyme involved in catabolism would also inhibit the reaction sequence in the anabolic direction. Catabolic and anabolic pathways that connect the same two end points (glucose →→ pyruvate, and pyruvate →→ glucose, for example) may employ many of the same enzymes; but, invariably, at least one of the steps is catalyzed by different enzymes in the catabolic and anabolic directions, and these enzymes are the sites of separate regulation. Moreover, for both anabolic and catabolic pathways to be essentially irreversible, the reactions unique to each direction must include at least one that is thermodynamically very favorable—in other words, a reaction for which the reverse reaction is very unfavorable.

As a further contribution to the separate regulation of catabolic and anabolic reaction sequences, paired catabolic and anabolic pathways commonly take place in different cellular compartments: for example, fatty acid catabolism occurs in animal mitochondria, whereas fatty acid synthesis occurs in the cytosol. The concentrations of intermediates, enzymes, and regulators can be maintained at different levels in these different compartments. Because metabolic pathways are subject to kinetic control by substrate concentration, separate pools of anabolic and catabolic intermediates also contribute to the control of metabolic rates. Devices that separate anabolic and catabolic processes will be of particular interest in our discussions of metabolism.

Metabolic pathways are regulated at several levels, from within the cell and from outside. A key enzyme in a pathway may be activated allosterically, or its amount may be changed by the rates of synthesis and breakdown of the enzyme. In multicellular organisms, the metabolic activities of different tissues are regulated and integrated by growth factors and hormones that act from outside the cell.

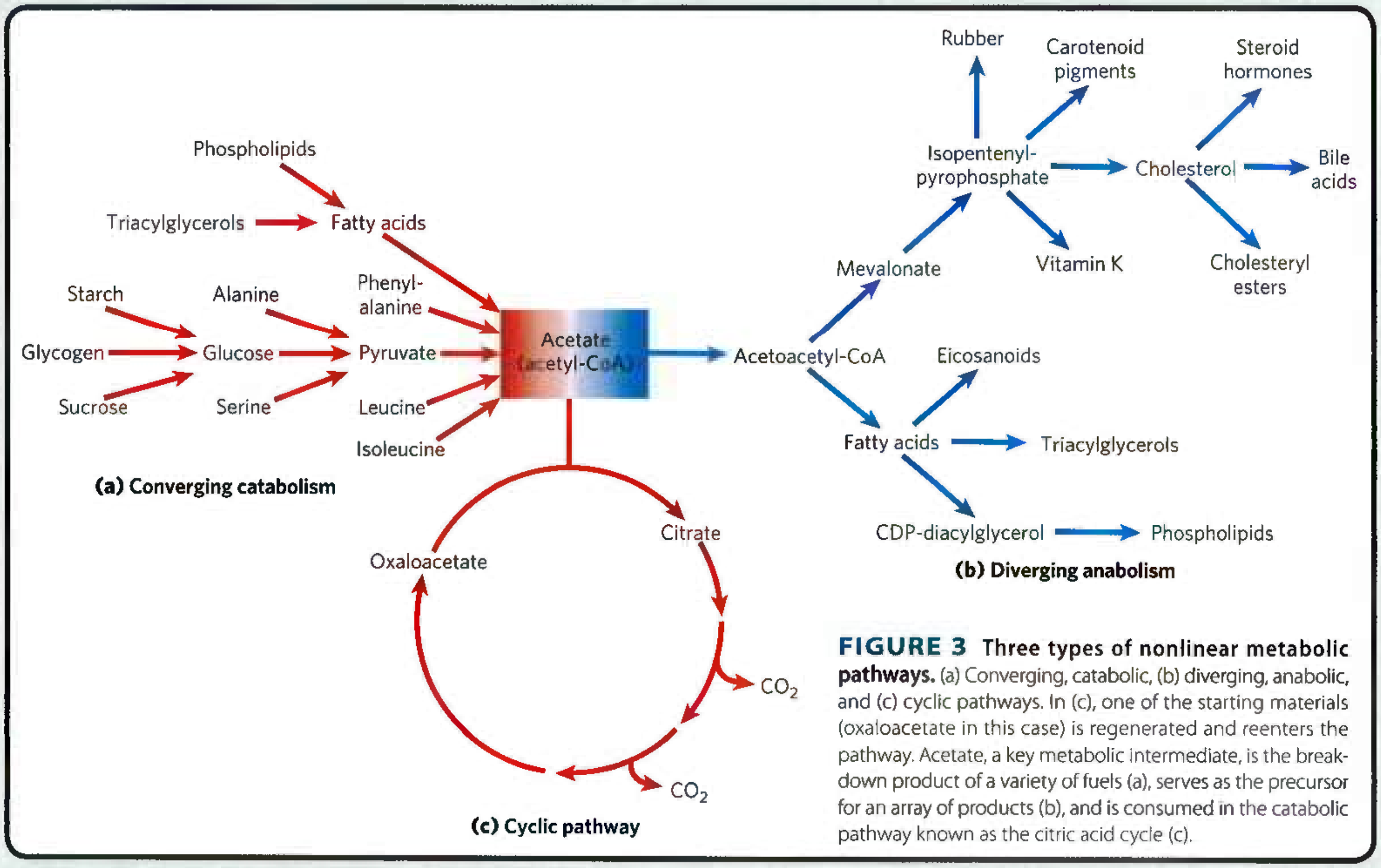

Phospholipids
Triacylglycerols
Fatty acids
Starch
Alanine
Phenyl-alanine
Glycogen
Glucose
Pyruvate
Sucrose
Serine
Leucine
Isoleucine
(a) Converging catabolism
Acetate (acetyl-CoA)
Acetoacetyl-CoA
Mevalonate
Isopentenyl-pyrophosphate
Rubber
Carotenoid pigments
Steroid hormones
Cholesterol
Bile acids
Vitamin K
Cholesteryl esters
Eicosanoids
Fatty acids
Triacylglycerols
CDP-diacylglycerol
Phospholipids
(b) Diverging anabolism
Citrate
Oxaloacetate
CO_2
CO_2
(c) Cyclic pathway

FIGURE 3 Three types of nonlinear metabolic pathways. (a) Converging, catabolic, (b) diverging, anabolic, and (c) cyclic pathways. In (c), one of the starting materials (oxaloacetate in this case) is regenerated and reenters the pathway. Acetate, a key metabolic intermediate, is the breakdown product of a variety of fuels (a), serves as the precursor for an array of products (b), and is consumed in the catabolic pathway known as the citric acid cycle (c).

We begin Part II with a discussion of the basic energetic principles that govern all metabolism, as well as a refresher on the reactions from organic chemistry that we will see in metabolism (Chapter 13). We then consider the major catabolic pathways by which cells obtain energy from the oxidation of various fuels (Chapters 14 through 20). Chapters 19 and 20 represent the pivotal point of our discussion of metabolism; they concern chemiosmotic energy coupling, a universal mechanism in which a transmembrane electrochemical potential, produced either by substrate oxidation or by light absorption, drives the synthesis of ATP.

Chapters 20 through 22 describe the major anabolic pathways by which cells use the energy in ATP to produce carbohydrates, lipids, amino acids, and nucleotides from simpler precursors. In Chapter 23 we step back from our detailed look at the metabolic pathways—as they occur in all organisms, from *E. coli* to humans—and consider how they are regulated and integrated in mammals by hormonal mechanisms.

As we undertake our study of intermediary metabolism, a final word. Keep in mind that the many reactions described in these pages take place in, and play crucial roles in, living organisms. As you encounter each reaction and each pathway, ask: Where does this piece fit in the big picture? What does this chemical transformation do for the organism? How does this pathway interconnect with the other pathways operating simultaneously in the same cell to produce the energy and products required for cell maintenance and growth? What would be the expected result of a defect in this enzyme or that pathway? Studied with this perspective, metabolism provides fascinating and revealing insights into life, with countless applications in medicine, agriculture, and biotechnology.

Chapter 13

INTRODUCTION TO METABOLISM

13.1 Bioenergetics and Thermodynamics *466*

13.2 Chemical Logic and Common Biochemical Reactions *472*

13.3 Phosphoryl Group Transfers and ATP *479*

13.4 Biological Oxidation-Reduction Reactions *488*

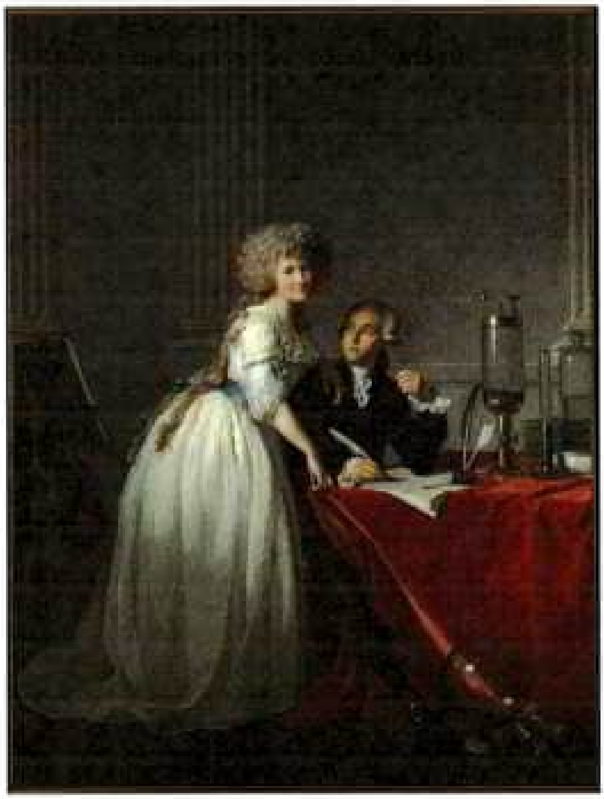
13.5 Regulation of Metabolic Pathways *496*

Living cells and organisms must perform work to stay alive, to grow, and to reproduce. The ability to harness energy and to channel it into biological work is a fundamental property of all living organisms; it must have been acquired very early in cellular evolution. Modern organisms carry out a remarkable variety of energy transductions, conversions of one form of energy to another. They use the chemical energy in fuels to bring about the synthesis of complex, highly ordered macromolecules from simple precursors. They also convert the chemical energy of fuels into concentration gradients and electrical gradients, into motion and heat, and, in a few organisms such as fireflies and some deep-sea fish, into light. Photosynthetic organisms transduce light energy into all these other forms of energy.

The chemical mechanisms that underlie biological energy transductions have fascinated and challenged biologists for centuries. The French chemist Antoine Lavoisier recognized that animals somehow transform chemical fuels (foods) into heat and that this process of respiration is essential to life. He observed that

> in general, respiration is nothing but a slow combustion of carbon and hydrogen, which is entirely similar to that which occurs in a lighted lamp or candle, and that, from this point of view, animals that respire are true combustible bodies that burn and consume themselves. . . . One may say that this analogy between combustion and respiration has not escaped the notice of the poets, or rather the philosophers of antiquity, and which they had expounded and interpreted. This fire stolen from heaven, this torch of Prometheus, does not only represent an ingenious and poetic idea, it is a faithful picture of the operations of nature, at least for animals that breathe; one may therefore say, with the ancients, that the torch of life lights itself at the moment the infant breathes for the first time, and it does not extinguish itself except at death.*

We now understand much of the chemistry underlying that "torch of life." Biological energy transductions



A portrait by Jacques Louis David of Antoine Lavoisier (1743–1794) in the laboratory with chemist Marie Anne Pierrette Paulze (1758–1836), his wife. [The Metropolitan Museum of Art, New York. Purchase, Mr. and Mrs. Charles Wrightsman Gift, in honor of Everett Fahy, 1977] obey the same chemical and physical laws that govern all other natural processes, and many of the types of chemical reactions that occur in living organisms have been long known to organic chemists. One unique feature of cellular chemistry is its exquisitely sensitive regulation by a variety of mechanisms that respond to changes in the external and internal circumstances of the cell and organism.

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* From a memoir by Armand Seguin and Antoine Lavoisier, dated 1789, quoted in A. Lavoisier, *Oeuvres de Lavoisier*, Imprimerie Impériale, Paris, 1862.

In this chapter we lay out the foundational principles for understanding the reactions of metabolism that follow in Part II. We first review the laws of thermodynamics and the quantitative relationships among free energy, enthalpy, and entropy. We then review the common types of biochemical reactions that occur in living cells, reactions that harness, store, transfer, and release the energy taken up by organisms from their surroundings. Our focus then shifts to reactions that have special roles in biological energy exchanges, particularly those involving the cofactors ATP (for phosphoryl transfers) and NADH (for electron transfers). Finally, we look at the most common of the strategies for regulating biochemical reactions. Watch for examples of these principles as you read this chapter:

P1 **The chemical changes and energy transductions in living organisms follow the laws of thermodynamics.**

P2 **The free-energy change is the maximum energy made available to do work when a chemical reaction occurs.** If two reactions can be combined to yield a third reaction, the overall free energy change is the sum of the two. Cells accomplish energy-requiring chemical work by coupling an energy-releasing (exergonic) reaction such as the cleavage of ATP to an endergonic reaction (which requires energy input).

P3 **Although thousands of different chemical reactions occur in the biosphere, most of them fall within a small set of reaction types.**

P4 **ATP is the universal energy currency in living organisms.** Transfer of its phosphoryl group to a water molecule or metabolic intermediates provides the energetic push for muscle contraction, the pumping of solutes against concentration gradients, and the synthesis of complex molecules.

P5 **Oxidation-reduction reactions indirectly provide much of the energy needed to make ATP.** Reduced substrates such as glucose are oxidized in several steps, with the energy of oxidation steps conserved in the form of a reduced cofactor, NADH. Energy stored in NADH is used to drive the synthesis of ATP.

P6 **To respond to changes in external circumstances, cells must regulate enzyme activities**, by changing either the number of enzyme molecules or the catalytic activity of preexisting enzyme molecules.

13.1 Bioenergetics and Thermodynamics

Bioenergetics is the quantitative study of **energy transductions**—changes of one form of energy into another—that occur in living cells, and of the nature and function of the chemical processes underlying these transductions. Although many of the principles of thermodynamics have been introduced in earlier chapters and may be familiar to you, a review of the quantitative aspects of these principles is useful here.

Biological Energy Transformations Obey the Laws of Thermodynamics

Many quantitative observations made by physicists and chemists on the interconversion of different forms of energy led, in the nineteenth century, to the formulation of two fundamental laws of thermodynamics. The first law is the principle of the conservation of energy: *for any physical or chemical change, the total amount of energy in the universe remains constant; energy may change form or it may be transported from one region to another, but it cannot be created or destroyed.* The second law of thermodynamics, which can be stated in several forms, says that the universe always tends toward increasing disorder: *in all natural processes, the entropy of the universe increases.*

FACTS OF LIFE

"Now, in the *second* law of thermodynamics . . ."

[ScienceCartoonsPlus.com]

Living organisms consist of collections of molecules much more highly organized than the surrounding materials from which they are constructed, and organisms maintain and produce order, seemingly immune to the second law of thermodynamics. But living organisms do not violate the second law; they operate strictly within it. To discuss the application of the second law to biological systems, we must first define those systems and their surroundings.

The *reacting system* is the collection of matter that is undergoing a particular chemical or physical process; it may be an organism, a cell, or two reacting compounds. The reacting system and its *surroundings* together constitute the *universe*. In the laboratory, some chemical or physical processes can be carried out in isolated or closed systems, in which no material or energy is exchanged with the surroundings. Living cells and organisms, however, are open systems, exchanging both material and energy with their surroundings; living systems are never at equilibrium with their surroundings, and the constant transactions between system and surroundings explain how organisms can create order within themselves while operating within the second law of thermodynamics.

In Chapter 1 (p. 21) we defined three thermodynamic quantities that describe the energy changes occurring in a chemical reaction:

> **Free energy**, ***G*** (for J. Willard Gibbs), expresses the amount of energy capable of doing work during a reaction at constant temperature and pressure. When a reaction proceeds with the release of free energy (that is, when the system changes so as to possess less free energy), the free-energy change, ΔG, has a negative value and the reaction is said to be **exergonic**. In **endergonic** reactions, the system gains free energy and ΔG is positive.
>
> **Enthalpy**, ***H***, is the heat content of the reacting system. It reflects the number and kinds of chemical bonds (covalent and noncovalent) in the reactants and products. When a chemical reaction releases heat, it is said to be **exothermic**; the heat content of the products is less than that of the reactants, and the change in enthalpy, ΔH, has, by convention, a negative value. Reacting systems that take up heat from their surroundings are **endothermic** and have positive values of ΔH.
>
> **Entropy**, ***S***, is a quantitative expression for the randomness or disorder in a system (see Box 1-3). When the products of a reacting system are less complex and more disordered than the reactants, the reaction is said to proceed with a gain in entropy.

The units of ΔG and ΔH are joules/mole or calories/mole (recall that 1 cal = 4.184 J); units of entropy are joules/mole • Kelvin (J/mol • K) (**Table 13-1**).

TABLE 13-1 Some Physical Constants and Units Used in Thermodynamics

Boltzmann constant, $\mathbf{k} = 1.381 \times 10^{-23}$ J/K
Avogadro's number, $N = 6.022 \times 10^{23}$ mol^{-1}
Faraday constant, $\mathcal{F} = 96{,}480$ J/V • mol
Gas constant, $R = 8.315$ J/mol • K
(= 1.987 cal/mol • K)

Units of ΔG and ΔH are J/mol (or cal/mol)
Units of ΔS are J/mol • K (or cal/mol • K)
1 cal = 4.184 J

Units of absolute temperature, T, are Kelvin, K
25 °C = 298 K
At 25 °C, $RT = 2.478$ kJ/mol
(= 0.592 kcal/mol)

Under the conditions existing in biological systems (including constant temperature and pressure), changes in free energy, enthalpy, and entropy are related to each other quantitatively by the equation

$$\Delta G = \Delta H - T\,\Delta S \qquad (13\text{-}1)$$

in which ΔG is the change in Gibbs free energy of the reacting system, ΔH is the change in enthalpy of the system, T is the absolute temperature, and ΔS is the change in entropy of the system. By convention, ΔS has a positive sign when entropy increases and ΔH, as noted above, has a negative sign when heat is released by the system to its surroundings. Either of these conditions, both of which are typical of energetically favorable processes, tends to make ΔG negative. In fact, ΔG of a spontaneously reacting system is always negative.

P1 The second law of thermodynamics states that the entropy *of the universe* increases during all chemical and physical processes, but it does not require that the entropy increase take place *in the reacting system* itself. The order produced within cells as they grow and divide is more than compensated for by the disorder they create in their surroundings in the course of growth and division (see Box 1-3, case 2). In short, living organisms preserve their internal order by taking from their surroundings free energy in the form of nutrients or sunlight, and returning to their surroundings an equal amount of energy as heat and entropy.

Cells are isothermal systems — they function at essentially constant temperature (and also function at constant pressure). Heat flow is not a source of energy for cells, because heat can do work only as it passes to a zone or an object at a lower temperature. The energy that cells can and must use is free energy, described by the Gibbs free-energy function G, which allows prediction of the direction of chemical reactions, their exact equilibrium position, and the amount of work they can (in theory) perform at constant temperature and pressure. Heterotrophic cells acquire free energy from nutrient molecules, and photosynthetic cells acquire it from absorbed solar radiation. Both kinds of cells transform this free energy into ATP and other energy-rich compounds capable of providing energy for biological work at constant temperature.

Standard Free-Energy Change Is Directly Related to the Equilibrium Constant

The composition of a reacting system (a mixture of chemical reactants and products) tends to continue changing until equilibrium is reached. (In the case of an organism, equilibrium is reached only after death and complete decay.) At the equilibrium concentration of reactants and products, the rates of the forward and reverse reactions are exactly equal and no further net change occurs in the system. The concentrations of reactants and products *at equilibrium* define the equilibrium constant, K_{eq} (p. 23). In the general reaction

$$aA + bB \rightleftharpoons cC + dD$$

where a, b, c, and d are the number of molecules of A, B, C, and D participating, the equilibrium constant is given by

$$K_{eq} = \frac{[C]^c_{eq}[D]^d_{eq}}{[A]^a_{eq}[B]^b_{eq}} \qquad (13\text{-}2)$$

where $[A]_{eq}$, $[B]_{eq}$, $[C]_{eq}$, and $[D]_{eq}$ are the molar concentrations of the reaction components *at the point of equilibrium*.

When a reacting system is not at equilibrium, the tendency to move toward equilibrium represents a driving force, the magnitude of which can be expressed as the free-energy change for the reaction, ΔG. Under standard conditions of temperature and pressure and when reactants and products are initially present at 1 M concentrations or, for gases, at partial pressures of 101.3 kilopascals (kPa), or 1 atm, the force driving the system toward equilibrium is defined as the standard free-energy change, $\Delta G°$. By this definition, the standard state for reactions that involve hydrogen ions is $[H^+] = 1$ M, or pH 0. Most biochemical reactions, however, occur in well-buffered aqueous solutions near pH 7; both the pH and the concentration of water (55.5 M) are essentially constant.

KEY CONVENTION For convenience of calculations, biochemists define a different standard state from that used in chemistry and physics: in the biochemical standard state, $[H^+]$ is 10^{-7} M (pH 7) and $[H_2O]$ is 55.5 M. For reactions that involve Mg^{2+} (which include most of those with ATP as a reactant), $[Mg^{2+}]$ in solution is commonly taken to be constant at 1 mM. ■

Physical constants based on this biochemical standard state are called **standard transformed constants** and are written with a prime (such as $\Delta G'°$ and K'_{eq}) to distinguish them from the untransformed constants used by chemists and physicists. (Note that most other textbooks use the symbol $\Delta G°'$ rather than $\Delta G'°$. Our use of $\Delta G'°$, recommended by an international committee of chemists and biochemists, is intended to emphasize that the transformed free-energy change, $\Delta G'°$, is the criterion for equilibrium.) For simplicity, we will hereafter refer to these transformed constants as **standard free-energy changes** and **standard equilibrium constants**.

KEY CONVENTION In another simplifying convention used by biochemists, when H_2O, H^+, and/or Mg^{2+} are reactants or products, their concentrations are not included in equations such as Equation 13-2 but are instead incorporated into the constants K'_{eq} and $\Delta G'°$. ■

Just as K'_{eq} is a physical constant characteristic for each reaction, so too is $\Delta G'°$ a constant. As we noted in Chapter 6, there is a simple relationship between K'_{eq} and $\Delta G'°$:

$$\Delta G'° = -RT \ln K'_{eq} \qquad (13\text{-}3)$$

P1 *The standard free-energy change of a chemical reaction is simply an alternative mathematical way of expressing its equilibrium constant.* **Table 13-2** shows the relationship between $\Delta G'°$ and K'_{eq}. If the equilibrium constant for a given chemical reaction is 1.0, the standard free-energy change of that reaction is 0.0 (the natural logarithm of 1.0 is zero). If K'_{eq} of a reaction is greater than 1.0, its $\Delta G'°$ is negative. If K'_{eq} is less than 1.0, $\Delta G'°$ is positive. Because the relationship between $\Delta G'°$ and K'_{eq} is exponential, relatively small changes in $\Delta G'°$ correspond to large changes in K'_{eq}.

It may be helpful to think of the standard free-energy change in another way. $\Delta G'°$ is the difference between the free-energy content of the products and the free-energy content of the reactants, under standard conditions. When $\Delta G'°$ is negative, the products contain less free energy than the reactants and the reaction will proceed spontaneously under standard conditions; all chemical reactions tend to go in the direction that results in a decrease in the free energy of the system. A positive value of $\Delta G'°$ means that the products of the reaction contain more free energy than the reactants, and this reaction will tend to go in the reverse direction

TABLE 13-2 Relationship between Equilibrium Constants and Standard Free-Energy Changes of Chemical Reactions

	$\Delta G'°$	
K'_{eq}	(kJ/mol)	(kcal/mol)[a]
10^3	−17.1	−4.1
10^2	−11.4	−2.7
10^1	−5.7	−1.4
1	0.0	0.0
10^{-1}	5.7	1.4
10^{-2}	11.4	2.7
10^{-3}	17.1	4.1
10^{-4}	22.8	5.5
10^{-5}	28.5	6.8
10^{-6}	34.2	8.2

[a] Although joules and kilojoules are the standard units of energy and are used throughout this text, biochemists and nutritionists sometimes express $\Delta G'°$ values in kilocalories per mole. We have therefore included values in both kilojoules and kilocalories in this table and in Tables 13-4 and 13-6. To convert kilojoules to kilocalories, divide the number of kilojoules by 4.184.

TABLE 13-3 Relationships among K'_{eq}, $\Delta G'^\circ$, and the Direction of Chemical Reactions

When K'_{eq} is...	$\Delta G'^\circ$ is...	Starting with all components at 1 M, the reaction...
>1.0	negative	proceeds forward
1.0	zero	is at equilibrium
<1.0	positive	proceeds in reverse

if we start with 1.0 M concentrations of all components (standard conditions). **Table 13-3** summarizes these points.

WORKED EXAMPLE 13-1 *Calculation of $\Delta G'^\circ$*

Calculate the standard free-energy change of the reaction catalyzed by the enzyme phosphoglucomutase,

$$\text{Glucose 1-phosphate} \rightleftharpoons \text{glucose 6-phosphate}$$

given that, starting with 20 mM glucose 1-phosphate and no glucose 6-phosphate, the final equilibrium mixture at 25 °C and pH 7.0 contains 1.0 mM glucose 1-phosphate and 19 mM glucose 6-phosphate. Does the reaction in the direction of glucose 6-phosphate formation proceed with a loss or a gain of free energy?

SOLUTION: First we calculate the equilibrium constant:

$$K'_{eq} = \frac{[\text{glucose 6-phosphate}]_{eq}}{[\text{glucose 1-phosphate}]_{eq}} = \frac{19\text{ mM}}{1.0\text{ mM}} = 19$$

We can now calculate the standard free-energy change:

$$\begin{aligned} \Delta G'^\circ &= -RT \ln K'_{eq} \\ &= -(8.315\text{ J/mol} \cdot \text{K})(298\text{ K})(\ln 19) \\ &= -7.3\text{ kJ/mol} \end{aligned}$$

Because the standard free-energy change is negative, the conversion of glucose 1-phosphate to glucose 6-phosphate proceeds with a loss (release) of free energy. (For the reverse reaction, $\Delta G'^\circ$ has the same magnitude but the *opposite* sign.)

Table 13-4 gives the standard free-energy changes for some representative chemical reactions. Note that hydrolysis of simple esters, amides, peptides, and glycosides, as well as rearrangements and eliminations, proceed with relatively small standard free-energy changes, whereas hydrolysis of acid anhydrides is accompanied by relatively

TABLE 13-4 Standard Free-Energy Changes of Some Chemical Reactions

	$\Delta G'^\circ$	
Reaction type	(kJ/mol)	(kcal/mol)
Hydrolysis reactions		
Acid anhydrides		
Acetic anhydride + $H_2O \longrightarrow$ 2 acetate	−91.1	−21.8
ATP + $H_2O \longrightarrow$ ADP + P_i	−30.5	−7.3
ATP + $H_2O \longrightarrow$ AMP + PP_i	−45.6	−10.9
$PP_i + H_2O \longrightarrow 2P_i$	−19.2	−4.6
UDP-glucose + $H_2O \longrightarrow$ UMP + glucose 1-phosphate	−43.0	−10.3
Esters		
Ethyl acetate + $H_2O \longrightarrow$ ethanol + acetate	−19.6	−4.7
Glucose 6-phosphate + $H_2O \longrightarrow$ glucose + P_i	−13.8	−3.3
Amides and peptides		
Glutamine + $H_2O \longrightarrow$ glutamate + NH_4^+	−14.2	−3.4
Glycylglycine + $H_2O \longrightarrow$ 2 glycine	−9.2	−2.2
Glycosides		
Maltose + $H_2O \longrightarrow$ 2 glucose	−15.5	−3.7
Lactose + $H_2O \longrightarrow$ glucose + galactose	−15.9	−3.8
Rearrangements		
Glucose 1-phosphate $\longrightarrow$ glucose 6-phosphate	−7.3	−1.7
Fructose 6-phosphate $\longrightarrow$ glucose 6-phosphate	−1.7	−0.4
Elimination of water		
Malate $\longrightarrow$ fumarate + H_2O	3.1	0.8
Oxidations with molecular oxygen		
Glucose + $6O_2 \longrightarrow 6CO_2 + 6H_2O$	−2,840	−686
Palmitate + $23O_2 \longrightarrow 16CO_2 + 16H_2O$	−9,770	−2,338

large decreases in standard free energy. The complete oxidation of organic compounds such as glucose or palmitate to CO_2 and H_2O, which in cells requires many steps, results in very large decreases in standard free energy. However, standard free-energy changes such as those in Table 13-4 indicate how much free energy is available from a reaction under *standard conditions.* To describe the energy released under the conditions existing in cells, an expression for the *actual* free-energy change is essential.

Actual Free-Energy Changes Depend on Reactant and Product Concentrations

We must be careful to distinguish between two different quantities: the actual free-energy change, ΔG, and the standard free-energy change, $\Delta G'^{\circ}$. Each chemical reaction has a characteristic standard free-energy change, which may be positive, negative, or zero, depending on the equilibrium constant of the reaction. P1 The standard free-energy change tells us in which direction and how far a given reaction must go to reach equilibrium *when the initial concentration of each component is 1.0 M,* the pH is 7.0, the temperature is 25 °C, and the pressure is 101.3 kPa (1 atm). Thus $\Delta G'^{\circ}$ is a constant: it has a characteristic, unchanging value for a given reaction. But the *actual* free-energy change, ΔG, is a function of reactant and product concentrations and of the temperature prevailing during the reaction, none of which will necessarily match the standard conditions as defined above. Moreover, the ΔG of any reaction proceeding spontaneously toward its equilibrium is always negative, becomes less negative as the reaction proceeds, and is zero at the point of equilibrium, indicating that no more work can be done by the reaction.

ΔG and $\Delta G'^{\circ}$ for any reaction $a\text{A} + b\text{B} \rightleftharpoons c\text{C} + d\text{D}$ are related by the equation

$$\Delta G = \Delta G'^{\circ} + RT \ln \frac{[\text{C}]^c[\text{D}]^d}{[\text{A}]^a[\text{B}]^b} \qquad (13\text{-}4)$$

in which the terms in red are those *actually prevailing* in the system under observation. The concentration terms in this equation express the effects commonly called mass action, and the term $[\text{C}]^c[\text{D}]^d/[\text{A}]^a[\text{B}]^b$ is called the **mass-action ratio, *Q***. Thus Equation 13-4 can be expressed as $\Delta G = \Delta G'^{\circ} + RT \ln Q$. As an example, let us suppose that the reaction $\text{A} + \text{B} \rightleftharpoons \text{C} + \text{D}$ is taking place under the standard conditions of temperature (25 °C) and pressure (101.3 kPa) but that the concentrations of A, B, C, and D are *not* equal and none of the components is present at the standard concentration of 1.0 M. To determine the actual free-energy change, ΔG, under these nonstandard conditions of concentration as the reaction proceeds from left to right, we simply enter the *actual* concentrations of A, B, C, and D in Equation 13-4; the values of R, T, and $\Delta G'^{\circ}$ are the standard values. ΔG is negative and approaches zero as the reaction proceeds, because the actual concentrations of A and B decrease and the concentrations of C and D increase. Notice that when a reaction is at equilibrium—when there is no force driving the reaction in either direction and ΔG is zero—Equation 13-4 reduces to

$$0 = \Delta G = \Delta G'^{\circ} + RT \ln \frac{[\text{C}]_{\text{eq}}[\text{D}]_{\text{eq}}}{[\text{A}]_{\text{eq}}[\text{B}]_{\text{eq}}}$$

or

$$\Delta G'^{\circ} = -RT \ln K'_{\text{eq}}$$

which is the equation relating the standard free-energy change and equilibrium constant (Eqn 13-3).

The criterion for spontaneity of a reaction is the value of ΔG, not $\Delta G'^{\circ}$. A reaction with a positive $\Delta G'^{\circ}$ can go in the forward direction *if ΔG is negative.* This is possible if the term $RT \ln$ ([products]/[reactants]) in Equation 13-4 is negative and has a larger absolute value than $\Delta G'^{\circ}$. For example, the immediate removal of the products of a reaction by an enzyme that degrades the product can keep the ratio [products]/[reactants] well below 1, such that the term $RT \ln$ ([products]/[reactants]) has a large, negative value. This is a quantitative expression of Le Chatelier's principle. $\Delta G'^{\circ}$ and ΔG are expressions of the *maximum* amount of free energy that a given reaction can *theoretically* deliver—an amount of energy that could be realized only if a perfectly efficient device were available to trap or harness it. Given that no such device is possible (some energy is always lost to entropy during any process), the amount of work done by the reaction at constant temperature and pressure is always less than the theoretical amount.

Another important point is that some thermodynamically favorable reactions (that is, reactions for which $\Delta G'^{\circ}$ is large and negative) do not occur at measurable rates. For example, combustion of firewood to CO_2 and H_2O is very favorable thermodynamically, but firewood remains stable for years because the activation energy (see Figs. 6-2, 6-3) for the combustion reaction is higher than the energy available at room temperature. If the necessary activation energy is provided (with a lighted match, for example), combustion will begin, converting the wood to the more stable products CO_2 and H_2O and releasing energy as heat and light. The heat released by this exothermic reaction provides the activation energy for combustion of neighboring regions of the firewood; the process is self-perpetuating. Thermodynamics allows us to predict which direction a process will tend to go; how fast it will go is the subject of kinetics.

In living cells, reactions that would be extremely slow *if uncatalyzed* are caused to proceed not by supplying additional heat but by lowering the activation energy through use of an enzyme catalyst. An enzyme provides an alternative reaction pathway with a lower activation energy than the uncatalyzed reaction, so that at body temperature a large fraction of the substrate molecules have enough thermal energy to overcome the activation barrier, and the reaction rate increases dramatically. *The free-energy change for a reaction is independent of* *the pathway by which the reaction occurs;* it depends only on the nature and concentration of the initial reactants and the final products. *Enzymes cannot, therefore, change equilibrium constants;* but they can and do increase the *rate* at which a reaction proceeds in the direction dictated by thermodynamics (see Section 6.2).

Standard Free-Energy Changes Are Additive

In the case of two sequential chemical reactions, $A \rightleftharpoons B$ and $B \rightleftharpoons C$, each reaction has its own equilibrium constant and each has its characteristic standard free-energy change, $\Delta G_1'^\circ$ and $\Delta G_2'^\circ$. As the two reactions are sequential, B cancels out to give the overall reaction $A \rightleftharpoons C$, which has its own equilibrium constant and thus its own standard free-energy change, $\Delta G'^\circ_{\text{Sum}}$. *The $\Delta G'^\circ$ values of sequential chemical reactions are additive.* For the overall reaction $A \rightleftharpoons C$, $\Delta G'^\circ_{\text{Sum}}$ is the sum of the individual standard free-energy changes, $\Delta G_1'^\circ$ and $\Delta G_2'^\circ$, of the two reactions: $\Delta G'^\circ_{\text{Sum}} = \Delta G_1'^\circ + \Delta G_2'^\circ$.

$$\begin{array}{lll} (1) & A \longrightarrow \cancel{B} & \Delta G_1'^\circ \\ (2) & \cancel{B} \longrightarrow C & \Delta G_2'^\circ \\ \hline \textit{Sum:} & A \longrightarrow C & \Delta G_1'^\circ + \Delta G_2'^\circ \end{array}$$

P2 This principle of bioenergetics explains how a thermodynamically unfavorable (endergonic) reaction can be driven in the forward direction by coupling it to a highly exergonic reaction. For example, in many organisms, the synthesis of glucose 6-phosphate is the first step in the utilization of glucose. In principle, the synthesis could be accomplished by this reaction:

$$\text{Glucose} + P_i \longrightarrow \text{glucose 6-phosphate} + H_2O \qquad \Delta G'^\circ = 13.8 \text{ kJ/mol}$$

But the positive value of $\Delta G'^\circ$ predicts that under standard conditions the reaction will tend not to proceed spontaneously in the direction written. Another cellular reaction, the hydrolysis of ATP to ADP and P_i, is highly exergonic:

$$\text{ATP} + H_2O \longrightarrow \text{ADP} + P_i \qquad \Delta G'^\circ = -30.5 \text{ kJ/mol}$$

These two reactions share the common intermediates P_i and H_2O and may be expressed as sequential reactions:

$$\begin{array}{ll} (1) & \text{Glucose} + \cancel{P_i} \longrightarrow \text{glucose 6-phosphate} + \cancel{H_2O} \\ (2) & \text{ATP} + \cancel{H_2O} \longrightarrow \text{ADP} + \cancel{P_i} \\ \hline \textit{Sum:} & \text{ATP} + \text{glucose} \longrightarrow \text{ADP} + \text{glucose 6-phosphate} \end{array}$$

The overall standard free-energy change is obtained by adding the $\Delta G'^\circ$ values for individual reactions:

$$\Delta G'^\circ_{\text{Sum}} = 13.8 \text{ kJ/mol} + (-30.5 \text{ kJ/mol}) = -16.7 \text{ kJ/mol}$$

P2 The overall reaction is exergonic. In this case, energy stored in ATP is used to drive the synthesis of glucose 6-phosphate, even though its formation from glucose and inorganic phosphate (P_i) is endergonic. The *pathway* of glucose 6-phosphate formation from glucose by phosphoryl transfer from ATP is different from reactions (1) and (2), but the net result is the same as the sum of the two reactions. The standard free-energy change is a state function. In thermodynamic calculations, all that matters is the state of the system at the beginning of the process and its state at the end; the route between the initial and final states is immaterial.

We have said that $\Delta G'^\circ$ is a way of expressing the equilibrium constant for a reaction. For reaction (1),

$$K'_{\text{eq}_1} = \frac{[\text{glucose 6-phosphate}]_{\text{eq}}}{[\text{glucose}]_{\text{eq}}[P_i]_{\text{eq}}} = 3.9 \times 10^{-3} \text{ M}^{-1}$$

Notice that H_2O is not included in this expression, as its concentration (55.5 M) is assumed to remain unchanged by the reaction. The equilibrium constant for the hydrolysis of ATP is

$$K'_{\text{eq}_2} = \frac{[\text{ADP}]_{\text{eq}}[P_i]_{\text{eq}}}{[\text{ATP}]_{\text{eq}}} = 2.0 \times 10^5 \text{ M}$$

The equilibrium constant for the two coupled reactions is

$$\begin{aligned} K'_{\text{eq}_3} &= \frac{[\text{glucose 6-phosphate}]_{\text{eq}}[\text{ADP}]_{\text{eq}}[P_i]_{\text{eq}}}{[\text{glucose}]_{\text{eq}}[P_i]_{\text{eq}}[\text{ATP}]_{\text{eq}}} \\ &= (K'_{\text{eq}_1})(K'_{\text{eq}_2}) = (3.9 \times 10^{-3} \text{ M}^{-1})(2.0 \times 10^5 \text{ M}) \\ &= 7.8 \times 10^2 \end{aligned}$$

This calculation illustrates an important point about equilibrium constants: although the $\Delta G'^\circ$ values for two reactions that sum to a third, overall reaction are *additive*, the K'_{eq} for the overall reaction is the *product* of the individual K'_{eq} values for the two reactions. Equilibrium constants are *multiplicative*. By coupling ATP hydrolysis to glucose 6-phosphate synthesis, the K'_{eq} for formation of glucose 6-phosphate from glucose has been raised by a factor of about 2×10^5 compared with the direct reaction between glucose and P_i.

This strategy of coupling endergonic processes to exergonic reactions that drive them is employed by all living cells in the synthesis of metabolic intermediates and cellular components. Obviously, the strategy works only if compounds such as ATP are continuously available. In the following chapters we consider several of the most important cellular pathways for producing ATP. For more practice in dealing with free-energy changes and equilibrium constants for coupled reactions, see Worked Examples 1-1, 1-2, and 1-3 in Chapter 1 (pp. 24–25).

SUMMARY 13.1 *Bioenergetics and Thermodynamics*

- Bioenergetics is the quantitative study of energy relationships and energy conversions in biological systems. Biological energy transformations obey the laws of thermodynamics.

- Living cells constantly perform work. They require energy for maintaining their highly organized structures, synthesizing cellular components, transporting small molecules and ions across membranes, and generating electric currents.
- All chemical reactions are influenced by two forces: the tendency to achieve the most stable bonding state (for which enthalpy, H, is a useful expression) and the tendency to achieve the highest degree of randomness, expressed as entropy, S. The driving force in a reaction is ΔG, the free-energy change, which represents the net effect of these two factors: $\Delta G = \Delta H - T\Delta S$.
- The standard transformed free-energy change, $\Delta G'^{\circ}$, is a physical constant that is characteristic for a given reaction and can be calculated from the equilibrium constant for the reaction: $\Delta G'^{\circ} = -RT \ln K'_{\text{eq}}$.
- The actual free-energy change, ΔG, is a variable that depends on $\Delta G'^{\circ}$ and on the concentrations of reactants and products: $\Delta G = \Delta G'^{\circ} + RT \ln([\text{products}]/[\text{reactants}])$. When ΔG is large and negative, the reaction tends to go in the forward direction; when ΔG is large and positive, the reaction tends to go in the reverse direction; and when $\Delta G = 0$, the system is at equilibrium.
- The free-energy change for a reaction is independent of the pathway by which the reaction occurs. Free-energy changes are additive; the net chemical reaction that results from successive reactions sharing a common intermediate has an overall free-energy change that is the sum of the ΔG values for the individual reactions.

13.2 Chemical Logic and Common Biochemical Reactions

The biological energy transductions we are concerned with in this book are chemical reactions. Cellular chemistry does not encompass every kind of reaction learned in a typical organic chemistry course. Which reactions take place in biological systems and which do not is determined by (1) their relevance to that particular metabolic system and (2) their rates. Both considerations play major roles in shaping the metabolic pathways we consider throughout the rest of the book. A relevant reaction is one that makes use of an available substrate and converts it to a useful product. However, even a potentially relevant reaction may not occur. Some chemical transformations are too slow (have activation energies that are too high) to contribute to living systems, even with the aid of powerful enzyme catalysts. The reactions that do occur in cells represent a toolbox that evolution has used to construct metabolic pathways that circumvent the "impossible" reactions. Learning to recognize the plausible reactions can be a great aid in developing a command of biochemistry.

Even so, the number of metabolic transformations taking place in a typical cell can seem overwhelming. Most cells have the capacity to carry out thousands of specific, enzyme-catalyzed reactions: for example, transformation of a simple nutrient such as glucose into amino acids, nucleotides, or lipids; extraction of energy from fuels by oxidation; and polymerization of monomeric subunits into macromolecules.

Biochemical Reactions Occur in Repeating Patterns

To study these reactions, some organization is essential. P3 There are patterns within the chemistry of life; you do not need to learn every individual reaction to comprehend the molecular logic of biochemistry. Most of the reactions in living cells fall into one of five general categories: (1) reactions that make or break carbon–carbon bonds; (2) internal rearrangements, isomerizations, and eliminations; (3) free-radical reactions; (4) group transfers; and (5) oxidation-reductions. We discuss each of these in more detail below and refer to some examples of each type in later chapters. Note that the five reaction types are not mutually exclusive; for example, an isomerization reaction may involve a free-radical intermediate.

Before proceeding, however, we should review two basic chemical principles. First, a covalent bond consists of a shared pair of electrons, and the bond can be broken in two general ways (**Fig. 13-1**). In **homolytic cleavage**, each atom leaves the bond as a **radical**, carrying one unpaired electron. In **heterolytic cleavage**, which is

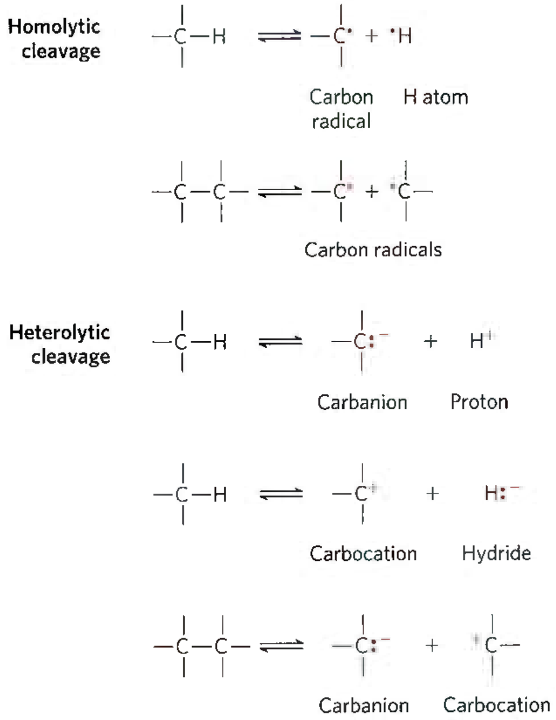


FIGURE 13-1 Two mechanisms for cleavage of a C—C or C—H bond. In a homolytic cleavage, each atom keeps one of the bonding electrons, resulting in the formation of carbon radicals (carbons having unpaired electrons) or uncharged hydrogen atoms. In a heterolytic cleavage, one of the atoms retains both bonding electrons. This can result in the formation of carbanions, carbocations, protons, or hydride ions.

more common, one atom retains both bonding electrons. The species most often generated when C—C and C—H bonds are cleaved are illustrated in Figure 13-1. Carbanions, carbocations, and hydride ions are highly unstable; this instability shapes the chemistry of these ions, as we shall see.

The second basic principle is that many biochemical reactions involve interactions between **nucleophiles** (functional groups rich in and capable of donating electrons) and **electrophiles** (electron-deficient functional groups that seek electrons). Nucleophiles combine with and give up electrons to electrophiles. Common biological nucleophiles and electrophiles are shown in **Figure 13-2**. Note that a carbon atom can act as either a nucleophile or an electrophile, depending on which bonds and functional groups surround it.

Reactions That Make or Break Carbon–Carbon Bonds Heterolytic cleavage of a C—C bond yields a **carbanion** and a **carbocation** (Fig. 13-1). Conversely, the formation of a C—C bond involves the combination of a nucleophilic carbanion and an electrophilic carbocation. Carbanions and carbocations are generally so unstable that their formation as reaction intermediates can be energetically unfeasible, even with enzyme catalysts. For the purpose of cellular biochemistry, they are impossible reactions — unless chemical assistance is provided in the form of functional groups containing electronegative atoms (O and N) that can alter the electronic structure of adjacent carbon atoms so as to stabilize and facilitate the formation of carbanion and carbocation intermediates.

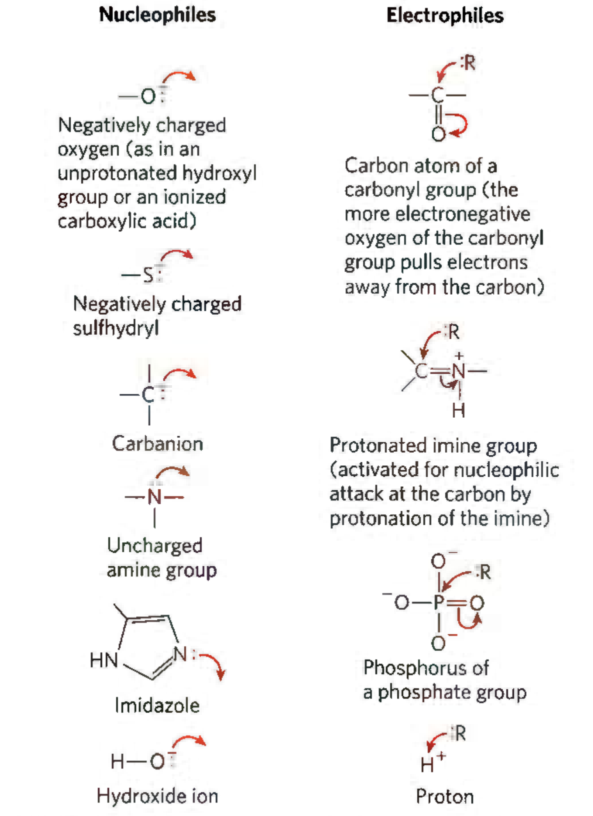

Nucleophiles
Electrophiles
Negatively charged oxygen (as in an unprotonated hydroxyl group or an ionized carboxylic acid)
Negatively charged sulfhydryl
Carbanion
Uncharged amine group
Imidazole
Hydroxide ion
Carbon atom of a carbonyl group (the more electronegative oxygen of the carbonyl group pulls electrons away from the carbon)
Protonated imine group (activated for nucleophilic attack at the carbon by protonation of the imine)
Phosphorus of a phosphate group
Proton

FIGURE 13-2 Common nucleophiles and electrophiles in biochemical reactions. Chemical reaction mechanisms, which trace the formation and breakage of covalent bonds, are communicated with dots and curved arrows, a convention known informally as "electron pushing." A covalent bond consists of a shared pair of electrons. Nonbonded electrons important to the reaction mechanism are designated by dots (:). Curved arrows (↷) represent the movement of electron pairs. For movement of a single electron (as in a free-radical reaction), a single-headed (fishhook-type) arrow is used (↷). Most reaction steps involve an unshared electron pair.

Carbonyl groups are particularly important in the chemical transformations of metabolic pathways. The carbon of a carbonyl group has a partial positive charge due to the electron-withdrawing property of the carbonyl oxygen, and so is an electrophilic carbon (**Fig. 13-3a**). A carbonyl group can thus facilitate the formation of a carbanion on an adjoining carbon by delocalizing the carbanion's negative charge (Fig. 13-3b). An imine group (see Fig. 1-14) can serve a similar function (Fig. 13-3c). The capacity of carbonyl and imine groups to delocalize electrons can be further enhanced by a general acid catalyst or by a metal ion (Me^{2+}) such as Mg^{2+} (Fig. 13-3d).

P3 The importance of a carbonyl group is evident in three major classes of reactions in which C—C bonds are formed or broken (**Fig. 13-4**): aldol condensations, Claisen ester condensations, and decarboxylations. In each type of reaction, a carbanion intermediate is stabilized by a carbonyl group, and in many cases another carbonyl provides the electrophile with which the nucleophilic carbanion reacts.

An **aldol condensation** is a common route to the formation of a C—C bond; the aldolase reaction, which converts a six-carbon compound to two three-carbon compounds in glycolysis, is an aldol condensation in reverse (see Fig. 14-5). In a **Claisen condensation**,

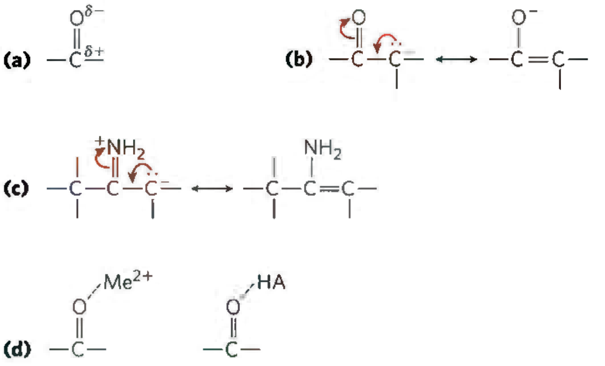


FIGURE 13-3 Chemical properties of carbonyl groups. (a) The carbon atom of a carbonyl group is an electrophile by virtue of the electron-withdrawing capacity of the electronegative oxygen atom, which results in a structure in which the carbon has a partial positive charge. (b) Within a molecule, delocalization of electrons into a carbonyl group stabilizes a carbanion on an adjacent carbon, facilitating its formation. (c) Imines function much like carbonyl groups in facilitating electron withdrawal. (d) Carbonyl groups do not always function alone; their capacity as electron sinks often is augmented by interaction with either a metal ion (Me^{2+}, such as Mg^{2+}) or a general acid (HA).

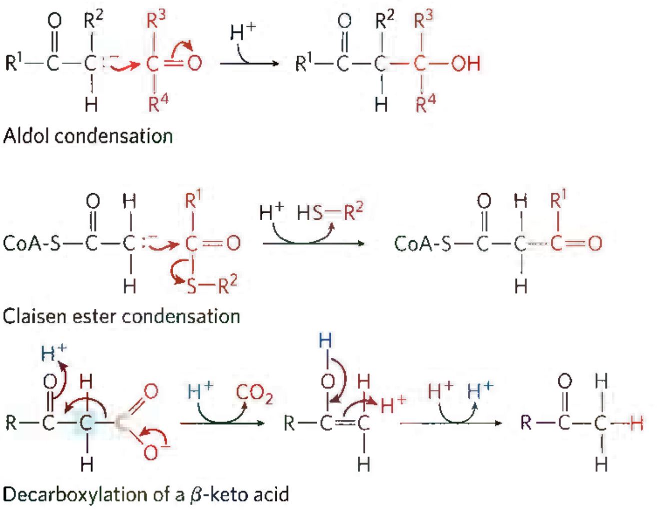


FIGURE 13-4 Some common reactions that form and break C—C bonds in biological systems. For both the aldol condensation and the Claisen condensation, a carbanion serves as nucleophile and the carbon of a carbonyl group serves as electrophile. The carbanion is stabilized in each case by another carbonyl at the adjoining carbon. In the decarboxylation reaction, a carbanion is formed on the carbon shaded blue as the CO_2 leaves. The reaction would not occur at an appreciable rate without the stabilizing effect of the carbonyl adjacent to the carbanion carbon. Wherever a carbanion is shown, a stabilizing resonance with the adjacent carbonyl, as shown in Figure 13-3b, is assumed. An imine (Fig. 13-3c) or other electron-withdrawing group (including certain enzymatic cofactors such as pyridoxal) can replace the carbonyl group in the stabilization of carbanions.

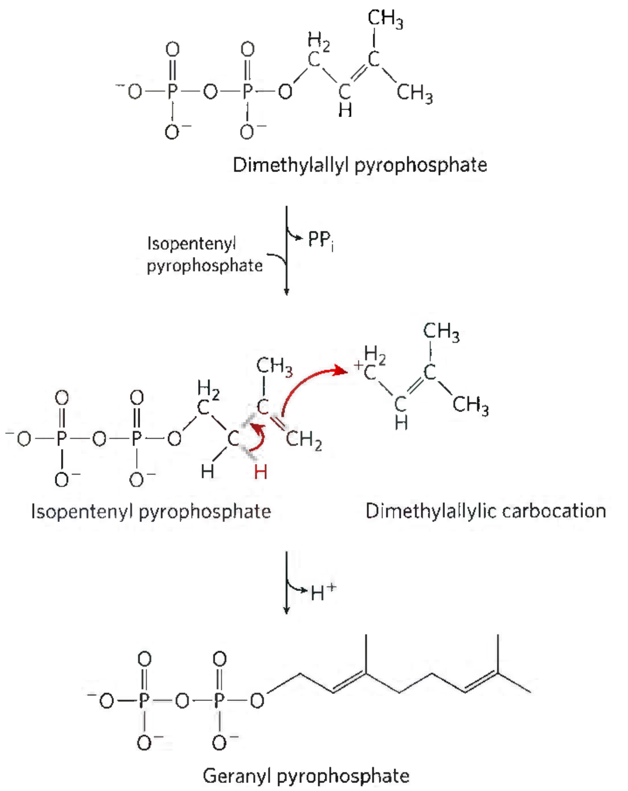

Dimethylallyl pyrophosphate
Isopentenyl pyrophosphate
PP_i
Isopentenyl pyrophosphate
Dimethylallylic carbocation
H^+
Geranyl pyrophosphate

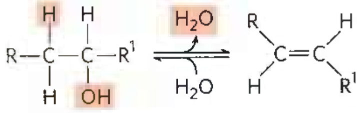
FIGURE 13-5 Carbocations in carbon–carbon bond formation. In one of the early steps in cholesterol biosynthesis, the enzyme prenyltransferase catalyzes condensation of isopentenyl pyrophosphate and dimethylallyl pyrophosphate to form geranyl pyrophosphate (see Fig. 21-36). The reaction is initiated by elimination of pyrophosphate from the dimethylallyl pyrophosphate to generate a carbocation, stabilized by resonance with the adjacent C═C bond.

the carbanion is stabilized by the carbonyl of an adjacent thioester; an example is the synthesis of citrate in the citric acid cycle (see Fig. 16-9). Decarboxylation also commonly involves the formation of a carbanion stabilized by a carbonyl group; the acetoacetate decarboxylase reaction that occurs in the formation of ketone bodies during fatty acid catabolism provides an example (see Fig. 17-16). Entire metabolic pathways are organized around the introduction of a carbonyl group in a particular location so that a nearby carbon–carbon bond can be formed or cleaved. In some reactions, an imine or a specialized cofactor such as pyridoxal phosphate plays the electron-withdrawing role, instead of a carbonyl group.

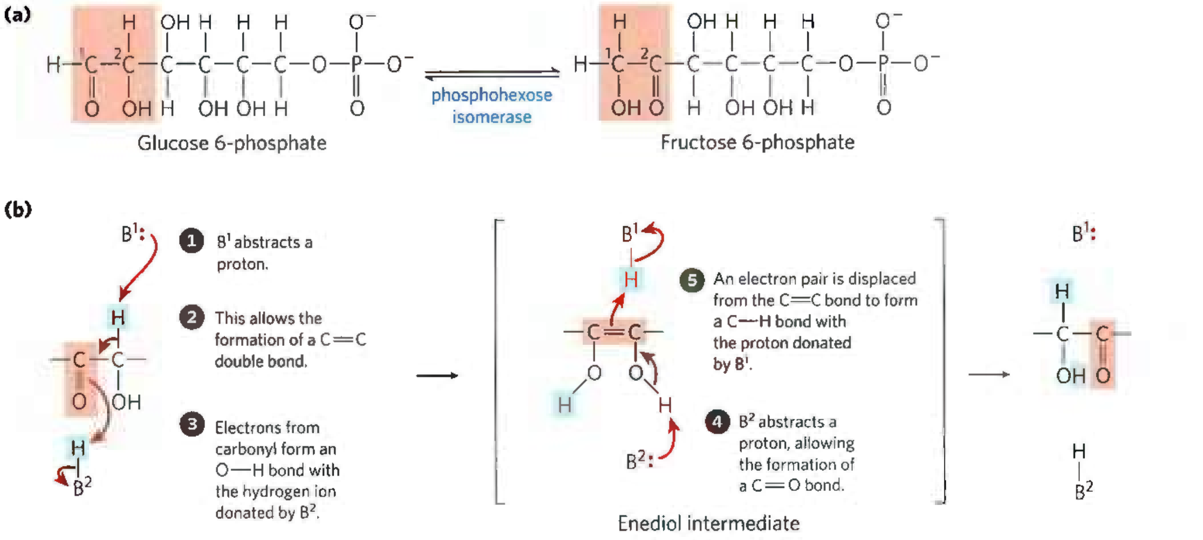
The carbocation intermediate occurring in some reactions that form or cleave C—C bonds is generated by the elimination of an excellent leaving group, such as pyrophosphate (see "Group Transfer Reactions" below). An example is the prenyltransferase reaction (**Fig. 13-5**), an early step in the pathway of cholesterol biosynthesis.

Internal Rearrangements, Isomerizations, and Eliminations Another common type of cellular reaction is an intramolecular rearrangement in which redistribution of electrons results in alterations of many different types without a change in the overall oxidation state of the molecule. For example, different groups in a molecule may undergo oxidation-reduction, with no net change in oxidation state of the molecule; groups at a double bond may undergo a cis-trans rearrangement; or the positions of double bonds may be transposed. An example of an isomerization entailing internal oxidation-reduction is the formation of fructose 6-phosphate from glucose 6-phosphate in glycolysis (**Fig. 13-6**; this reaction is discussed in detail in Chapter 14): C-1 is reduced (aldehyde to alcohol) and C-2 is oxidized (alcohol to ketone). Figure 13-6b shows the details of the electron movements in this type of isomerization. A cis-trans rearrangement is illustrated by the prolyl cis-trans isomerase reaction in the folding of certain proteins (see p. 133). A simple transposition of a C═C bond occurs during metabolism of oleic acid, a common fatty acid (see Fig. 17-10). Some spectacular examples of double-bond repositioning occur in the biosynthesis of cholesterol (see Fig. 21-37).

An example of an elimination reaction that does not affect overall oxidation state is the loss of water from an alcohol, resulting in the introduction of a C═C bond:



Similar reactions can result from eliminations in amines.



① B^1 abstracts a proton.

② This allows the formation of a C═C double bond.

③ Electrons from carbonyl form an O—H bond with the hydrogen ion donated by B^2.

④ B^2 abstracts a proton, allowing the formation of a C═O bond.

⑤ An electron pair is displaced from the C═C bond to form a C—H bond with the proton donated by B^1.

FIGURE 13-6 Isomerization and elimination reactions. (a) The conversion of glucose 6-phosphate to fructose 6-phosphate, a reaction of sugar metabolism catalyzed by phosphohexose isomerase. (b) This reaction proceeds through an enediol intermediate. Light red screens follow the path of oxidation from left to right. B^1 and B^2 are ionizable groups on the enzyme; they are capable of donating and accepting protons (acting as general acids or general bases) as the reaction proceeds.

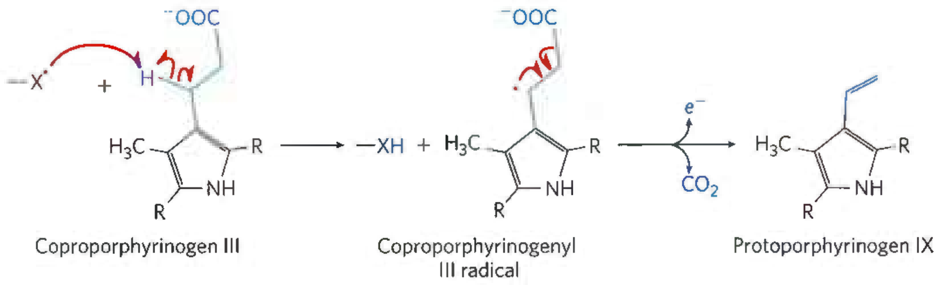
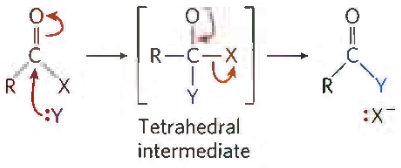


FIGURE 13-7 A free radical–initiated decarboxylation reaction. The biosynthesis of heme in *Escherichia coli* includes a decarboxylation step in which propionyl side chains on the coproporphyrinogen III intermediate are converted to the vinyl side chains of protoporphyrinogen IX. When the bacteria are grown anaerobically the enzyme oxygen-independent coproporphyrinogen III oxidase, also called HemN protein, promotes decarboxylation via the free-radical mechanism shown here. The acceptor of the released electron is not known. For simplicity, only the relevant portions of the large coproporphyrinogen III and protoporphyrinogen molecules are shown; the entire structures are given in Figure 22-26. When *E. coli* is grown in the presence of oxygen, this reaction is an oxidative decarboxylation and is catalyzed by a different enzyme. (Information from G. Layer et al., *Curr. Opin. Chem. Biol.* 8:468, 2004, Fig. 4.)

Free-Radical Reactions Once thought to be rare, the homolytic cleavage of covalent bonds to generate free radicals has now been found in a wide range of biochemical processes. These include isomerizations that make use of adenosylcobalamin (vitamin B_{12}) or *S*-adenosylmethionine, which are initiated with a 5′-deoxyadenosyl radical (see the methylmalonyl-CoA mutase reaction in Box 17-2); certain radical-initiated decarboxylation reactions (**Fig. 13-7**); some reductase reactions, such as that catalyzed by ribonucleotide reductase (see Fig. 22-43); and some rearrangement reactions, such as that catalyzed by DNA photolyase (see Fig. 25-25).

Group Transfer Reactions The transfer of acyl, glycosyl, and phosphoryl groups from one nucleophile to another is common in living cells. Acyl group transfer generally involves the addition of a nucleophile to the carbonyl carbon of an acyl group to form a tetrahedral intermediate:



The chymotrypsin reaction is one example of acyl group transfer (see Fig. 6-27). Glycosyl group transfers involve nucleophilic substitution at C-1 of a sugar ring, which is the central atom of an acetal. In principle, the substitution could proceed by an S_N1 or S_N2 pathway.

Phosphoryl group transfers play a special role in metabolic pathways, and these transfer reactions are discussed in detail in Section 13.3. **P3** A general theme in metabolism is the attachment of a good leaving group to a metabolic intermediate to "activate" the intermediate for subsequent reaction. Among the better leaving groups in nucleophilic substitution reactions are inorganic orthophosphate (the ionized form of H_3PO_4 at neutral pH, a mixture of $H_2PO_4^-$ and HPO_4^{2-}, commonly abbreviated P_i) and inorganic pyrophosphate ($P_2O_7^{4-}$, abbreviated PP_i); esters and anhydrides of phosphoric acid are effectively activated for reaction. Nucleophilic substitution is made more favorable by the attachment of a phosphoryl group to an otherwise poor leaving group such as —OH. Nucleophilic substitutions in which the phosphoryl group ($—PO_3^{2-}$) serves as a leaving group occur in hundreds of metabolic reactions.

Phosphorus can form five covalent bonds. The conventional representation of P_i (**Fig. 13-8a**), with three P—O bonds and one P═O bond, is a convenient but inaccurate picture. In P_i, four equivalent phosphorus–oxygen bonds share some double-bond character, and the anion has a tetrahedral structure (Fig. 13-8b). Because oxygen is more electronegative than phosphorus, the sharing of electrons is unequal: the central phosphorus bears a partial positive charge and can therefore act as an electrophile. In a great many metabolic reactions, a phosphoryl group ($—PO_3^{2-}$) is transferred from ATP to an alcohol, forming a phosphate ester (Fig. 13-8c), or to a carboxylic acid, forming a mixed anhydride. When a nucleophile attacks the electrophilic phosphorus atom in ATP, a relatively stable pentacovalent structure forms as a reaction intermediate (Fig. 13-8d). With departure of the leaving group (ADP), the transfer of a phosphoryl group is complete. The large family of enzymes that catalyze phosphoryl group transfers with ATP as donor are called **kinases** (Greek *kinein*, "to move"). Hexokinase, for example, "moves" a phosphoryl group from ATP to the hexose glucose. **Box 13-1** offers a primer on some of the broad classes of enzymes (including kinases) that you will encounter in your study of metabolism.

Phosphoryl groups are not the only groups that activate molecules for reaction. Thioalcohols (thiols), in which the oxygen atom of an alcohol is replaced with a sulfur atom, are also good leaving groups. Thiols activate carboxylic acids by forming thioesters (thiol esters). In later chapters we discuss several reactions, including those catalyzed by the fatty acyl synthases in lipid synthesis (see Fig. 21-2), in which nucleophilic substitution at the carbonyl carbon of a thioester results in transfer of the acyl group to another moiety.

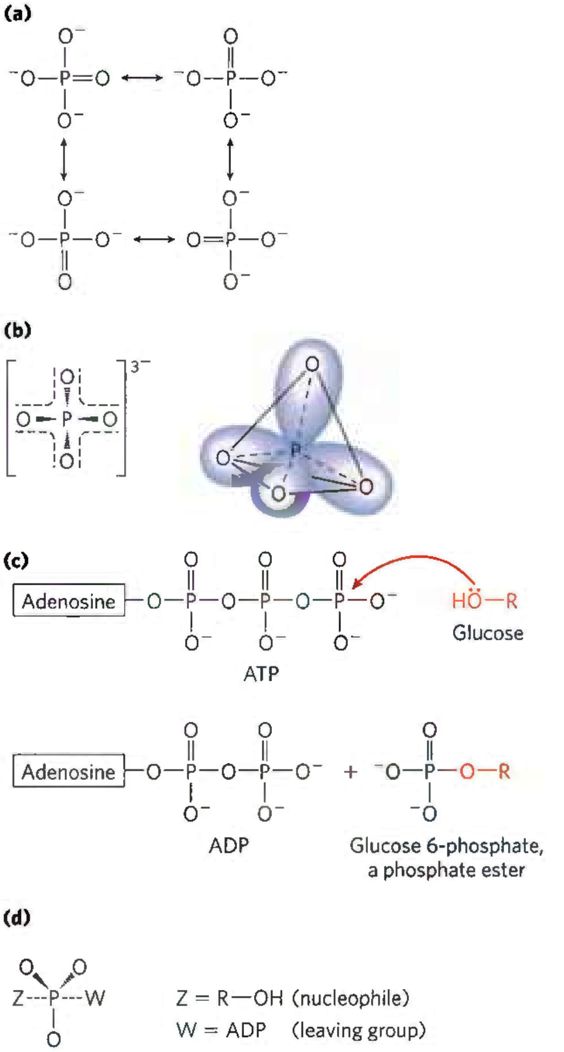


FIGURE 13-8 Phosphoryl group transfers: some of the participants. (a) In one (inadequate) representation of P_i, three oxygens are single-bonded to phosphorus, and the fourth is double-bonded, allowing the four different resonance structures shown here. (b) The resonance structures of P_i can be represented more accurately by showing all four phosphorus–oxygen bonds with some double-bond character; the hybrid orbitals so represented are arranged in a tetrahedron with P at its center. (c) When a nucleophile Z (in this case, the —OH on C-6 of glucose) attacks ATP, it displaces ADP (W). In this S_N2 reaction, a pentacovalent intermediate (d) forms transiently.

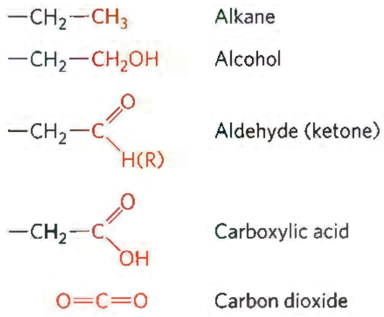


FIGURE 13-9 The oxidation levels of carbon in biomolecules. Each compound is formed by oxidation of the carbon shown in red in the compound immediately above. Carbon dioxide is the most highly oxidized form of carbon found in living systems.

Oxidation-Reduction Reactions We will encounter carbon atoms in five oxidation states, depending on the elements with which they share electrons (**Fig. 13-9**), and transitions between these states are of crucial importance in metabolism (oxidation-reduction reactions are the topic of Section 13.4). In many biological oxidations, a compound loses two electrons and two hydrogen ions (that is, two hydrogen atoms); these reactions are commonly called dehydrogenations, and the enzymes that catalyze them are called dehydrogenases (**Fig. 13-10**). In some, but not all, biological oxidations, a

BOX 13-1

A Primer on Enzyme Names

The name **kinase** is applied to enzymes that transfer a phosphoryl group from a nucleoside triphosphate such as ATP to an acceptor molecule—a sugar (as in hexokinase and glucokinase), a protein (as in glycogen phosphorylase kinase), another nucleotide (as in nucleoside diphosphate kinase), or a metabolic intermediate such as oxaloacetate (as in PEP carboxykinase). The reaction catalyzed by a kinase is a phosphorylation. On the other hand, phosphorolysis is a displacement reaction in which phosphate is the attacking species and becomes covalently attached at the point of bond breakage. Such reactions are catalyzed by **phosphorylases**. Glycogen phosphorylase, for example, catalyzes the phosphorolysis of glycogen, producing glucose 1-phosphate. Dephosphorylation, the removal of a phosphoryl group from a phosphate ester, is catalyzed by phosphatases, with water as the attacking species. Fructose bisphosphatase-1 converts fructose 1,6-bisphosphate to fructose 6-phosphate in gluconeogenesis, and phosphorylase *a* phosphatase removes phosphoryl groups from phosphoserine in phosphorylated glycogen phosphorylase. Whew!

Citrate synthase, the first enzyme in the citric acid cycle (see Fig. 16-7), is one of many enzymes that catalyze condensation reactions, yielding a product more chemically complex than its precursors. **Synthases** catalyze condensation reactions in which no nucleoside triphosphate (ATP, GTP, and so forth) is required as an energy source. **Synthetases** catalyze condensations that do use ATP or another nucleoside triphosphate as a source of energy for the synthetic reaction. Succinyl-CoA synthetase is such an enzyme. **Ligases** (from the Latin *ligare*, "to tie together") are enzymes that catalyze condensation reactions in which two atoms are joined, using ATP or another energy source. (Thus, synthetases are ligases.) DNA ligase, for example, closes breaks in DNA molecules, using energy supplied by either ATP or NAD$^+$; it is widely used in joining DNA pieces for genetic engineering. Ligases are not to be confused with **lyases**, enzymes that catalyze cleavages (or, in the reverse direction, additions) in which electronic rearrangements occur. The PDH complex, which oxidatively cleaves CO_2 from pyruvate, is a member of the large class of lyases.

In some biological oxidation reactions, molecular oxygen is the electron acceptor. If the oxygen atoms *do not* appear in the oxidized product, the enzyme is an **oxidase.** If one or both of the oxygen atoms *do* appear in the oxidized product, as a new hydroxyl or carboxyl group, for example, the enzyme is an **oxygenase.** Both classes are further subdivided. **Mixed-function oxidases** oxidize two different substrates simultaneously. **Monooxygenases** and **dioxygenases** catalyze reactions in which one or two oxygen atoms, respectively, are incorporated into the organic product. These enzymes are particularly important in biosynthetic pathways of fatty acids and eicosanoids (see Box 21-1). **Dehydrogenases** catalyze oxidation-reductase reactions in which NAD$^+$ is electron acceptor, and molecular oxygen is generally not involved.

Unfortunately, these descriptions of enzyme types overlap, and many enzymes have two or more common names. Succinyl-CoA synthetase, for example, is also called succinate thiokinase; the enzyme is both a synthetase in the citric acid cycle and a kinase when acting in the direction of succinyl-CoA synthesis. This raises another source of confusion in the naming of enzymes. An enzyme may have been discovered by the use of an assay in which, say, A is converted to B. The enzyme is then named for that reaction. Later work may show, however, that in the cell, the enzyme functions primarily in converting B to A. Commonly, the first name continues to be used, although the metabolic role of the enzyme would be better described by naming it for the reverse reaction. The glycolytic enzyme pyruvate kinase illustrates this situation (p. 521). To a beginner in biochemistry, this duplication in nomenclature can be bewildering. International committees have made heroic efforts to systematize the nomenclature of enzymes (see Table 6-3 for a brief summary of the system), but some systematic names have proved too long and cumbersome and are not frequently used in biochemical conversation.

We have tried throughout this book to use the enzyme name most commonly employed by working biochemists and to point out cases in which an enzyme has more than one widely used name.

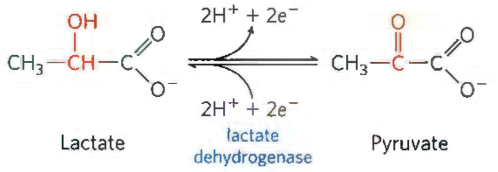


FIGURE 13-10 An oxidation-reduction reaction. Shown here is the oxidation of lactate to pyruvate. In this dehydrogenation, two electrons and two hydrogen ions (the equivalent of two hydrogen atoms) are removed from C-2 of lactate, an alcohol, to form pyruvate, a ketone. In cells the reaction is catalyzed by lactate dehydrogenase and the electrons are transferred to the cofactor nicotinamide adenine dinucleotide (NAD$^+$). This reaction is fully reversible; pyruvate can be reduced by electrons transferred from the cofactor.

carbon atom becomes covalently bonded to an oxygen atom. The enzymes that catalyze oxidations with oxygen as electron acceptor are generally called oxidases or, if the oxygen atom is derived directly from molecular oxygen (O_2) and incorporated into the product, oxygenases.

Every oxidation must be accompanied by a reduction, in which an electron acceptor acquires the electrons removed by oxidation. Oxidation reactions generally release energy (think of campfires: the compounds in wood are oxidized by oxygen molecules in the air). P5 Most living cells obtain the energy needed for cellular work by oxidizing metabolic fuels such as carbohydrates or fat (photosynthetic organisms can also trap and use the energy of sunlight). The catabolic (energy-yielding) pathways described in Chapters 14 through 19 are oxidative reaction sequences that result in the transfer of electrons from fuel molecules, through a series of electron carriers, to oxygen. The high affinity of O_2 for electrons makes the overall electron-transfer process highly exergonic, providing the energy that drives ATP synthesis—the central goal of catabolism.

Many of the reactions within these five classes are facilitated by cofactors, in the form of coenzymes and metal ions (vitamin B_{12}, *S*-adenosylmethionine, folate, nicotinamide, and Fe^{2+} are some examples). Cofactors bind to enzymes—in some cases reversibly, in other cases almost irreversibly—and give them the capacity to promote a particular kind of chemistry (p. 178). Most cofactors participate in a narrow range of closely related reactions. In the following chapters, we introduce and discuss each important cofactor at the point where we first encounter its function. The cofactors provide another way to organize the study of biochemical processes, given that the reactions facilitated by a given cofactor generally are mechanistically related.

Biochemical and Chemical Equations Are Not Identical

Biochemists write metabolic equations in a simplified way, and this is particularly evident for reactions involving ATP. Phosphorylated compounds can exist in several ionization states and, as we have noted, the different species can bind Mg^{2+}. For example, at pH 7 and 2 mM Mg^{2+}, ATP exists as an equilibrium distribution of the forms ATP^{4-}, $HATP^{3-}$, H_2ATP^{2-}, $MgHATP^-$, and Mg_2ATP. In thinking about the biological role of ATP, however, we are not always interested in all this detail, and so we consider ATP as an entity made up of a sum of species, and we write its hydrolysis as the biochemical equation

$$\text{ATP} + H_2O \longrightarrow \text{ADP} + P_i$$

where ATP, ADP, and P_i are sums of species. The corresponding standard transformed equilibrium constant, $K'_{eq} = [\text{ADP}]_{eq}[P_i]_{eq}/[\text{ATP}]_{eq}$, depends on the pH and the concentration of free Mg^{2+}. Note that H^+ and Mg^{2+} do not appear in the biochemical equation, because their concentrations are not significantly changed by the reaction. Thus a biochemical equation does not necessarily balance H, Mg, or charge, although it does balance all other elements involved in the reaction (C, N, O, and P in the equation above).

We can write a chemical equation that *does* balance for all elements and for charge. For example, when ATP is hydrolyzed at a pH above 8.5 in the absence of Mg^{2+}, the chemical reaction is represented by

$$\text{ATP}^{4-} + H_2O \longrightarrow \text{ADP}^{3-} + HPO_4^{2-} + H^+$$

The corresponding equilibrium constant, $K_{eq} = [\text{ADP}^{3-}]_{eq}[HPO_4^{2-}]_{eq}[H^+]_{eq}/[\text{ATP}^{4-}]_{eq}$, depends only on temperature, pressure, and ionic strength.

Both ways of writing a metabolic reaction have value in biochemistry. Chemical equations are needed when we want to account for all atoms and charges in a reaction, as when we are considering the mechanism of a chemical reaction. Biochemical equations are used to determine in which direction a reaction will proceed spontaneously, given a specified pH and $[Mg^{2+}]$, or to calculate the equilibrium constant of such a reaction.

Throughout this book we use biochemical equations, unless the focus is on chemical mechanism, and we use values of $\Delta G'^\circ$ and K'_{eq} as determined at pH 7 and 1 mM Mg^{2+}.

SUMMARY 13.2 *Chemical Logic and Common Biochemical Reactions*

- Living systems make use of a large number of chemical reactions that can be classified into five general types: reactions that make or break carbon–carbon bonds; internal rearrangements and eliminations; free-radical reactions; group transfers; and oxidation-reduction reactions. Heterolytic cleavages occur often in reactions that make or break C—C bonds.
- Carbonyl groups play a special role in reactions that form or cleave C—C bonds. Carbanion intermediates are common and are stabilized by adjacent carbonyl groups or, less often, by imines or certain cofactors.
- A redistribution of electrons can produce internal rearrangements, isomerizations, and eliminations. Such reactions include intramolecular oxidation-reduction, change in cis-trans arrangement at a double bond, and transposition of double bonds.
- Homolytic cleavage of covalent bonds to generate free radicals occurs in some pathways.
- Phosphoryl transfer reactions are an especially important type of group transfer in cells, required for the activation of molecules for reactions that would otherwise be highly unfavorable.
- Oxidation-reduction reactions involve the loss or gain of electrons: one reactant gains electrons and is reduced, while the other loses electrons and is oxidized. Oxidation reactions generally release energy and are important in catabolism.
- Biochemists often write reaction equations that are not balanced for H^+ and don't attempt to describe the state of phosphate ionization.

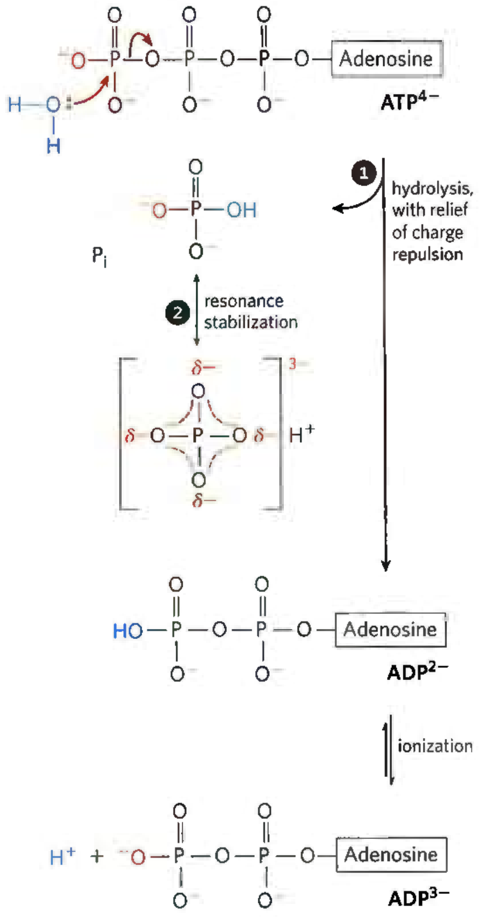
13.3 Phosphoryl Group Transfers and ATP

Having developed some fundamental principles of energy changes in chemical systems and reviewed the common classes of reactions, we can now examine the energy cycle in cells and the special role of ATP as the energy currency that links catabolism and anabolism (see Fig. 1-28). Heterotrophic cells obtain free energy in a chemical form by the catabolism of nutrient molecules, and they use that energy to make ATP from ADP and P_i. ATP then donates some of its chemical energy to endergonic processes such as the synthesis of metabolic intermediates and macromolecules from smaller precursors, the transport of substances across membranes against concentration gradients, and mechanical motion. This donation of energy from ATP generally involves the covalent participation of ATP in the reaction that is to be driven, with the eventual result that ATP is converted to ADP and P_i or, in some reactions, to AMP and 2 P_i. We discuss here the chemical basis for the large free-energy changes that accompany hydrolysis of ATP and other high-energy phosphate compounds, and we show that most cases of energy donation by ATP involve group transfer, not simple hydrolysis of ATP. To illustrate the range of energy transductions in which ATP provides the energy, we consider the synthesis of information-rich macromolecules, the transport of solutes across membranes, and motion produced by muscle contraction.

The Free-Energy Change for ATP Hydrolysis Is Large and Negative

Figure 13-11 summarizes the chemical basis for the relatively large, negative, standard free energy of hydrolysis of ATP. The hydrolytic cleavage of the terminal phosphoric acid anhydride (phosphoanhydride) bond in ATP separates one of the three negatively charged phosphates and thus relieves some of the internal electrostatic repulsion in ATP; the P_i released is stabilized by the formation of several resonance forms not possible in ATP.

The free-energy change for ATP hydrolysis is −30.5 kJ/mol under standard conditions, but the *actual* free energy of hydrolysis (ΔG) of ATP in living cells is very different: the cellular concentrations of ATP, ADP, and P_i are not identical and are much lower than the 1.0 M of standard conditions (**Table 13-5**). Furthermore, Mg^{2+} in the cytosol binds to ATP and ADP (**Fig. 13-12**), and for most enzymatic reactions that involve ATP as phosphoryl group donor, the true substrate is $MgATP^{2-}$. The relevant $\Delta G'^{\circ}$ is therefore that for $MgATP^{2-}$ hydrolysis. We can calculate ΔG for ATP hydrolysis using data such as those in Table 13-5. The actual free energy of hydrolysis of ATP under intracellular conditions is often called its **phosphorylation potential**, ΔG_p, for reasons we will explain.


Adenosine
ATP⁴⁻
H
❶ hydrolysis, with relief of charge repulsion
Pᵢ
❷ resonance stabilization
δ−
3−
H⁺
HO
Adenosine
ADP²⁻
ionization
H⁺ +
Adenosine
ADP³⁻

$$ATP^{4-} + H_2O \longrightarrow ADP^{3-} + HPO_4^{2-} + H^+$$
$$\Delta G'^{\circ} = -30.5 \text{ kJ/mol}$$

FIGURE 13-11 Chemical basis for the large free-energy change associated with ATP hydrolysis. ❶ The charge separation that results from hydrolysis relieves electrostatic repulsion among the four negative charges on ATP. ❷ The product inorganic phosphate (P_i) is stabilized by formation of a resonance hybrid, in which each of the four phosphorus–oxygen bonds has the same degree of double-bond character and the hydrogen ion is not permanently associated with any one of the oxygens. (Some degree of resonance stabilization also occurs in phosphates involved in ester or anhydride linkages, but fewer resonance forms are possible than for P_i.) A third factor (not shown) that favors ATP hydrolysis is the greater degree of solvation (hydration) of the products P_i and ADP relative to ATP, which further stabilizes the products relative to the reactants.

Because the concentrations of ATP, ADP, and P_i differ from one cell type to another, ΔG_p for ATP likewise differs among cells. Moreover, in any given cell, ΔG_p can vary from time to time, depending on the metabolic conditions and how they influence the concentrations of ATP, ADP, P_i, and H^+ (pH). We can calculate the actual free-energy change for any given metabolic reaction as it occurs in a cell, provided we know the concentrations of all the reactants and products and other factors (such as pH, temperature, and [Mg^{2+}]) that may affect the actual free-energy change.

TABLE 13-5 Total Concentrations of Adenine Nucleotides, Inorganic Phosphate, and Phosphocreatine in Some Cells

	Concentration (mM)[a]				
Cell type	ATP	ADP[b]	AMP	P_i	PCr
Rat hepatocyte	3.38	1.32	0.29	4.8	0
Rat myocyte	8.05	0.93	0.04	8.05	27
Rat neuron	2.59	0.73	0.06	2.72	4.7
Human erythrocyte	2.25	0.25	0.02	1.65	0
E. coli cell	9.6	0.56	0.28	—	—

[a] For erythrocytes, the concentrations are those of the cytosol (human erythrocytes lack a nucleus and mitochondria). In the other types of cells, the data are for the entire cell contents, although the cytosol and the mitochondria have very different concentrations of ADP. PCr is phosphocreatine, discussed on p. 487.

[b] This value reflects total concentration; the true value for free ADP may be much lower (Worked Example 13-2).

Mammalian data from R. L. Veech et al., *J. Biol. Chem.* 254:6538, 1979. *E. coli* data from B. D. Bennett et al., *Nat. Chem. Biol.* 5:593, 2009.

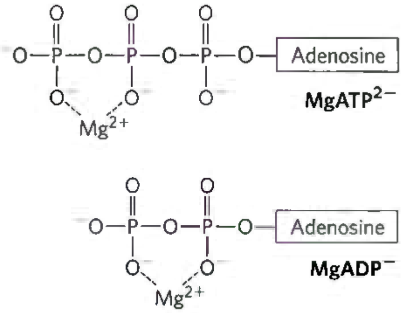


FIGURE 13-12 **Mg^{2+} and ATP.** Formation of Mg^{2+} complexes partially shields the negative charges and influences the conformation of the phosphate groups in nucleotides such as ATP and ADP.

WORKED EXAMPLE 13-2 *Calculation of ΔG_p*

Calculate the actual free energy of hydrolysis of ATP, ΔG_p, in human erythrocytes. The standard free energy of hydrolysis of ATP is −30.5 kJ/mol, and the concentrations of ATP, ADP, and P_i in erythrocytes are as shown in Table 13-5. Assume that the pH is 7.0 and the temperature is 37 °C (human body temperature). What does this reveal about the amount of energy required to *synthesize* ATP under the same cellular conditions?

SOLUTION: The concentrations of ATP, ADP, and P_i in human erythrocytes are 2.25, 0.25, and 1.65 mM, respectively. The actual free energy of hydrolysis of ATP under these conditions is given by the relationship (see Eqn 13-4)

$$\Delta G_p = \Delta G'^\circ + RT \ln \frac{[\text{ADP}][\text{P}_i]}{[\text{ATP}]}$$

Substituting the appropriate values, we get

$$\begin{aligned}\Delta G_p &= -30.5\ \text{kJ/mol} + \left[(8.315\ \text{J/mol}\cdot\text{K})(310\ \text{K}) \ln \frac{(0.25\times10^{-3})(1.65\times10^{-3})}{(2.25\times10^{-3})}\right]\\ &= -30.5\ \text{kJ/mol} + (2.58\ \text{kJ/mol}) \ln 1.8\times10^{-4}\\ &= -30.5\ \text{kJ/mol} + (2.58\ \text{kJ/mol})(-8.6)\\ &= -30.5\ \text{kJ/mol} - 22\ \text{kJ/mol}\\ &= -52\ \text{kJ/mol}\end{aligned}$$

Thus ΔG_p, the actual free-energy change for ATP hydrolysis in the intact erythrocyte (−52 kJ/mol), is much larger than the standard free-energy change (−30.5 kJ/mol). Similarly, the free energy required to *synthesize* ATP from ADP and P_i under the conditions prevailing in the erythrocyte would be 52 kJ/mol.

To further complicate the issue, the *total* concentrations of ATP, ADP, and P_i (and H^+) in a cell—such as the values given in Table 13-5—may be substantially higher than the *free* concentrations, which are the thermodynamically relevant values. The difference is due to tight binding of ATP, ADP, and P_i to cellular proteins. For example, the free [ADP] in resting muscle has been variously estimated at between 1 and 37 μM. Using the value 25 μM in Worked Example 13-2, we would get a ΔG_p of −58 kJ/mol. Calculation of the exact value of ΔG_p, however, is perhaps less instructive than the generalization we can make about actual free-energy changes: in vivo, the energy released by ATP hydrolysis is greater than the standard free-energy change, $\Delta G'^\circ$.

In the following discussions we use the $\Delta G'^\circ$ value for ATP hydrolysis because this allows comparisons, on the same basis, with the energetics of other cellular reactions. Always keep in mind, however, that in living cells ΔG is the relevant quantity—for ATP hydrolysis and all other reactions—and may be quite different from $\Delta G'^\circ$.

Here we must make an important point about cellular ATP levels. We have shown (and will discuss further) how the chemical properties of ATP make it a suitable form of energy currency in cells. But it is not merely the molecule's intrinsic chemical properties that give it this ability to drive metabolic reactions and other energy-requiring processes. Even more important is that, **P4** in the course of evolution, there has been a very strong selective pressure for regulatory mechanisms that *hold cellular ATP concentrations far above the equilibrium concentrations* for the hydrolysis reaction. When the ATP level drops, not only does the *amount* of fuel decrease, but the fuel itself *loses its potency:* ΔG for its hydrolysis (that is, its phosphorylation potential, ΔG_p) is diminished. As our discussions of the metabolic pathways that produce and consume ATP will show, living cells have developed elaborate mechanisms—often at what might seem to us the expense of efficiency—to maintain high concentrations of ATP.

Other Phosphorylated Compounds and Thioesters Also Have Large, Negative Free Energies of Hydrolysis

ATP is not the only biological compound with a large, negative free energy of hydrolysis. **Table 13-6** lists the standard free energies of hydrolysis for some biologically important phosphorylated compounds. In all of these phosphate-releasing reactions, a few of which we describe below, the several resonance forms available to P_i (Fig. 13-11) stabilize this product relative to the reactant, contributing to an already negative free-energy change.

TABLE 13-6 Standard Free Energies of Hydrolysis of Some Phosphorylated Compounds and Acetyl-CoA (a Thioester)

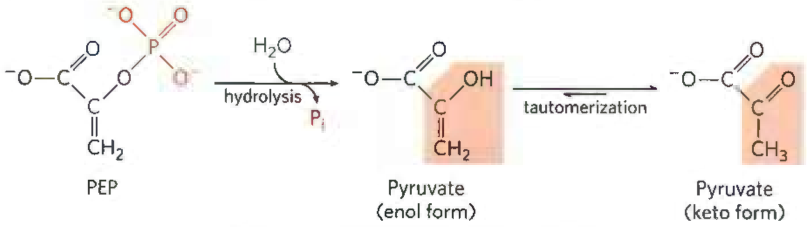
	$\Delta G'^\circ$	
Compounds and Acetyl-CoA	**(kJ/mol)**	**(kcal/mol)**
Phosphoenolpyruvate	−61.9	−14.8
1,3-Bisphosphoglycerate (→ 3-phosphoglycerate + P_i)	−49.3	−11.8
Phosphocreatine	−43.0	−10.3
ADP (→ AMP + P_i)	−32.8	−7.8
ATP (→ ADP + P_i)	−30.5	−7.3
ATP (→ AMP + PP_i)	−45.6	−10.9
AMP (→ adenosine + P_i)	−14.2	−3.4
PP_i(→ 2P_i)	−19.2	−4.0
Glucose 3-phosphate	−20.9	−5.0
Fructose 6-phosphate	−15.9	−3.8
Glucose 6-phosphate	−13.8	−3.3
Glycerol 3-phosphate	−9.2	−2.2
Acetyl-CoA	−31.4	−7.5

Data mostly from W. P. Jencks, in *Handbook of Biochemistry and Molecular Biology*, 3rd edn (G. D. Fasman, ed.), *Physical and Chemical Data*, Vol. 1, p. 296, CRC Press, 1976. Value for the free energy of hydrolysis of PP_i from P. A. Frey and A. Arabshahi, *Biochemistry* 34:11,307, 1995.

Phosphoenolpyruvate (PEP; **Fig. 13-13**), a central intermediate in the energy-conserving process of glycolysis (Chapter 14), contains a phosphate ester bond that undergoes hydrolysis to yield the enol form of pyruvate, and this direct product can tautomerize to the more stable keto form. Because the reactant (PEP) has only one form (enol) and the product (pyruvate) has two possible forms, the reaction occurs with a gain in entropy, and the product is therefore stabilized relative to the reactant. This is a major contributor to the high standard free energy of hydrolysis of phosphoenolpyruvate: $\Delta G'^\circ = -61.9$ kJ/mol. A second contributor is the greater resonance stabilization in the P_i released by cleavage of PEP.

Another intermediate in glycolysis, the three-carbon compound 1,3-bisphosphoglycerate (**Fig. 13-14**), contains an anhydride bond between the C-1 carboxyl group and phosphoric acid. Hydrolysis of this acyl phosphate is accompanied by a large, negative, standard free-energy change ($\Delta G'^\circ = -49.3$ kJ/mol), which can, again, be explained in terms of the structure of reactant and products. When H_2O is added across the anhydride bond of 1,3-bisphosphoglycerate, one of the direct products, 3-phosphoglyceric acid, can lose a proton to give the carboxylate ion, 3-phosphoglycerate, which has two equally probable resonance forms. Removal of the direct product (3-phosphoglyceric acid) by its further metabolism and formation of the resonance-stabilized ion both favor the forward reaction.

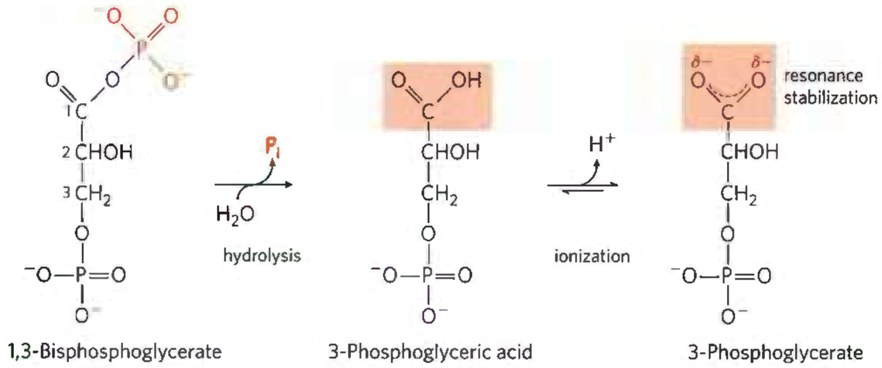
In phosphocreatine (**Fig. 13-15**), which is used in muscle tissue to replenish ATP after its use in contraction, the P—N bond can be hydrolyzed to generate free creatine and P_i. The release of P_i and the resonance stabilization of creatine favor the forward reaction. The standard free-energy change of phosphocreatine hydrolysis is therefore large: −43.0 kJ/mol.



$$PEP^{3-} + H_2O \longrightarrow \text{pyruvate}^- + HPO_4^{2-}$$
$$\Delta G'^\circ = -61.9 \text{ kJ/mol}$$

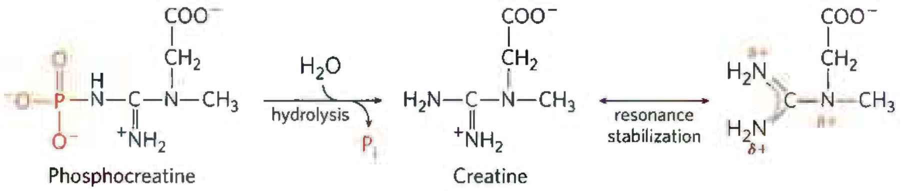
FIGURE 13-13 Hydrolysis of phosphoenolpyruvate (PEP). Catalyzed by pyruvate kinase, this reaction is followed by spontaneous tautomerization of the product, pyruvate. Tautomerization is not possible in PEP, and thus the products of hydrolysis are stabilized relative to the reactants. Resonance stabilization of P_i also occurs, as shown in Figure 13-11.

FIGURE 13-14 Hydrolysis of 1,3-bisphosphoglycerate. The direct product of hydrolysis is 3-phosphoglyceric acid, with an undissociated carboxylic acid. Its dissociation allows resonance structures that stabilize the product relative to the reactants. Resonance stabilization of P_i further contributes to the negative free-energy change.



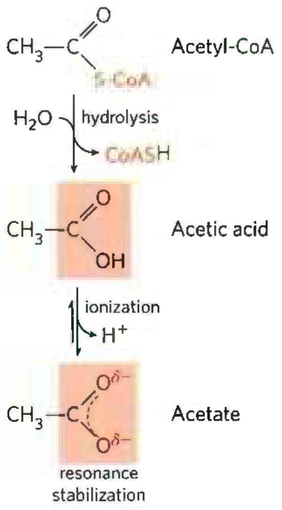
$$\text{1,3-Bisphosphoglycerate}^{4-} + H_2O \longrightarrow \text{3-phosphoglycerate}^{3-} + HPO_4^{2-} + H^+$$
$$\Delta G'^\circ = -49.3\ \text{kJ/mol}$$

FIGURE 13-15 Hydrolysis of phosphocreatine. Breakage of the P—N bond in phosphocreatine produces creatine, which is stabilized by formation of a resonance hybrid. The other product, P_i, is also resonance stabilized.



$$\text{Phosphocreatine}^{2-} + H_2O \longrightarrow \text{creatine} + HPO_4^{2-}$$
$$\Delta G'^\circ = -43.0\ \text{kJ/mol}$$

Thioesters, in which a sulfur atom replaces the usual oxygen in the ester bond, also have large, negative, standard free energies of hydrolysis. Acetyl-coenzyme A, or acetyl-CoA (**Fig. 13-16**), is one of many thioesters important in metabolism. The acyl group in these compounds is activated for transacylation and condensation. Thioesters undergo much less resonance stabilization than do oxygen esters; consequently, the difference in free energy between the reactant and its hydrolysis products, which *are* resonance-stabilized, is greater for thioesters than for comparable oxygen esters (**Fig. 13-17**). In both cases, hydrolysis of the ester generates a carboxylic acid, which can ionize and assume several resonance forms. Together, these factors result in the large, negative $\Delta G'^\circ$ (−31.4 kJ/mol) for acetyl-CoA hydrolysis.



$$\text{Acetyl-CoA} + H_2O \longrightarrow \text{acetate}^- + \text{CoA-SH} + H^+$$
$$\Delta G'^\circ = -31.4\ \text{kJ/mol}$$

FIGURE 13-16 Hydrolysis of acetyl-coenzyme A. Acetyl-CoA is a thioester with a large, negative, standard free energy of hydrolysis. Thioesters contain a sulfur atom in the position occupied by an oxygen atom in oxygen esters. The complete structure of coenzyme A (CoA, or CoASH) is shown in Figure 8-41.

To summarize, for hydrolysis reactions with large, negative, standard free-energy changes, the products are more stable than the reactants for one or more of the following reasons: (1) the bond strain in reactants due to electrostatic repulsion is relieved by charge separation, as for ATP; (2) the products are stabilized by ionization, as for ATP, acyl phosphates, and thioesters; (3) the products are stabilized by isomerization (tautomerization), as for PEP; and/or (4) the products are stabilized by resonance, as for creatine released from phosphocreatine, carboxylate ion released from acyl phosphates and thioesters, and phosphate (P_i) released from anhydride or ester linkages.

ATP Provides Energy by Group Transfers, Not by Simple Hydrolysis

Throughout this book you will encounter reactions or processes for which ATP supplies energy, and the contribution of ATP to these reactions is commonly indicated

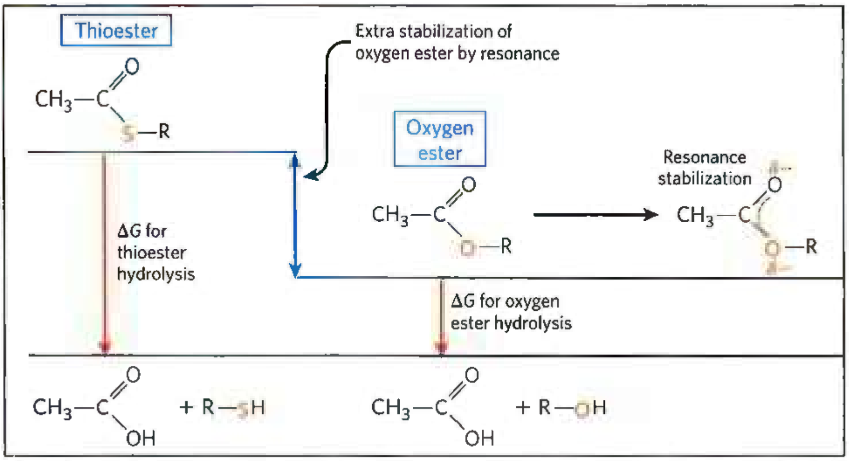


FIGURE 13-17 Free energy of hydrolysis for thioesters and oxygen esters. The *products* of both types of hydrolysis reaction have about the same free-energy content (G), but the thioester has a higher free-energy content than the oxygen ester. Orbital overlap between the O and C atoms allows resonance stabilization in oxygen esters; orbital overlap between S and C atoms is poorer and provides little resonance stabilization.

as in **Figure 13-18a**, with a single arrow showing the conversion of ATP to ADP and P_i (or, in some cases, of ATP to AMP and pyrophosphate, PP_i). When written this way, these reactions of ATP seem to be simple hydrolysis reactions in which water displaces P_i (or PP_i), and one is tempted to say that an ATP-dependent reaction is "driven by the hydrolysis of ATP." This is *not* the case. ATP hydrolysis per se usually accomplishes nothing but the liberation of heat, which cannot drive a chemical process in an isothermal system. A single reaction arrow such as that in Figure 13-18a almost invariably represents a two-step process (Fig. 13-18b) in which part of the ATP molecule, a phosphoryl or pyrophosphoryl group or the adenylate moiety (AMP), is first transferred to a substrate molecule or to an amino acid residue in an enzyme, becoming covalently attached to the substrate or the enzyme and raising its free-energy content (activating it). Then, in a second step, the phosphate-containing moiety transferred in the first step is displaced, generating P_i, PP_i, or AMP as the leaving group. Thus, ATP participates *covalently* in the enzyme-catalyzed reaction to which it contributes free energy.

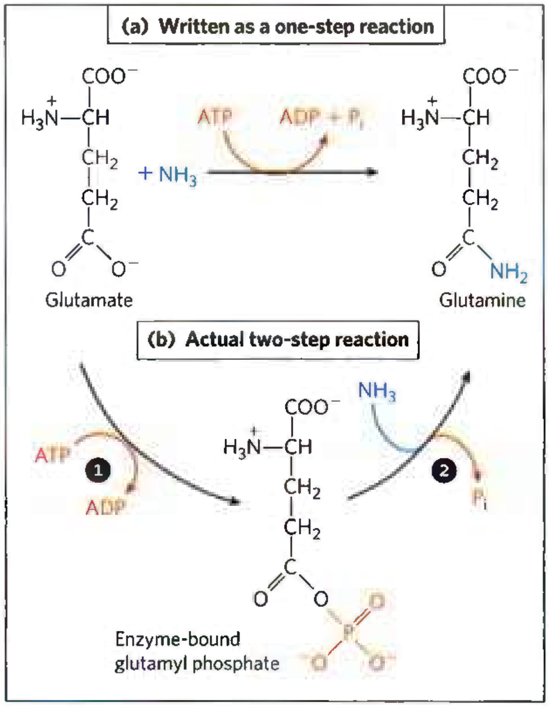

(a) Written as a one-step reaction
Glutamate
+ NH_3
ATP
ADP + P_i
Glutamine
(b) Actual two-step reaction
ATP
①
ADP
Enzyme-bound glutamyl phosphate
NH_3
②
P_i

FIGURE 13-18 ATP hydrolysis in two steps. (a) The contribution of ATP to a reaction is often shown as a single step but is almost always a two-step process. (b) Shown here is the reaction catalyzed by ATP-dependent glutamine synthetase. ① A phosphoryl group is transferred from ATP to glutamate, then ② the phosphoryl group is displaced by NH_3 and released as P_i.

Some processes *do* involve direct hydrolysis of ATP (or the related GTP, guanosine triphosphate), however. For example, noncovalent binding of ATP (or GTP), followed by its hydrolysis to ADP (or GDP, guanosine diphosphate) and P_i, can provide the energy to cycle some proteins between two conformations, producing mechanical motion. This occurs in muscle contraction (see Fig. 5-29) and in the movement of enzymes along DNA (see Fig. 25-30) or of ribosomes along messenger RNA (see Fig. 27-30). The energy-dependent reactions catalyzed by helicases, RecA protein, and some topoisomerases (Chapter 25) also involve direct hydrolysis of phosphoanhydride bonds. The AAA+ ATPases involved in DNA replication and other processes described in Chapter 25 use ATP hydrolysis to cycle associated proteins between active and inactive forms. GTP-binding proteins that act in signaling pathways directly hydrolyze GTP to drive conformational changes that terminate signals triggered by hormones or by other extracellular factors (Chapter 12).

The phosphate compounds found in living organisms can be divided, somewhat arbitrarily, into two groups, based on their standard free energies of hydrolysis (**Fig. 13-19**). **P4** "High-energy" compounds have a $\Delta G'^\circ$ of hydrolysis more negative than –25 kJ/mol; "low-energy" compounds have a less negative $\Delta G'^\circ$. Based on this criterion, ATP, with a $\Delta G'^\circ$ of hydrolysis of –30.5 kJ/mol (–7.3 kcal/mol), is a high-energy compound; glucose 6-phosphate, with a $\Delta G'^\circ$ of hydrolysis of –13.8 kJ/mol (–3.3 kcal/mol), is a low-energy compound.

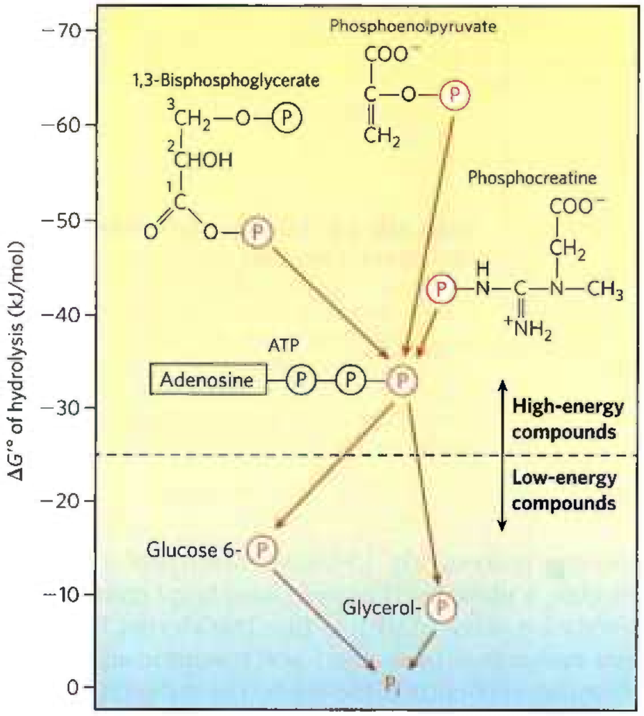


FIGURE 13-19 Ranking of biological phosphate compounds by standard free energies of hydrolysis. Phosphoryl groups, represented by Ⓟ, flow from high-energy phosphoryl group donors via ATP to acceptor molecules (such as glucose and glycerol) to form their low-energy phosphate derivatives. (The location of each compound's donor phosphoryl group along the scale is an approximate indication of the compound's $\Delta G'^\circ$ of hydrolysis.) This flow of phosphoryl groups, catalyzed by kinases, proceeds with an overall loss of free energy under intracellular conditions. Hydrolysis of low-energy phosphate compounds releases P_i, which has an even lower phosphoryl group transfer potential.

The term "high-energy phosphate bond," long used by biochemists to describe the P—O bond broken in hydrolysis reactions, is incorrect and misleading, as it wrongly suggests that the bond itself contains the energy. In fact, the breaking of all chemical bonds requires an *input* of energy. The free energy released by hydrolysis of phosphate compounds does not come from the specific bond that is broken; it results from the products of the reaction having a lower free-energy content than the reactants. For simplicity, we sometimes use the term "high-energy phosphate compound" when referring to ATP or other phosphate compounds with a large, negative, standard free energy of hydrolysis.

P2 As is evident from the additivity of free-energy changes of sequential reactions (see Section 13.1), any phosphorylated compound can be synthesized by coupling the synthesis to the breakdown of another phosphorylated compound with a more negative free energy of hydrolysis. For example, because cleavage of P_i from phosphoenolpyruvate releases more energy than is needed to drive the condensation of P_i with ADP, the direct donation of a phosphoryl group from PEP to ADP is thermodynamically feasible:

$$\begin{array}{llr} & & \Delta G'^\circ\ \text{(kJ/mol)} \\ (1) & \text{PEP} + H_2O \longrightarrow \text{Pyruvate} + P_i & -61.9 \\ (2) & \text{ADP} + P_i \longrightarrow \text{ATP} + H_2O & +30.5 \\ \hline \textit{Sum:} & \text{PEP} + \text{ADP} \longrightarrow \text{Pyruvate} + \text{ATP} & -31.4 \end{array}$$

Notice that although the actual reaction is represented as the algebraic sum of the first two reactions, the actual reaction is a third, distinct reaction that does not involve P_i; PEP donates a *phosphoryl* group *directly* to ADP. We can describe phosphorylated compounds as having a high or low *phosphoryl group transfer potential*, on the basis of their standard free energies of hydrolysis (as listed in Table 13-6). The phosphoryl group transfer potential of PEP is very high, that of ATP is high, and that of glucose 6-phosphate is low (Fig. 13-19).

Much of catabolism is directed toward the synthesis of high-energy phosphate compounds, but their formation is not an end in itself; they are the means of activating a wide variety of compounds for further chemical transformation. The transfer of a phosphoryl group to a compound effectively puts free energy into that compound, so that it has more free energy to give up during subsequent metabolic transformations. We described above how the synthesis of glucose 6-phosphate is accomplished by phosphoryl group transfer from ATP. In the next chapter we see how this phosphorylation of glucose activates, or "primes," the glucose for catabolic reactions that occur in nearly every living cell. Because of its intermediate position on the scale of group transfer potential, ATP can carry energy from high-energy phosphate compounds produced by catabolism (phosphoenolpyruvate, for example) to compounds such as glucose, converting them into more reactive species with better leaving groups. ATP thus serves as the universal energy currency in all living cells.

One more chemical feature of ATP is crucial to its role in metabolism: although, in aqueous solution, ATP is thermodynamically unstable and is therefore a good phosphoryl group donor, it is *kinetically* stable. Because of the huge activation energies (200 to 400 kJ/mol) required for uncatalyzed cleavage of its phosphoanhydride bonds, ATP does not spontaneously donate phosphoryl groups to water or to the hundreds of other potential acceptors in the cell. Only when specific enzymes are present to lower the energy of activation does phosphoryl group transfer from ATP proceed. The cell is therefore able to regulate the disposition of the energy carried by ATP by regulating the various enzymes that act on it.

ATP Donates Phosphoryl, Pyrophosphoryl, and Adenylyl Groups

The reactions of ATP are generally S_N2 nucleophilic displacements (see Section 13.2) in which the nucleophile may be, for example, the oxygen of an alcohol or carboxylate, or a nitrogen of creatine or of the side chain

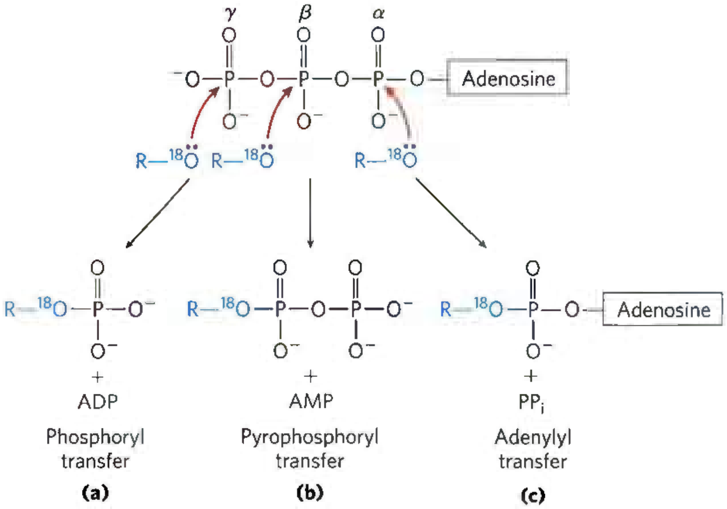


FIGURE 13-20 Three positions on ATP for attack by the nucleophile R—$^{18}\ddot{O}$. Any of the three P atoms (α, β, or γ) may serve as the electrophilic target for nucleophilic attack, in this case by the labeled nucleophile R—$^{18}\ddot{O}$. The nucleophile may be an alcohol (ROH), a carboxyl group ($RCOO^-$), or a phosphoanhydride (a nucleoside mono- or diphosphate, for example). (a) When the oxygen of the nucleophile attacks the γ position, the bridge oxygen of the product is labeled, indicating that the group transferred from ATP is a phosphoryl ($—PO_3^{2-}$), not a phosphate ($—OPO_3^{2-}$). (b) Attack on the β position displaces AMP and leads to the transfer of a pyrophosphoryl (not pyrophosphate) group to the nucleophile. (c) Attack on the α position displaces PP_i and transfers the adenylyl group to the nucleophile.

of arginine or histidine. Each of the three phosphates of ATP is susceptible to nucleophilic attack (**Fig. 13-20**), and each position of attack yields a different type of product.

Nucleophilic attack by an alcohol on the γ phosphate (Fig. 13-20a) displaces ADP and produces a new phosphate ester. Studies with $^{18}\ddot{O}$-labeled reactants have shown that the bridge oxygen in the new compound is derived from the alcohol, not from ATP; the group transferred from ATP is therefore a phosphoryl ($-PO_3^{2-}$), not a phosphate ($-OPO_3^{2-}$). Phosphoryl group transfer from ATP to glutamate (Fig. 13-18) or to glucose (p. 209) involves attack at the γ position of the ATP molecule.

Attack at the β phosphate of ATP displaces AMP and transfers a pyrophosphoryl (not pyrophosphate) group to the attacking nucleophile (Fig. 13-20b). For example, the formation of 5-phosphoribosyl-1-pyrophosphate (Chapter 22), a key intermediate in nucleotide synthesis, results from attack of an —OH of the ribose on the β phosphate.

Nucleophilic attack at the α position of ATP displaces PP_i and transfers adenylate (5′-AMP) as an adenylyl group (Fig. 13-20c); the reaction is an **adenylylation** (a-den′-i-li-la′-shun, one of the most ungainly words in the biochemical language). Notice that hydrolysis of the α–β phosphoanhydride bond releases considerably more energy (~46 kJ/mol) than hydrolysis of the β–γ bond (~31 kJ/mol) (Table 13-6). Furthermore, the PP_i formed as a byproduct of the adenylylation is hydrolyzed to two P_i by the ubiquitous enzyme **inorganic pyrophosphatase**, releasing 19 kJ/mol and thereby providing a further energy "push" for the adenylylation reaction. In effect, both phosphoanhydride bonds of ATP are split in the overall reaction. Adenylylation reactions are therefore thermodynamically very favorable. **P4** When the energy of ATP is used to drive a particularly unfavorable metabolic reaction, adenylylation is often the mechanism of energy coupling. Fatty acid activation is a good example of this energy-coupling strategy.

The first step in the activation of a fatty acid—either for energy-yielding oxidation or for use in the synthesis of more complex lipids—is the formation of its thiol ester (see Fig. 17-5). The direct condensation of a fatty acid with coenzyme A is endergonic, but the formation of a fatty acyl–CoA is made exergonic by stepwise removal of *two* phosphoryl groups from ATP. First, adenylate (AMP) is transferred from ATP to the carboxyl group of the fatty acid, forming a mixed anhydride (fatty acyl adenylate) and liberating PP_i. The thiol group of coenzyme A then displaces the adenylyl group and forms a thioester with the fatty acid. The sum of these two reactions is energetically equivalent to the exergonic hydrolysis of ATP to AMP and PP_i ($\Delta G'^\circ = -45.6$ kJ/mol) and the endergonic formation of fatty acyl–CoA. The formation of fatty acyl–CoA ($\Delta G'^\circ = -31.4$ kJ/mol) is made energetically favorable by hydrolysis of the PP_i by inorganic pyrophosphatase. Thus, in the activation of a fatty acid, both phosphoanhydride bonds of ATP are broken. The resulting $\Delta G'^\circ$ is the sum of the $\Delta G'^\circ$ values for the breakage of these bonds, or −45.6 kJ/mol + (−19.2) kJ/mol:

$$ATP + 2H_2O \longrightarrow AMP + 2P_i \qquad \Delta G'^\circ = -64.8 \text{ kJ/mol}$$

The activation of amino acids before their polymerization into proteins (see Fig. 27-19) is accomplished by an analogous set of reactions in which a transfer RNA molecule takes the place of coenzyme A. An interesting use of the cleavage of ATP to AMP and PP_i occurs in the firefly, which uses ATP as an energy source to produce flashes of light (**Box 13-2**).

Assembly of Informational Macromolecules Requires Energy

When simple precursors are assembled into high molecular weight polymers with defined sequences (DNA, RNA, proteins), as described in detail in Part III, energy is required both for the condensation of monomeric units and for the creation of *ordered* sequences. The precursors for DNA and RNA synthesis are nucleoside triphosphates, and polymerization is accompanied by cleavage

BOX 13-2

Firefly Flashes: Glowing Reports of ATP

Bioluminescence requires considerable amounts of energy. The firefly uses ATP to convert chemical energy into light energy. Males emit a flash of light to attract females, who flash in return to signal their interest. In the 1950s, from many thousands of fireflies collected by children in and around Baltimore, William McElroy and his colleagues at Johns Hopkins University isolated the principal biochemical components: luciferin, a complex carboxylic acid, and luciferase, an enzyme. Activation of luciferin by an enzymatic reaction involving pyrophosphate cleavage of ATP to form luciferyl adenylate generates the light flash (Fig. 1). In the presence of molecular oxygen and luciferase, the luciferin undergoes a multistep oxidative decarboxylation to oxyluciferin. This process is accompanied by emission of light. The color of the light flash differs from one firefly species to another and seems to be determined by differences in the structure of the luciferase. Luciferin is regenerated from oxyluciferin in a subsequent series of reactions.

In the laboratory, pure firefly luciferin and luciferase are used to measure minute quantities of ATP by the intensity of the light flash produced. As little as a few picomoles (10^{-12} mol) of ATP can be measured in this way.

The firefly, a beetle of the *Lampyridae* family.
[Cathy Keifer/Fotolia]

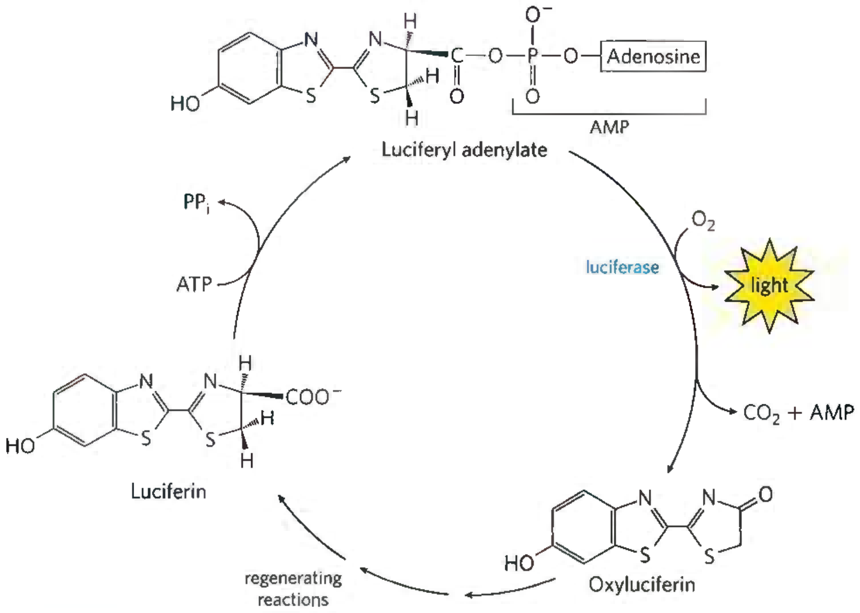


FIGURE 1 Important components in the firefly bioluminescence cycle.

of the phosphoanhydride linkage between the α and β phosphates, with the release of PP_i (Fig. 13-20). The moieties transferred to the growing polymer in these reactions are adenylate (AMP), guanylate (GMP), cytidylate (CMP), or uridylate (UMP) for RNA synthesis, and their deoxy analogs (with TMP in place of UMP) for DNA synthesis. As noted above, the activation of amino acids for protein synthesis involves the donation of adenylyl groups from ATP, and we shall see in Chapter 27 that several steps of protein synthesis on the ribosome are also accompanied by GTP hydrolysis. In all these cases, the exergonic breakdown of a nucleoside triphosphate is coupled to the endergonic process of synthesizing a polymer of a specific sequence.

ATP can supply the energy for transporting an ion or a molecule across a membrane into another aqueous compartment where its concentration is higher (see Fig. 11-39). Transport processes are major consumers of energy; in human kidney and brain, for example, as much as two-thirds of the energy consumed at rest is used to pump Na^+ and K^+ across plasma membranes via the Na^+K^+ ATPase. The transport of Na^+ and K^+ is driven by cyclic phosphorylation and dephosphorylation of the transporter protein, with ATP as the phosphoryl group donor. Na^+-dependent phosphorylation of the Na^+K^+ ATPase forces a change in the protein's conformation, and K^+-dependent dephosphorylation favors return to the original conformation. Each cycle in the transport process results in the conversion of ATP to ADP and P_i, and it is the free-energy change of ATP hydrolysis that drives the cyclic changes in protein conformation that result in the electrogenic pumping of Na^+ and K^+. Note that in this case, ATP interacts covalently by phosphoryl group transfer to the *enzyme*, not to the *substrate*.

In the contractile system of skeletal muscle cells, myosin and actin are specialized to transduce the chemical energy of ATP into motion (see Fig. 5-29). ATP binds tightly but noncovalently to one conformation of myosin, holding the protein in that conformation. When myosin catalyzes the hydrolysis of its bound ATP, the ADP and P_i dissociate from the protein, allowing it to relax into a second conformation until another molecule of ATP binds. The binding and subsequent hydrolysis of ATP (by myosin ATPase) provide the energy that forces cyclic changes in the conformation of the myosin head. The change in conformation of many individual myosin molecules results in the sliding of myosin fibrils along actin filaments (see Fig. 5-28), which translates into macroscopic contraction of the muscle fiber. As we noted earlier, this production of mechanical motion at the expense of ATP is one of the few cases in which ATP hydrolysis per se, rather than group transfer from ATP, is the source of the chemical energy in a coupled process.

Transphosphorylations between Nucleotides Occur in All Cell Types

P4 Although we have focused on ATP as the cell's energy currency and donor of phosphoryl groups, all other nucleoside triphosphates (GTP, UTP, and CTP) and all deoxynucleoside triphosphates (dATP, dGTP, dTTP, and dCTP) are energetically equivalent to ATP. The standard free-energy changes associated with hydrolysis of their phosphoanhydride linkages are very nearly identical with those shown in Table 13-6 for ATP. In preparation for their various biological roles, these other nucleotides are generated and maintained as the nucleoside triphosphate (NTP) forms by phosphoryl group transfer to the corresponding nucleoside diphosphates (NDPs) and monophosphates (NMPs).

ATP is the primary high-energy phosphate compound produced by catabolism, in the processes of glycolysis, oxidative phosphorylation, and, in photosynthetic cells, photophosphorylation. Several enzymes then carry phosphoryl groups from ATP to the other nucleotides. **Nucleoside diphosphate kinase**, found in all cells, catalyzes the reaction

$$\text{ATP} + \text{NDP (or dNDP)} \xrightleftharpoons{\text{Mg}^{2+}} \text{ADP} + \text{NTP (or dNTP)} \qquad \Delta G'^{\circ} \approx 0$$

Although this reaction is fully reversible, the relatively high [ATP]/[ADP] ratio in cells normally drives the reaction to the right, with the net formation of NTPs and dNTPs. The enzyme actually catalyzes a two-step phosphoryl group transfer, which is a classic case of a double-displacement (Ping-Pong) mechanism (**Fig. 13-21**; see also Fig. 6-15b). First, phosphoryl group transfer from ATP to an active-site His residue produces a phosphoenzyme intermediate; then the phosphoryl group is transferred from the Ⓟ–His residue to an NDP acceptor. Because the enzyme is nonspecific for the base in the NDP and works equally well on dNDPs and NDPs, it can synthesize all NTPs and dNTPs, given the corresponding NDPs and a supply of ATP.

Phosphoryl group transfers from ATP result in an accumulation of ADP; for example, when muscle is contracting vigorously, ADP accumulates and interferes with ATP-dependent contraction. During periods of intense demand for ATP, the cell lowers the ADP concentration, and at the same time replenishes ATP, by the action of **adenylate kinase**:

$$2\text{ADP} \xrightleftharpoons{\text{Mg}^{2+}} \text{ATP} + \text{AMP} \qquad \Delta G'^{\circ} \approx 0$$

This reaction is fully reversible, so, after the intense demand for ATP ends, the enzyme can recycle AMP by converting it to ADP, which can then be phosphorylated to ATP in mitochondria. A similar enzyme, guanylate kinase, converts GMP to GDP at the expense of ATP. By pathways such as these, energy conserved in the catabolic production of ATP is used to supply the cell with all required NTPs and dNTPs.

Phosphocreatine (PCr; Fig. 13-15), also called creatine phosphate, serves as a ready source of phosphoryl groups for the quick synthesis of ATP from ADP. The PCr concentration in skeletal muscle is approximately 30 mM, nearly 10 times the concentration of ATP, and in other tissues such as smooth muscle, brain, and kidney, [PCr] is 5 to 10 mM. The enzyme **creatine kinase** catalyzes the reversible reaction

$$\text{ADP} + \text{PCr} \xrightleftharpoons{\text{Mg}^{2+}} \text{ATP} + \text{Cr} \qquad \Delta G'^{\circ} = -12.5 \text{ kJ/mol}$$

When a sudden demand for energy depletes ATP, the PCr reservoir is used to replenish ATP at a rate considerably faster than ATP can be synthesized by catabolic pathways. When the demand for energy slackens, ATP

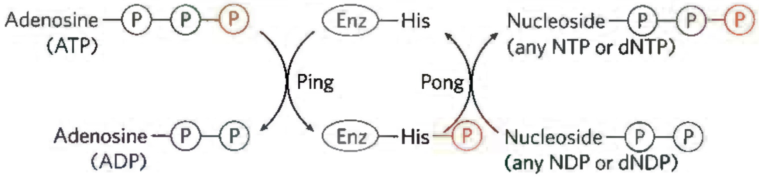


FIGURE 13-21 **Ping-Pong mechanism of nucleoside diphosphate kinase.** The enzyme binds its first substrate (ATP in our example), and a phosphoryl group is transferred to the side chain of a His residue. ADP departs, another nucleoside (or deoxynucleoside) diphosphate replaces it, and this is converted to the corresponding triphosphate by transfer of the phosphoryl group from the phosphohistidine residue.

produced by catabolism is used to replenish the PCr reservoir by reversal of the creatine kinase reaction (see Box 23-1). Organisms in the lower phyla employ other PCr-like molecules (collectively called **phosphagens**) as phosphoryl reservoirs.

SUMMARY 13.3 *Phosphoryl Group Transfers and ATP*

- ATP is the chemical link between catabolism and anabolism. It is the energy currency of the living cell. The exergonic conversion of ATP to ADP and P_i, or to AMP and PP_i, is coupled to many endergonic reactions and processes.
- The free-energy change for ATP hydrolysis under cellular conditions is its phosphorylation potential, ΔG_p.
- Direct hydrolysis of ATP is the source of energy in some processes driven by conformational changes. In general, however, it is not ATP hydrolysis but the transfer of a phosphoryl group from ATP to a substrate or an enzyme that couples the energy of ATP breakdown to endergonic transformations of substrates.
- Phosphate compounds with high free energies of hydrolysis can donate their phosphoryl group to form another phosphate compound with a smaller free energy of hydrolysis.
- ATP can also donate a pyrophosphoryl (PP_i) or adenylyl (AMP) group to a variety of metabolic intermediates, activating them for nucleophilic displacement reactions.
- Through these group transfer reactions, ATP provides the energy for a large number of anabolic reactions, including the synthesis of informational macromolecules, and for the transport of molecules and ions across membranes against concentration gradients and electrical potential gradients. Muscle contraction is also powered by ATP.
- To maintain its high group transfer potential, ATP concentration must be held far above the equilibrium concentration by energy-yielding reactions of catabolism.
- ATP can donate a phosphoryl group to nucleoside diphosphates by transphosphorylation to keep the levels of GTP, UTP, CTP, and the deoxynucleotides far above their equilibrium concentrations.

13.4 Biological Oxidation-Reduction Reactions

The transfer of phosphoryl groups is a central feature of metabolism. Equally important is another kind of transfer: electron transfer in oxidation-reduction reactions, sometimes referred to as redox reactions. These reactions involve the loss of electrons by one chemical species, which is thereby oxidized, and the gain of electrons by another, which is reduced. P5 The flow of electrons in oxidation-reduction reactions is responsible, directly or indirectly, for all work done by living organisms. In nonphotosynthetic organisms, the sources of electrons are reduced compounds (foods); in photosynthetic organisms, the initial electron donor is a chemical species excited by the absorption of light. The path of electron flow in metabolism is complex. Electrons move from various metabolic intermediates to specialized electron carriers in enzyme-catalyzed reactions. The carriers, in turn, donate electrons to acceptors with higher electron affinities, with the release of energy. Cells possess a variety of molecular energy transducers, which convert the energy of electron flow into useful work.

We begin by discussing how work can be accomplished by an electromotive force (emf), then consider the theoretical and experimental basis for measuring energy changes in oxidation reactions in terms of emf and the relationship between this force, expressed in volts, and the free-energy change, expressed in joules. We also describe the structures and oxidation-reduction chemistry of the most common of the specialized electron carriers, which you will encounter repeatedly in later chapters.

The Flow of Electrons Can Do Biological Work

Every time we use a motor, an electric light or heater, or a spark to ignite gasoline in a car engine, we use the flow of electrons to accomplish work. In the circuit that powers a motor, the source of electrons can be a battery containing two chemical species that differ in affinity for electrons. Electrical wires provide a pathway for electron flow from the chemical species at one pole of the battery, through the motor, to the chemical species at the other pole of the battery. Because the two chemical species differ in their affinity for electrons, electrons flow spontaneously through the circuit, driven by a force proportional to the difference in electron affinity, the **electromotive force, emf**. The emf (typically a few volts) can accomplish work if an appropriate energy transducer—in this case a motor—is placed in the circuit. The motor can be coupled to a variety of mechanical devices to do useful work.

P5 Living cells have an analogous biological "circuit," with a relatively reduced compound such as glucose as the source of electrons. As glucose is enzymatically oxidized, the released electrons flow spontaneously through a series of electron-carrier intermediates to another chemical species, such as O_2. This electron flow is exergonic, because O_2 has a higher affinity for electrons than do the electron-carrier intermediates. The resulting emf provides energy to a variety of molecular energy transducers (enzymes and other proteins) that do biological work. In the mitochondrion, for example, membrane-bound enzymes couple electron flow to the production of a transmembrane pH difference and a transmembrane electrical potential, accomplishing chemiosmotic and electrical work. The proton gradient thus formed has potential energy, sometimes called the proton-motive force by analogy with electromotive force. Another enzyme, ATP synthase in the inner mitochondrial membrane, uses the proton-motive force to do chemical work: synthesis of ATP from ADP and P_i as protons flow spontaneously across the membrane. Similarly, membrane-localized enzymes in *Escherichia coli* convert emf to proton-motive force, which is then used to power flagellar motion. The principles of electrochemistry that govern energy changes in the macroscopic circuit with a motor and battery apply with equal validity to the molecular processes accompanying electron flow in living cells.

Oxidation-Reductions Can Be Described as Half-Reactions

Although oxidation and reduction must occur together, it is convenient when describing electron transfers to consider the two halves of an oxidation-reduction reaction separately. For example, the oxidation of ferrous ion by cupric ion,

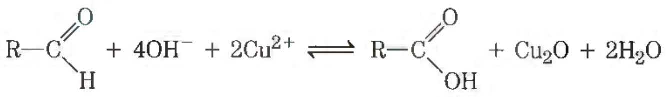
$$Fe^{2+} + Cu^{2+} \rightleftharpoons Fe^{3+} + Cu^{+}$$

can be described in terms of two half-reactions:

$$(1)\ Fe^{2+} \rightleftharpoons Fe^{3+} + e^{-}$$
$$(2)\ Cu^{2+} + e^{-} \rightleftharpoons Cu^{+}$$

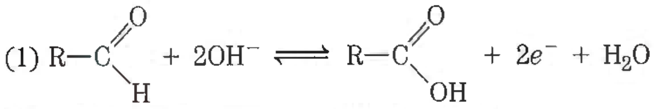
The electron-donating molecule in an oxidation-reduction reaction is called the reducing agent or reductant; the electron-accepting molecule is the oxidizing agent or oxidant. A given agent, such as an iron cation existing in the ferrous (Fe^{2+}) or ferric (Fe^{3+}) state, functions as a conjugate reductant-oxidant pair (redox pair), just as an acid and corresponding base function as a conjugate acid-base pair. Recall from Chapter 2 that in acid-base reactions we can write a general equation: proton donor $\rightleftharpoons$ H^+ + proton acceptor. In redox reactions we can write a similar general equation: electron donor (reductant) $\rightleftharpoons$ e^- + electron acceptor (oxidant). In the reversible half-reaction (1) above, Fe^{2+} is the electron donor and Fe^{3+} is the electron acceptor; together, Fe^{2+} and Fe^{3+} constitute a **conjugate redox pair**. The mnemonic OIL RIG—*o*xidation *i*s *l*osing, *r*eduction *i*s *g*aining—may be helpful in remembering what happens to electrons in redox reactions.

The electron transfers in the oxidation-reduction reactions of organic compounds are not fundamentally different from those of inorganic species. Consider the oxidation of a reducing sugar (an aldehyde or a ketone) by cupric ion:



$$R{-}C(=O){-}H + 4OH^{-} + 2Cu^{2+} \rightleftharpoons R{-}C(=O){-}OH + Cu_2O + 2H_2O$$

This overall reaction can be expressed as two half-reactions:



$$(1)\ R{-}C(=O){-}H + 2OH^{-} \rightleftharpoons R{-}C(=O){-}OH + 2e^{-} + H_2O$$

$$(2)\ 2Cu^{2+} + 2e^{-} + 2OH^{-} \rightleftharpoons Cu_2O + H_2O$$

Notice that because two electrons are removed from the aldehyde carbon, the second half-reaction (the one-electron reduction of cupric to cuprous ion) must be doubled to balance the overall equation.

Biological Oxidations Often Involve Dehydrogenation

The carbon in living cells exists in a range of oxidation states (**Fig. 13-22**). When a carbon atom shares an electron pair with another atom (typically H, C, S, N, or O), the sharing is unequal, in favor of the more electronegative atom. The order of increasing electronegativity is $H < C < S < N < O$. In oversimplified but useful terms, the more electronegative atom "owns" the bonding electrons it shares with another atom. For example, in methane (CH_4), carbon is more electronegative than the four hydrogens bonded to it, and the C atom therefore owns all eight bonding electrons (Fig. 13-22). In ethane, the electrons in the C—C bond are shared equally, so each C atom owns only seven of its eight bonding electrons. In ethanol, C-1 is less electronegative than the oxygen to which it is bonded, and the O atom therefore owns both electrons of the C—O bond, leaving C-1 with only five bonding electrons. With each formal loss of "owned" electrons, the carbon atom has undergone oxidation—even when no oxygen is involved, as in the conversion of an alkane ($—CH_2—CH_2—$) to an alkene ($—CH{=}CH—$). In this case, oxidation (loss of electrons) is coincident with the loss of hydrogen. In biological systems, as we noted earlier in the chapter, oxidation is often synonymous with **dehydrogenation**, and many enzymes that catalyze oxidation reactions are **dehydrogenases**. Notice that the more reduced compounds in Figure 13-22 (top) are richer in hydrogen than in oxygen, whereas the more oxidized compounds (bottom) have more oxygen and less hydrogen.

Not all biological oxidation-reduction reactions involve carbon. For example, in the conversion of molecular nitrogen to ammonia, $6H^+ + 6e^- + N_2 \rightarrow 2NH_3$, the nitrogen atoms are reduced.

Electrons are transferred from one molecule (electron donor) to another (electron acceptor) in one of four ways:

1. Directly as *electrons*. For example, the Fe^{2+}/Fe^{3+} redox pair can transfer an electron to the Cu^{+}/Cu^{2+} redox pair:

$$Fe^{2+} + Cu^{2+} \rightleftharpoons Fe^{3+} + Cu^{+}$$

2. As *hydrogen atoms*. Recall that a hydrogen atom consists of a proton (H^+) and a single electron (e^-). In this case we can write the general equation

$$AH_2 \rightleftharpoons A + 2e^{-} + 2H^{+}$$

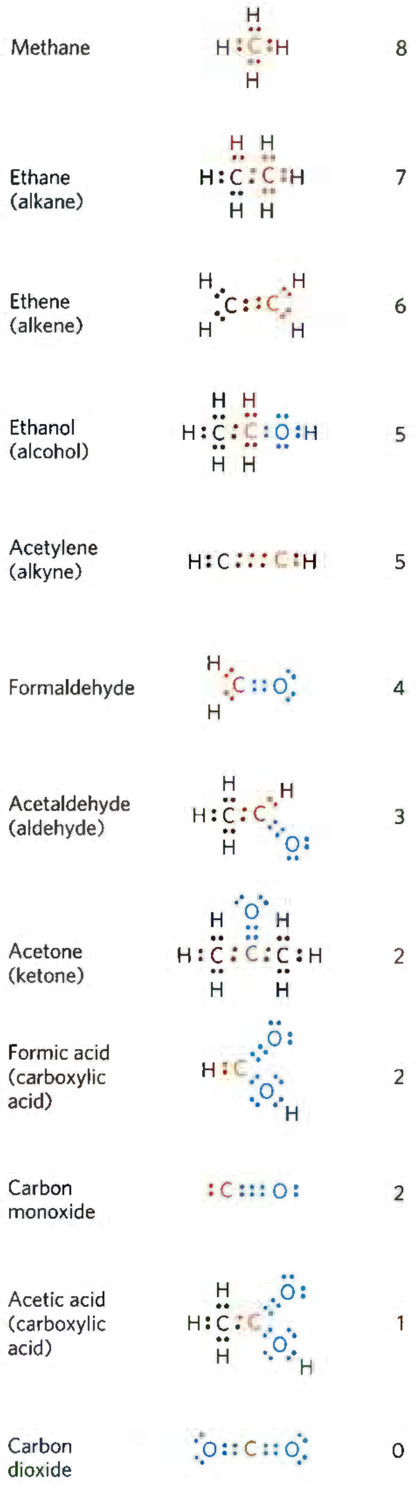


FIGURE 13-22 Different levels of oxidation of carbon compounds in the biosphere. To approximate the level of oxidation of these compounds, focus on the red carbon atom and its bonding electrons. When this carbon is bonded to the less electronegative H atom, both bonding electrons (red) are assigned to the carbon. When carbon is bonded to another carbon, bonding electrons are shared equally, so one of the two electrons is assigned to the red carbon. When the red carbon is bonded to the more electronegative O atom, the bonding electrons are assigned to the oxygen. The number to the right of each compound is the number of electrons "owned" by the red carbon, a rough expression of the degree of oxidation of that compound. As the red carbon undergoes oxidation (loses electrons), the number gets smaller.

where AH_2 is the hydrogen/electron donor. (Do not mistake the above reaction for an acid dissociation, which involves a proton and no electron.) AH_2 and A together constitute a conjugate redox pair (A/AH_2), which can reduce another compound B (or redox pair, B/BH_2) by transfer of hydrogen atoms:

$$AH_2 + B \rightleftharpoons A + BH_2$$

3. As a *hydride ion* (:H^-), which has two electrons. This occurs in the case of NAD-linked dehydrogenases, described below.

4. Through direct *combination with oxygen*. In this case, oxygen combines with an organic reductant and is covalently incorporated in the product, as in the oxidation of a hydrocarbon to an alcohol:

$$R{-}CH_3 + \tfrac{1}{2}O_2 \longrightarrow R{-}CH_2{-}OH$$

The hydrocarbon is the electron donor, and the oxygen atom is the electron acceptor.

All four types of electron transfer occur in cells. The neutral term **reducing equivalent** is commonly used to designate a single electron equivalent participating in an oxidation-reduction reaction, no matter whether this equivalent is an electron per se or is part of a hydrogen atom or a hydride ion, or whether the electron transfer takes place in a reaction with oxygen to yield an oxygenated product.

Reduction Potentials Measure Affinity for Electrons

When two conjugate redox pairs are together in solution, electron transfer from the electron donor of one pair to the electron acceptor of the other may proceed spontaneously. The tendency for such a reaction depends on the relative affinity of the electron acceptor of each redox pair for electrons. The **standard reduction potential, $E°$**, a measure (in volts) of this affinity, can be determined in an experiment such as that described in **Figure 13-23**. Electrochemists have chosen as a standard of reference the half-reaction

$$H^+ + e^- \longrightarrow \tfrac{1}{2}H_2$$

The electrode at which this half-reaction occurs (called a half-cell) is arbitrarily assigned an $E°$ of 0.00 V. When this hydrogen electrode is connected through an external circuit to another half-cell in which an oxidized species and its corresponding reduced species are present at standard concentrations (at 25 °C, each solute at 1 M, each gas at 101.3 kPa), electrons tend to flow through the external circuit from the half-cell of lower $E°$ to the half-cell of higher $E°$. By convention, a half-cell that takes electrons from the standard hydrogen cell is assigned a positive value of $E°$, and one that donates electrons to the hydrogen cell, a negative value. When any two half-cells are connected, that with the larger

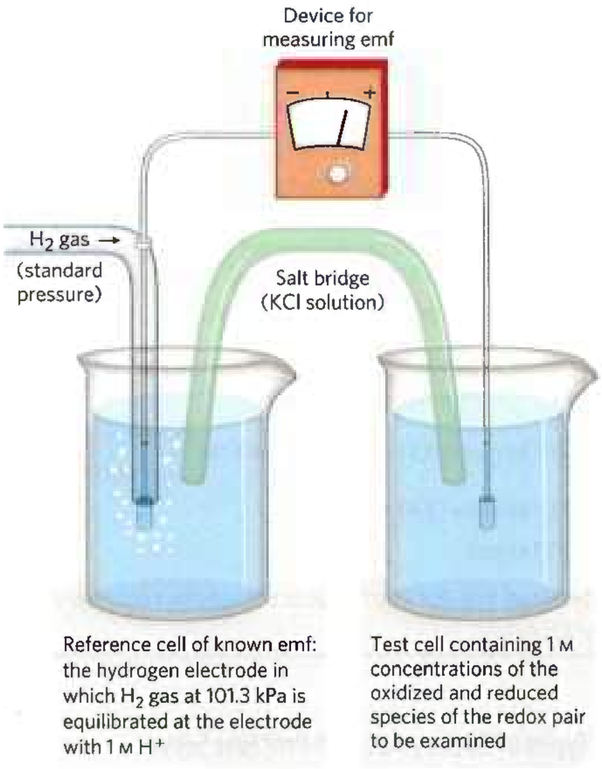

Device for measuring emf
−
+
H_2 gas →
(standard pressure)
Salt bridge (KCl solution)
Reference cell of known emf: the hydrogen electrode in which H_2 gas at 101.3 kPa is equilibrated at the electrode with 1 M H^+
Test cell containing 1 M concentrations of the oxidized and reduced species of the redox pair to be examined

FIGURE 13-23 Measurement of the standard reduction potential ($E'°$) of a redox pair. Electrons flow from the test electrode to the reference electrode, or vice versa. The ultimate reference half-cell is the hydrogen electrode, as shown here, at pH 0. The electromotive force (emf) of this electrode is designated 0.00 V. At pH 7 in the test cell (at 25 °C), $E'°$ for the hydrogen electrode is −0.414 V. The direction of electron flow depends on the relative electron "pressure" or potential of the two cells. A salt bridge containing a saturated KCl solution provides a path for counter-ion movement between the test cell and the reference cell. From the observed emf and the known emf of the reference cell, the experimenter can find the emf of the test cell containing the redox pair. The cell that gains electrons has, by convention, the more positive reduction potential.

(more positive) $E°$ will be reduced; it has the greater reduction potential.

The reduction potential of a half-cell depends not only on the chemical species present but also on their activities, approximated by their concentrations. The Nernst equation relates standard reduction potential ($E°$) to the actual reduction potential (E) at any concentration of oxidized and reduced species in a living cell:

$$E = E° + \frac{RT}{nF} \ln \frac{[\text{electron acceptor}]}{[\text{electron donor}]} \qquad (13\text{-}5)$$

where R and T have their usual meanings, n is the number of electrons transferred per molecule, and F is the Faraday constant, a proportionality constant that converts volts to joules (Table 13-1). At 298 K (25 °C), this expression reduces to

$$E = E° + \frac{0.026\ \text{V}}{n} \ln \frac{[\text{electron acceptor}]}{[\text{electron donor}]} \qquad (13\text{-}6)$$

KEY CONVENTION **Many half-reactions of interest to biochemists involve protons. As in the definition of $\Delta G'°$, biochemists define the standard state for oxidation-reduction reactions as pH 7 and express a standard transformed reduction potential, $E'°$, the standard reduction potential at pH 7 and 25 °C. By convention, $\Delta E'°$ for any redox reaction is given as $E'°$ of the electron acceptor minus $E'°$ of the electron donor.** ■

The standard reduction potentials given in **Table 13-7** and used throughout this book are values for $E'°$ and are therefore valid only for systems at neutral pH. Each value represents the potential difference when the conjugate redox pair, at 1 M concentrations, 25 °C, and pH 7, is connected with the standard (pH 0) hydrogen electrode. Notice in Table 13-7 that when the conjugate pair $2H^+/H_2$ at pH 7 is connected with the standard hydrogen

TABLE 13-7 Standard Reduction Potentials of Some Biologically Important Half-Reactions

Half-reaction	$E'°$ (V)
$\frac{1}{2}O_2 + 2H^+ + 2e^- \longrightarrow H_2O$	0.816
$Fe^{3+} + e^- \longrightarrow Fe^{2+}$	0.771
$NO_3^- + 2H^+ + 2e^- \longrightarrow NO_2^- + H_2O$	0.421
Cytochrome f (Fe^{3+}) + e^- $\longrightarrow$ cytochrome f (Fe^{2+})	0.365
$Fe(CN)_6^{3-}$ (ferricyanide) + e^- $\longrightarrow$ $Fe(CN)_6^{4-}$	0.36
Cytochrome a_3 (Fe^{3+}) + e^- $\longrightarrow$ cytochrome a_3 (Fe^{2+})	0.35
$O_2 + 2H^+ + 2e^- \longrightarrow H_2O_2$	0.295
Cytochrome a (Fe^{3+}) + e^- $\longrightarrow$ cytochrome a (Fe^{2+})	0.29
Cytochrome c (Fe^{3+}) + e^- $\longrightarrow$ cytochrome c (Fe^{2+})	0.254
Cytochrome c_1 (Fe^{3+}) + e^- $\longrightarrow$ cytochrome c_1 (Fe^{2+})	0.22
Cytochrome b (Fe^{3+}) + e^- $\longrightarrow$ cytochrome b (Fe^{2+})	0.077
Ubiquinone + $2H^+ + 2e^-$ $\longrightarrow$ ubiquinol	0.045
Fumarate^{2-} + $2H^+ + 2e^-$ $\longrightarrow$ succinate^{2-}	0.031
$2H^+ + 2e^- \longrightarrow H_2$ (at standard conditions, pH 0)	0.000
Crotonyl-CoA + $2H^+ + 2e^-$ $\longrightarrow$ butyryl-CoA	−0.015
Oxaloacetate^{2-} + $2H^+ + 2e^-$ $\longrightarrow$ malate^{2-}	−0.166
Pyruvate$^-$ + $2H^+ + 2e^-$ $\longrightarrow$ lactate$^-$	−0.185
Acetaldehyde + $2H^+ + 2e^-$ $\longrightarrow$ ethanol	−0.197
FAD + $2H^+ + 2e^-$ $\longrightarrow$ $FADH_2$	−0.219[a]
Glutathione + $2H^+ + 2e^-$ $\longrightarrow$ 2 reduced glutathione	−0.23
$S + 2H^+ + 2e^- \longrightarrow H_2S$	−0.243
Lipoic acid + $2H^+ + 2e^-$ $\longrightarrow$ dihydrolipoic acid	−0.29
$NAD^+ + H^+ + 2e^-$ $\longrightarrow$ NADH	−0.320
$NADP^+ + H^+ + 2e^-$ $\longrightarrow$ NADPH	−0.324
Acetoacetate + $2H^+ + 2e^-$ $\longrightarrow$ β-hydroxybutyrate	−0.346
α-Ketoglutarate + $CO_2 + 2H^+ + 2e^-$ $\longrightarrow$ isocitrate	−0.38
$2H^+ + 2e^- \longrightarrow H_2$ (at pH 7)	−0.414
Ferredoxin (Fe^{3+}) + e^- $\longrightarrow$ ferredoxin (Fe^{2+})	−0.432

Data mostly from R. A. Loach, in *Handbook of Biochemistry and Molecular Biology*, 3rd edn (G. D. Fasman, ed.), *Physical and Chemical Data*, Vol. 1, p. 122, CRC Press, 1976.

[a] This is the value for free FAD; FAD bound to a specific flavoprotein (e.g., succinate dehydrogenase) has a different $E'°$ that depends on its protein environment.

electrode (pH 0), electrons tend to flow from the pH 7 cell to the standard (pH 0) cell; the measured E'° for the $2H^+/H_2$ pair is −0.414 V.

Standard Reduction Potentials Can Be Used to Calculate Free-Energy Change

Why are reduction potentials so useful to the biochemist? When E values have been determined for any two half-cells, relative to the standard hydrogen electrode, we also know their reduction potentials relative to each other. We can then predict the direction in which electrons will tend to flow when the two half-cells are connected through an external circuit or when components of both half-cells are present in the same solution. Electrons tend to flow to the half-cell with the more positive E, and the strength of that tendency is proportional to ΔE, the difference in reduction potential. The energy made available by this spontaneous electron flow (the free-energy change, ΔG, for the oxidation-reduction reaction) is proportional to ΔE:

$$\Delta G = -nF\Delta E \quad \text{or} \quad \Delta G'^{\circ} = -nF\Delta E'^{\circ} \qquad (13\text{-}7)$$

where n is the number of electrons transferred in the reaction. P5 With this equation we can calculate the actual free-energy change for any oxidation-reduction reaction from the values of $\Delta E'^{\circ}$ in a table of reduction potentials (Table 13-7) and the concentrations of reacting species.

WORKED EXAMPLE 13-3 *Calculation of $\Delta G'^{\circ}$ and ΔG of a Redox Reaction*

Calculate the standard free-energy change, $\Delta G'^{\circ}$, for the reaction in which acetaldehyde is reduced by the biological electron carrier NADH:

$$\text{Acetaldehyde} + \text{NADH} + \text{H}^+ \longrightarrow \text{ethanol} + \text{NAD}^+$$

Then calculate the *actual* free-energy change, ΔG, when [acetaldehyde] and [NADH] are 1.00 M, and [ethanol] and [NAD^+] are 0.100 M. The relevant half-reactions and their E'° values are

(1) $\text{Acetaldehyde} + 2\text{H}^+ + 2e^- \longrightarrow \text{ethanol} \qquad E'^{\circ} = -0.197\ \text{V}$

(2) $\text{NAD}^+ + 2\text{H}^+ + 2e^- \longrightarrow \text{NADH} + \text{H}^+ \qquad E'^{\circ} = -0.320\ \text{V}$

Remember that, by convention, $\Delta E'^{\circ}$ is E'° of the electron acceptor minus E'° of the electron donor. It represents the difference between the electron affinities of the two half-reactions in the table of reduction potentials (Table 13-7). Note that the more widely separated the two half-reactions in the table, the more energetic the electron-transfer reaction when the two half-reactions occur together. By convention, in tables of reduction potentials, all half-reactions are represented as reductions, but when two half-reactions occur together, one of them must be an oxidation. Although that half-reaction will go in the opposite direction from that shown in Table 13-7, we *do not change the sign* of that half-reaction before calculating $\Delta E'^{\circ}$, because $\Delta E'^{\circ}$ is *defined* as a difference of reduction potentials.

SOLUTION: Because acetaldehyde is accepting electrons ($n = 2$) from NADH, $\Delta E'^{\circ} = -0.197\ \text{V} - (-0.320\ \text{V}) = 0.123\ \text{V}$. Therefore,

$$\Delta G'^{\circ} = -nF\Delta E'^{\circ} = -2(96.5\ \text{kJ/V}\cdot\text{mol})(0.123\ \text{V}) = -23.7\ \text{kJ/mol}$$

This is the free-energy change for the oxidation-reduction reaction at 25 °C and pH 7, when acetaldehyde, ethanol, NAD^+, and NADH are all present at 1.00 M concentrations.

To calculate ΔG when [acetaldehyde] and [NADH] are 1.00 M, and [ethanol] and [NAD^+] are 0.100 M, we can use Equation 13-4 and the standard free-energy change calculated above:

$$\begin{aligned}\Delta G &= \Delta G'^{\circ} + RT \ln \frac{[\text{ethanol}][\text{NAD}^+]}{[\text{acetaldehyde}][\text{NADH}]}\\ &= -23.7\ \text{kJ/mol} + (8.315\ \text{J/mol}\cdot\text{K})(298\ \text{K}) \ln \frac{(0.100\ \text{M})(0.100\ \text{M})}{(1.00\ \text{M})(1.00\ \text{M})}\\ &= -23.7\ \text{kJ/mol} + (2.48\ \text{J/mol}) \ln 0.01\\ &= -35.1\ \text{kJ/mol}\end{aligned}$$

This is the actual free-energy change at the specified concentrations of the redox pairs.

A Few Types of Coenzymes and Proteins Serve as Universal Electron Carriers

The principles of oxidation-reduction energetics described above apply to the many metabolic reactions that involve electron transfers. For example, in many organisms, the oxidation of glucose supplies energy for the production of ATP. The complete oxidation of glucose

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

has a $\Delta G'^{\circ}$ of −2,840 kJ/mol. This is a much larger release of free energy than is required for ATP synthesis in cells (50 to 60 kJ/mol; see Worked Example 13-2). Cells convert glucose to CO_2 not in a single, high-energy-releasing reaction but rather in a series of controlled reactions, some of which are oxidations. The free energy released in these oxidation steps is of the same order of magnitude as that required for ATP synthesis from ADP, with some energy to spare. Electrons removed in these oxidation steps are transferred to coenzymes specialized for carrying electrons, such as NAD^+ and FAD (described below).

The multitude of enzymes that catalyze cellular oxidations channel electrons from their hundreds of different substrates into just a few types of universal electron carriers. The reduction of these carriers in catabolic processes results in the conservation of free energy released by substrate oxidation. NAD, NADP, FMN, and FAD are water-soluble coenzymes that undergo reversible oxidation and reduction in many of the electron-transfer reactions of metabolism. The nucleotides NAD and NADP move readily from one enzyme to another; the flavin nucleotides FMN and FAD are usually very tightly bound to the enzymes, called flavoproteins, for which they serve as prosthetic groups. Lipid-soluble quinones such as ubiquinone and plastoquinone act as electron carriers and proton donors

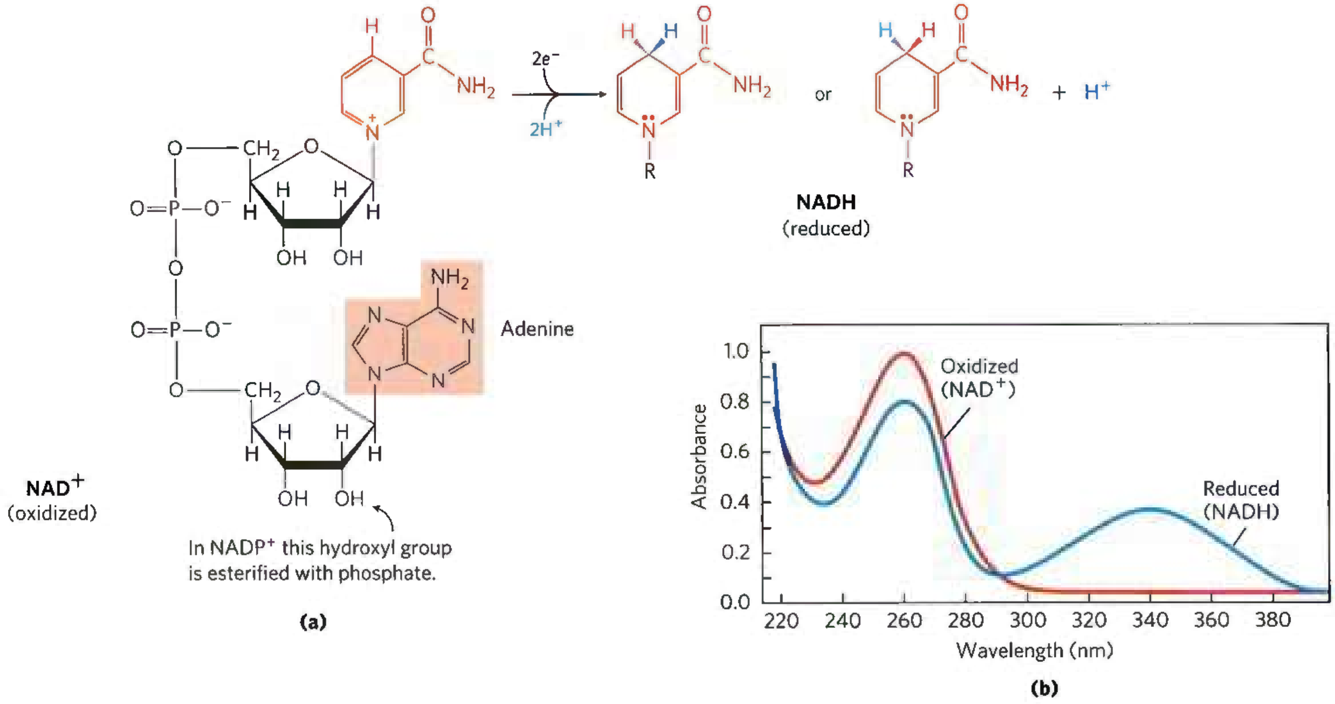


FIGURE 13-24 **NAD and NADP.** (a) Nicotinamide adenine dinucleotide, NAD$^+$, and its phosphorylated analog, NADP$^+$, undergo reduction to NADH and NADPH, accepting a hydride ion (two electrons and one proton) from an oxidizable substrate. The hydride ion is added to either the front or the back of the planar nicotinamide ring. (b) The UV absorption spectra of NAD$^+$ and NADH. Reduction of the nicotinamide ring produces a new, broad absorption band with a maximum at 340 nm. The production of NADH during an enzyme-catalyzed reaction can be conveniently followed by observing the appearance of the absorbance at 340 nm (molar extinction coefficient $\varepsilon_{340} = 6{,}200\ \text{M}^{-1}\ \text{cm}^{-1}$).

in the nonaqueous environment of membranes. Iron-sulfur proteins and cytochromes, which have tightly bound prosthetic groups that undergo reversible oxidation and reduction, also serve as electron carriers in many oxidation-reduction reactions. Some of these proteins are water-soluble, but others are peripheral or integral membrane proteins. The oxidation-reduction chemistry of quinones, iron-sulfur proteins, and cytochromes is discussed in Chapters 19 and 20.

Nicotinamide adenine dinucleotide (NAD; NAD$^+$ in its oxidized form) and its close analog nicotinamide adenine dinucleotide phosphate (NADP; NADP$^+$ when oxidized) are composed of two nucleotides joined through their phosphate groups by a phosphoanhydride bond (**Fig. 13-24a**). Because the nicotinamide ring resembles pyridine, these compounds are sometimes called **pyridine nucleotides**. The vitamin niacin is the source of the nicotinamide moiety in nicotinamide nucleotides.

Both coenzymes undergo reversible reduction of the nicotinamide ring (Fig. 13-24). As a substrate molecule undergoes oxidation (dehydrogenation), giving up two hydrogen atoms, the oxidized form of the nucleotide (NAD$^+$ or NADP$^+$) accepts a hydride ion (:H$^-$, the equivalent of a proton and two electrons) and is reduced (to NADH or NADPH). The second proton removed from the substrate is released to the aqueous solvent. The half-reactions for these nucleotide cofactors are

$$\text{NAD}^+ + 2e^- + 2\text{H}^+ \longrightarrow \text{NADH} + \text{H}^+$$

$$\text{NADP}^+ + 2e^- + 2\text{H}^+ \longrightarrow \text{NADPH} + \text{H}^+$$

Reduction of NAD$^+$ or NADP$^+$ converts the benzenoid ring of the nicotinamide moiety (with a fixed positive charge on the ring nitrogen) to the quinonoid form (with no charge on the nitrogen). The reduced nucleotides absorb light at 340 nm; the oxidized forms do not (Fig. 13-24b). Biochemists use this difference in absorption to assay reactions involving these coenzymes. Note that the plus sign in the abbreviations NAD$^+$ and NADP$^+$ does *not* indicate the net charge on these molecules (in fact, both are negatively charged); rather, it indicates that the nicotinamide ring is in its oxidized form, with a positive charge on the nitrogen atom. In the abbreviations NADH and NADPH, the "H" denotes the added hydride ion. To refer to these nucleotides without specifying their oxidation state, we use NAD and NADP.

The total concentration of NAD$^+$ + NADH in most tissues is about 10^{-5} M; that of NADP$^+$ + NADPH is about 10^{-6} M. In many cells and tissues, the ratio of NAD$^+$ (oxidized) to NADH (reduced) is high, favoring hydride transfer from a substrate *to* NAD$^+$ to form NADH. By contrast, NADPH is generally present at a higher concentration than NADP$^+$, favoring hydride transfer *from* NADPH to a substrate. This reflects the specialized metabolic roles of the two coenzymes: NAD$^+$ generally functions in oxidations—usually as part of a catabolic reaction; NADPH is the usual coenzyme in reductions—nearly always as part of an anabolic reaction. A few enzymes can use either coenzyme, but most show a strong preference for one over the other. Also, the processes in which these two cofactors function are segregated in eukaryotic cells: for example, oxidations of fuels such as pyruvate, fatty acids, and α-keto acids derived from amino acids occur in the mitochondrial matrix, whereas reductive biosynthetic processes such as fatty acid synthesis take place in the cytosol. This functional and spatial specialization allows a cell to maintain two distinct pools of electron carriers, with two distinct functions.

More than 200 enzymes are known to catalyze reactions in which NAD^+ (or $NADP^+$) accepts a hydride ion from a reduced substrate, or NADPH (or NADH) donates a hydride ion to an oxidized substrate. The general reactions are

$$AH_2 + NAD^+ \longrightarrow A + NADH + H^+$$
$$A + NADPH + H^+ \longrightarrow AH_2 + NADP^+$$

where AH_2 is the reduced substrate and A is the oxidized substrate. The general name for an enzyme of this type is **oxidoreductase**; they are also commonly called dehydrogenases. For example, alcohol dehydrogenase catalyzes the first step in the catabolism of ethanol, in which ethanol is oxidized to acetaldehyde:

$$\underset{\text{Ethanol}}{CH_3CH_2OH} + NAD^+ \longrightarrow \underset{\text{Acetaldehyde}}{CH_3CHO} + NADH + H^+$$

Notice that one of the carbon atoms in ethanol has lost a hydrogen; the compound has been oxidized from an alcohol to an aldehyde (refer again to Fig. 13-22 for the oxidation states of carbon).

The association between a dehydrogenase and NAD or NADP is relatively loose; the coenzyme readily diffuses from one enzyme to another, acting as a water-soluble carrier of electrons from one metabolite to another. For example, in the production of alcohol during fermentation of glucose by yeast cells, a hydride ion is removed from glyceraldehyde 3-phosphate by one enzyme (glyceraldehyde 3-phosphate dehydrogenase) and transferred to NAD^+. The NADH produced then leaves the enzyme surface and diffuses to another enzyme (alcohol dehydrogenase), which transfers a hydride ion to acetaldehyde, producing ethanol:

$$\begin{aligned}
&(1)\quad \text{Glyceraldehyde 3-phosphate} + NAD^+ \longrightarrow \\
&\qquad\qquad \text{3-phosphoglycerate} + NADH + H^+ \\
&(2)\quad \text{Acetaldehyde} + NADH + H^+ \longrightarrow \text{ethanol} + NAD^+ \\
\hline
&\textit{Sum:}\ \text{Glyceraldehyde 3-phosphate} + \text{acetaldehyde} \longrightarrow \\
&\qquad\qquad \text{3-phosphoglycerate} + \text{ethanol}
\end{aligned}$$

Notice that in the overall reaction there is no net production or consumption of NAD^+ or NADH; the coenzymes function catalytically and are recycled repeatedly without a net change in the total amount of $NAD^+ + NADH$.

Both reduced and oxidized forms of NAD and NADP serve as allosteric effectors of proteins in catabolic pathways. As we describe in later chapters, the ratios $NAD^+/NADH$ and $NADP^+/NADPH$ serve as sensitive gauges of a cell's fuel supply, allowing rapid, appropriate changes in energy-yielding and energy-dependent metabolism.

NAD Has Important Functions in Addition to Electron Transfer

Some key cellular functions are regulated by enzymes that use NAD^+ not as a redox cofactor but as a substrate in a coupled reaction in which the availability of NAD^+ can be an indicator of the cell's energy status. In DNA replication and repair, the enzyme DNA ligase is adenylylated and then transfers the AMP to a 5′ phosphate in a nicked DNA (see Fig. 25-15); in bacteria, NAD^+ serves as the source of the activating AMP group. A family of proteins called sirtuins regulate the activity of proteins in diverse cellular pathways by deacetylating the ε-amino group of an acetylated Lys residue. The deacetylation is coupled to NAD^+ hydrolysis, yielding *O*-acetyl-ADP-ribose and nicotinamide. Among the cellular processes regulated by sirtuins are inflammation, apoptosis, aging, and DNA transcription; deacetylation by a sirtuin alters the charge on histones, influencing which genes are expressed. The availability of NAD^+ for these types of reactions may indicate that the cell is undergoing stress and that pathways designed to respond to stress should be activated.

NAD^+ also plays an important role in cholera pathology (see Section 12.2). Cholera toxin has an enzymatic activity that transfers ADP-ribose from NAD^+ to a G protein involved in regulating ion fluxes in the cells lining the gut. This ADP-ribosylation blocks water retention, causing the diarrhea and dehydration characteristic of cholera.

Dietary deficiency of niacin, the vitamin form of NAD and NADP, causes pellagra (**Fig. 13-25**). The pyridine-like rings of NAD and NADP are derived from the vitamin niacin (nicotinic acid; **Fig. 13-26**), which is synthesized from tryptophan. Humans generally cannot synthesize sufficient quantities of niacin, and this is especially so for individuals with diets low in tryptophan (maize, for example, has a low tryptophan content). Niacin deficiency, which affects all the NAD(P)-dependent dehydrogenases, causes the serious human disease pellagra (Italian for "rough skin") and a related disease in dogs, called black tongue. Pellagra is characterized by the "three Ds": dermatitis, diarrhea, and dementia, followed in many cases by death. A century ago, pellagra was a common human disease; in the southern United States, where maize was a dietary staple, about 100,000 people were afflicted and about 10,000 died as a result of this disease between 1912 and 1916. In 1920, Joseph Goldberger showed pellagra to be caused by a dietary insufficiency, and in 1937, Frank Strong, D. Wayne Woolley, and Conrad Elvehjem identified niacin as the curative agent for the dog version of pellagra, black tongue. Supplementation of the human diet with this inexpensive compound has nearly eradicated pellagra in the populations of the

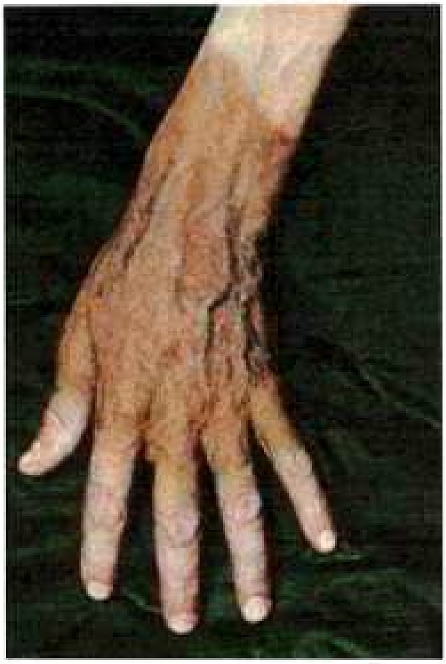


FIGURE 13-25 Dermatitis associated with pellagra. Dermatitis involving the face, hands, and feet is an early sign of pellagra, a serious human disease that results from insufficient niacin in the diet. Untreated, pellagra leads to dementia and ultimately is fatal. [Double Vision/Science Source]

developed world, with one significant exception: people who drink excessive amounts of alcohol. In these individuals, intestinal absorption of niacin is much reduced, and caloric needs are often met with distilled spirits that are virtually devoid of vitamins, including niacin. ■

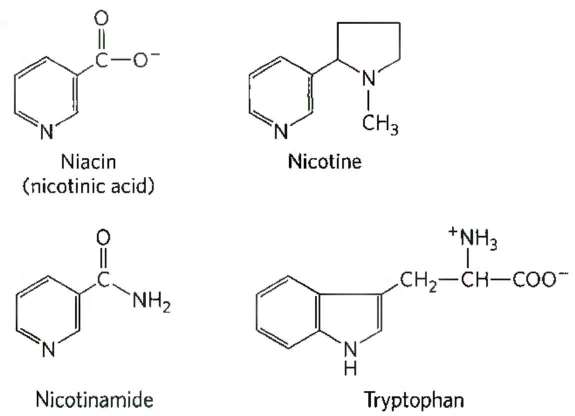


FIGURE 13-26 Niacin (nicotinic acid) and its derivative nicotinamide. The biosynthetic precursor of these compounds is tryptophan. In the laboratory, nicotinic acid was first produced by oxidation of the natural product nicotine—thus the name. Both nicotinic acid and nicotinamide cure pellagra, but nicotine (from cigarettes or elsewhere) has no curative activity.

Flavin Nucleotides Are Tightly Bound in Flavoproteins

Flavoproteins are enzymes that catalyze oxidation-reduction reactions using either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) as coenzyme (**Fig. 13-27**). These coenzymes, the **flavin nucleotides**, are derived from the vitamin riboflavin. The fused ring structure of flavin nucleotides (the isoalloxazine ring) undergoes reversible reduction, accepting either one or two electrons in the form of one or two hydrogen atoms (each atom an electron plus a proton) from a reduced substrate. The fully reduced forms are abbreviated $FADH_2$ and $FMNH_2$. When a fully oxidized

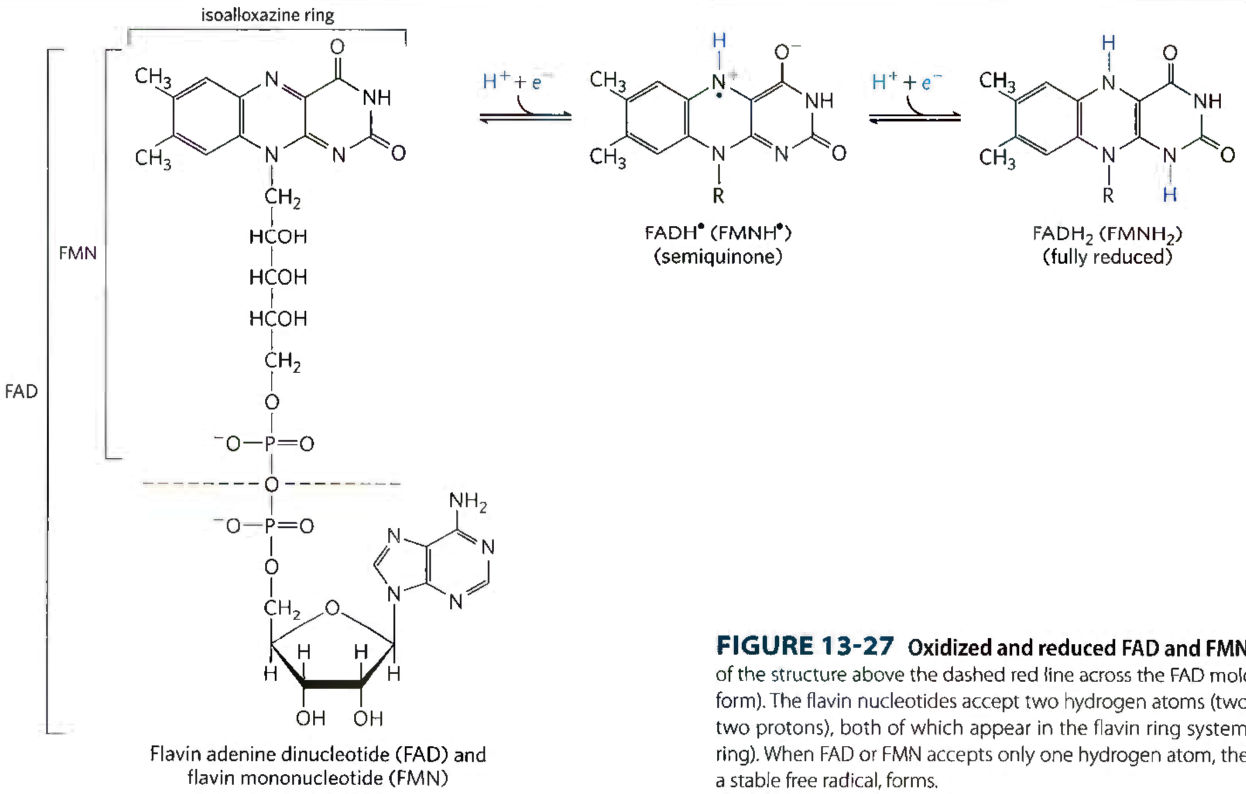


FIGURE 13-27 Oxidized and reduced FAD and FMN. FMN consists of the structure above the dashed red line across the FAD molecule (oxidized form). The flavin nucleotides accept two hydrogen atoms (two electrons and two protons), both of which appear in the flavin ring system (isoalloxazine ring). When FAD or FMN accepts only one hydrogen atom, the semiquinone, a stable free radical, forms.

flavin nucleotide accepts only one electron (one hydrogen atom), the semiquinone form of the isoalloxazine ring is produced, abbreviated FADH• and FMNH•. Because flavin nucleotides have a chemical specialty that is slightly different from that of the nicotinamide coenzymes—the ability to participate in either one- or two-electron transfers—flavoproteins are involved in a greater diversity of reactions than the NAD(P)-linked dehydrogenases.

Like the nicotinamide coenzymes (Fig. 13-24), the flavin nucleotides undergo a shift in a major absorption band on reduction (again, useful to biochemists who want to monitor reactions involving these coenzymes). Flavoproteins that are fully reduced (two electrons accepted) generally have an absorption maximum near 360 nm. When partially reduced (one electron), they acquire another absorption maximum at about 450 nm; when fully oxidized, the flavin has maxima at 370 and 440 nm.

The flavin nucleotide in most flavoproteins is bound rather tightly to the protein, and in some enzymes, such as succinate dehydrogenase, it is bound covalently. Such tightly bound coenzymes are properly called prosthetic groups. They do not transfer electrons by diffusing from one enzyme to another; rather, they provide a means by which the flavoprotein can temporarily hold electrons while it catalyzes electron transfer from a reduced substrate to an electron acceptor. One important feature of the flavoproteins is the variability in the standard reduction potential (E'°) of the bound flavin nucleotide. Tight association between the enzyme and prosthetic group confers on the flavin ring a reduction potential typical of that particular flavoprotein, sometimes quite different from the reduction potential of the free flavin nucleotide. FAD bound to succinate dehydrogenase, for example, has an E'° close to 0.0 V, compared with –0.219 V for free FAD; E'° for other flavoproteins ranges from –0.40 V to +0.06 V. Flavoproteins are often very complex; some have, in addition to a flavin nucleotide, tightly bound inorganic ions (iron or molybdenum, for example) capable of participating in electron transfers.

We examine the function of flavoproteins as electron carriers in Chapters 19 and 20, when we consider their roles in oxidative phosphorylation (in mitochondria) and photophosphorylation (in chloroplasts).

SUMMARY 13.4 *Biological Oxidation-Reduction Reactions*

- In many organisms, a central energy-conserving process is the stepwise oxidation of glucose to CO_2, in which some of the energy of oxidation is conserved in ATP as electrons are passed to O_2.
- Biological oxidation-reduction reactions can be described in terms of two half-reactions, each with a characteristic standard reduction potential, E'°.
- Many biological oxidation reactions are dehydrogenations in which one or two hydrogen atoms ($H^+ + e^-$) are transferred from a substrate to a hydrogen acceptor. In some biological redox reactions, the substrate loses both electrons and protons, the equivalent of losing hydrogen. The many enzymes that catalyze such reactions are called dehydrogenases.
- When two electrochemical half-cells, each containing the components of a half-reaction, are connected, electrons tend to flow to the half-cell with the higher reduction potential. The strength of this tendency is proportional to the difference between the two reduction potentials (ΔE) and is a function of the concentrations of oxidized and reduced species.
- The standard free-energy change for an oxidation-reduction reaction is directly proportional to the difference in standard reduction potentials of the two half-cells: $\Delta G'^{\circ} = -nF\Delta E'^{\circ}$.
- Oxidation-reduction reactions in living cells involve specialized electron carriers. NAD and NADP are the freely diffusible coenzymes of many dehydrogenases. Both NAD^+ and $NADP^+$ accept two electrons and one proton. In addition to its role in oxidation-reduction reactions, NAD^+ is the source of AMP in the bacterial DNA ligase reaction and of ADP-ribose in the cholera toxin reaction, and it is hydrolyzed in the deacetylation of proteins by some sirtuins.
- Lack of the vitamin niacin prevents NAD synthesis and leads to pellagra.
- FAD and FMN, the flavin nucleotides, serve as tightly bound prosthetic groups of flavoproteins. They can accept either one or two electrons and one or two protons. Their reduction potentials depend on the flavoprotein with which they are associated.

13.5 Regulation of Metabolic Pathways

Metabolic regulation is one of the most remarkable features of living organisms. Of the thousands of enzyme-catalyzed reactions that can take place in a cell, there is probably not one that escapes some form of regulation. This need to regulate every aspect of cellular metabolism becomes clear as one examines the complexity of metabolic reaction sequences. Although it is convenient for the student of biochemistry to divide metabolic processes into "pathways" that play discrete roles in the cell's economy, no such separation exists in the living cell. Rather, every pathway we discuss in this book is inextricably intertwined with all the other cellular pathways in a multidimensional network of reactions (**Fig. 13-28**).

For example, in Chapter 14 we will discuss four possible fates for **glucose 6-phosphate** in a hepatocyte: breakdown by glycolysis for the production of ATP, breakdown in the pentose phosphate pathway for the production of NADPH and pentose phosphates, use in

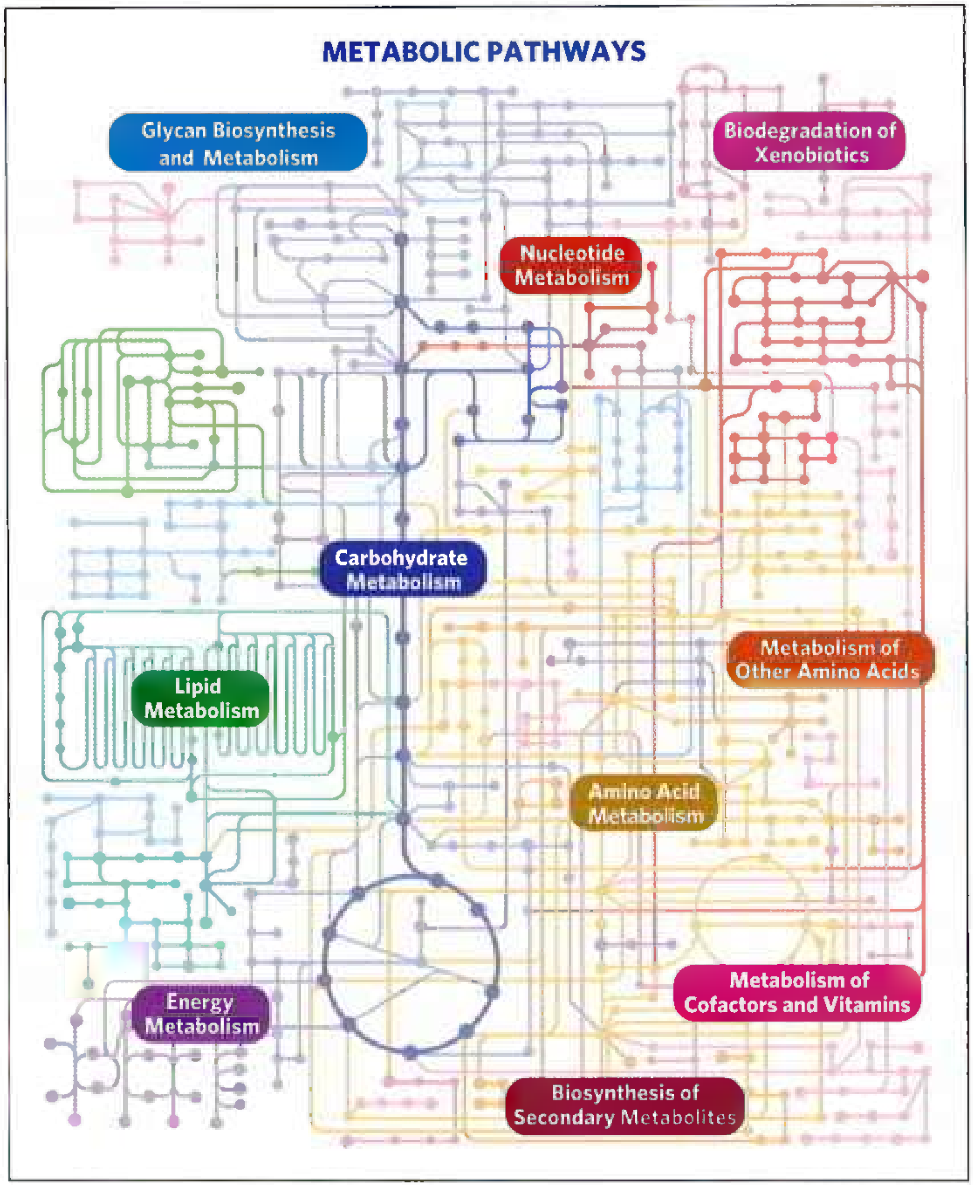

METABOLIC PATHWAYS
Glycan Biosynthesis and Metabolism
Biodegradation of Xenobiotics
Nucleotide Metabolism
Carbohydrate Metabolism
Lipid Metabolism
Metabolism of Other Amino Acids
Amino Acid Metabolism
Energy Metabolism
Metabolism of Cofactors and Vitamins
Biosynthesis of Secondary Metabolites

FIGURE 13-28 Metabolism as a three-dimensional meshwork. A typical eukaryotic cell has the capacity to make about 30,000 different proteins, which catalyze thousands of different reactions involving many hundreds of metabolites, most shared by more than one "pathway." In this much-simplified overview of metabolic pathways, each dot represents an intermediate compound and each connecting line represents an enzymatic reaction. For a more realistic and far more complex diagram of metabolism, see the online KEGG PATHWAY database (www.genome.ad.jp/kegg/pathway/map/map01100.html); in this interactive map, you can click on each dot to obtain extensive data about the compound and the enzymes for which it is a substrate. [www.genome.ad.jp/kegg/pathway/map/map01100.html]

the synthesis of complex polysaccharides of the extracellular matrix, or hydrolysis to glucose and phosphate to replenish blood glucose. In fact, glucose 6-phosphate has other possible fates in hepatocytes, too; it may, for example, be used to synthesize other sugars, such as glucosamine, galactose, galactosamine, fucose, and neuraminic acid, for use in protein glycosylation, or it may be partially degraded to provide acetyl-CoA for fatty acid and sterol synthesis. And *E. coli* can use glucose to produce the carbon skeleton of every one of its several thousand types of molecules. When any cell uses glucose 6-phosphate for one purpose, that "decision" affects all the other pathways for which glucose 6-phosphate is a precursor or intermediate: any change in the allocation of glucose 6-phosphate to one pathway affects, directly or indirectly, the flow of metabolites through all the others.

Cells and Organisms Maintain a Dynamic Steady State

The pathways of glucose metabolism provide, in the catabolic direction, the energy essential to oppose the forces of entropy and, in the anabolic direction, biosynthetic precursors and a storage form of metabolic energy. These reactions are so important to survival that very complex regulatory mechanisms have evolved to ensure that metabolites move through each pathway in the correct direction and at the correct rate to match exactly the cell's or the organism's changing circumstances. By a variety of mechanisms operating on different time scales, adjustments are made in the rate of metabolite flow through an entire pathway when external circumstances change.

Circumstances do change, sometimes dramatically. The availability of oxygen may decrease due to hypoxia (diminished delivery of oxygen to tissues) or ischemia (diminished flow of blood to tissues). Wound healing requires huge amounts of energy and biosynthetic precursors. The relative proportions of carbohydrate, fat, and protein in the diet vary from meal to meal, and the supply of fuels obtained in the diet is intermittent, requiring metabolic adjustments between meals and during periods of starvation.

Fuels such as glucose enter a cell, and waste products such as CO_2 leave, but the mass and the gross composition of a typical cell, organ, or adult animal do not change appreciably over time; cells and organisms exist in a dynamic steady state. For each metabolic reaction in a pathway, the substrate is provided by the preceding reaction at the same rate at which it is converted to product. Thus, although the rate (v) of metabolite flow, or **flux**, through this step of the pathway may be high and variable, the concentration of substrate, S, remains constant. So, for the two-step reaction

$$\mathrm{A} \xrightarrow{v_1} \mathrm{S} \xrightarrow{v_2} \mathrm{P}$$

when $v_1 = v_2$, [S] is constant. **P6** For example, changes in v_1 for the entry of glucose from various sources into the blood are balanced by changes in v_2 for the uptake of glucose from the blood into various tissues, so the concentration of glucose in the blood ([S]) is held nearly constant at 5 mM. This is **homeostasis** for blood glucose. The failure of homeostatic mechanisms is often at the root of human disease. In diabetes mellitus, for example, the regulation of blood glucose concentration is defective as a result of the lack of or insensitivity to insulin, with profound medical consequences.

In the course of evolution, organisms have acquired a remarkable collection of regulatory mechanisms for maintaining homeostasis at the molecular, cellular, and organismal levels, as reflected in the proportion of genes that encode regulatory machinery. In humans, about 2,500 genes (~12% of all genes) encode regulatory proteins, including a variety of receptors, regulators of gene expression, and more than 500 different protein kinases! In many cases, the regulatory mechanisms overlap: one enzyme is subject to regulation by several different mechanisms.

Both the Amount and the Catalytic Activity of an Enzyme Can Be Regulated

The flux through an enzyme-catalyzed reaction can be modulated by changes in the *number* of enzyme molecules or by changes in the *catalytic activity* of each enzyme molecule already present. Such changes occur on time scales from milliseconds to many hours, in response to signals from within or outside the cell. Very rapid allosteric changes in enzyme activity are generally triggered locally, by changes in the local concentration of a small molecule—a substrate of the pathway in which that reaction is a step (say, glucose for glycolysis), a product of the pathway (ATP from glycolysis), or a key metabolite or cofactor (such as NADH) that indicates the cell's metabolic state. Second messengers (such as cyclic AMP and Ca^{2+}) generated intracellularly in response to extracellular signals (hormones, cytokines, and so forth) also mediate allosteric regulation, on a slightly slower time scale set by the rate of the signal-transduction mechanism (see Chapter 12).

Extracellular signals (**Fig. 13-29**, ❶) may be hormonal (insulin or epinephrine, for example) or neuronal (acetylcholine), or may be growth factors or cytokines. The number of molecules of a given enzyme in a cell is a function of the relative rates of synthesis and degradation of that enzyme. The rate of synthesis can be adjusted by the activation (in response to some outside signal) of a transcription factor (Fig. 13-29, ❷; described in more detail in Chapter 28). **Transcription factors** are nuclear proteins that, when activated, bind specific DNA regions (**response elements**) near a gene's promoter (its transcriptional starting point) and activate or repress the transcription of that gene, leading to increased or decreased synthesis of the encoded protein. Activation of a transcription factor is sometimes the result of its binding of a specific ligand and sometimes the result of its phosphorylation or dephosphorylation. Each gene is controlled by one or more response elements that are recognized by specific transcription factors. Genes that have several response elements are therefore controlled by several different transcription factors responding to several different signals. Groups of genes encoding proteins that act together, such as the enzymes of glycolysis, often share common response element sequences, so that a single signal, acting through a particular transcription factor, turns all of these genes on and off together.

The stability of messenger RNAs—their resistance to degradation by cellular ribonucleases (Fig. 13-29, ❸)—varies, and the amount of a given mRNA in the cell is a function of its rates of synthesis and degradation (Chapter 26). The rate at which an mRNA is translated into a protein by ribosomes (Fig. 13-29, ❹) is also regulated, and depends on several factors described in detail in Chapter 27. Note that an n-fold increase in an mRNA does not always mean an n-fold increase in its protein product.

Once synthesized, protein molecules have a finite lifetime, which may range from minutes to many days (**Table 13-8**). The rate of protein degradation (Fig. 13-29, ❺) differs from one protein to another and depends on the conditions in the cell. Some proteins are tagged by

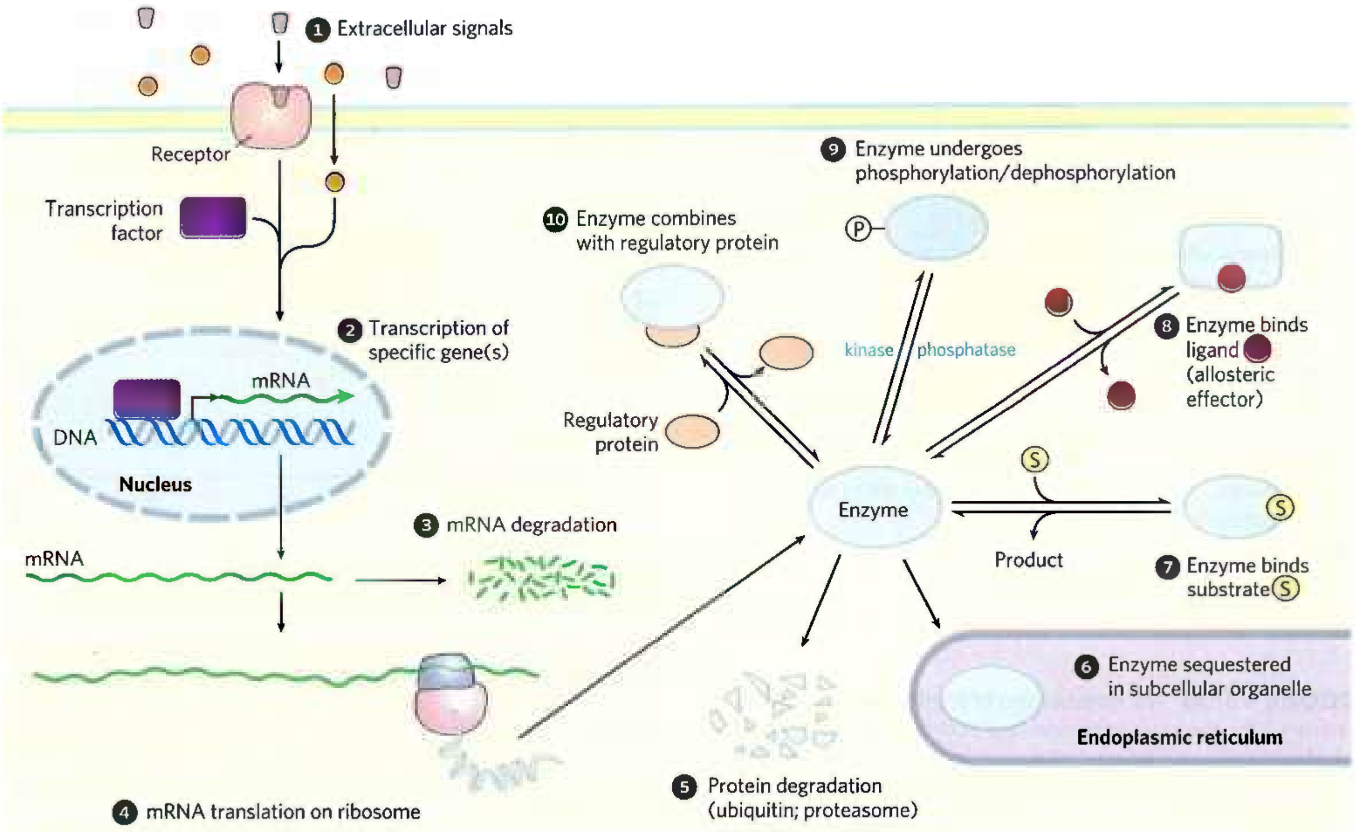

① Extracellular signals
Receptor
Transcription factor
② Transcription of specific gene(s)
mRNA
DNA
Nucleus
mRNA
③ mRNA degradation
④ mRNA translation on ribosome
Regulatory protein
⑩ Enzyme combines with regulatory protein
⑨ Enzyme undergoes phosphorylation/dephosphorylation
P
kinase
phosphatase
⑧ Enzyme binds ligand ● (allosteric effector)
S
Enzyme
Product
⑦ Enzyme binds substrate S
⑥ Enzyme sequestered in subcellular organelle
Endoplasmic reticulum
⑤ Protein degradation (ubiquitin; proteasome)

FIGURE 13-29 Factors affecting the activity of enzymes. The total activity of an enzyme can be changed by altering the *number* of its molecules in the cell, or its *effective* activity in a subcellular compartment (① through ⑥), or by modulating the *activity* of existing molecules (⑦ through ⑩), as detailed in the text. An enzyme may be influenced by a combination of such factors.

TABLE 13-8 Average Half-Life of Proteins in Mammalian Tissues

Tissue	Average half-life (days)
Liver	0.9
Kidney	1.7
Heart	4.1
Brain	4.6
Muscle	10.7

the covalent attachment of ubiquitin for degradation in proteasomes, as discussed in Chapter 27 (see, for example, the case of cyclin, in Fig. 12-38). P6 Rapid **turnover** (synthesis followed by degradation) is energetically expensive, but proteins with a short half-life can reach new steady-state levels much faster than those with a long half-life, and the benefit of this quick responsiveness must balance or outweigh the cost to the cell.

Yet another way to alter the *effective* activity of an enzyme is to sequester the enzyme and its substrate in different compartments (Fig. 13-29, ⑥). In muscle, for example, hexokinase cannot act on glucose until the sugar enters the myocyte from the blood, and the rate at which it enters depends on the activity of glucose transporters (see Table 11-1) in the plasma membrane. Within cells, membrane-bounded compartments segregate certain enzymes and enzyme systems, and the transport of substrate across these intracellular membranes may be the limiting factor in enzyme action.

By these several mechanisms for regulating enzyme level, cells can dramatically change their complement of enzymes in response to changes in metabolic circumstances. In vertebrates, liver is the most adaptable tissue; a change from a high-carbohydrate diet to a high-lipid diet, for example, affects the transcription of hundreds of genes and thus the levels of hundreds of proteins. These global changes in gene expression can be quantified in the entire complement of mRNAs (the **transcriptome**) or protein (**proteome**) of a cell type or organ, offering great insights into metabolic regulation. The effect of changes in the proteome is often a change in the total ensemble of low molecular weight metabolites, the **metabolome** (**Fig. 13-30**). The metabolome of *E. coli* growing on glucose is dominated by a few classes of metabolites: glutamate (49%); nucleotides (mainly ribonucleoside triphosphates) (15%); intermediates of glycolysis, the citric acid cycle, and the pentose phosphate pathway (central pathways of carbon metabolism) (15%); and redox cofactors and glutathione (9%).

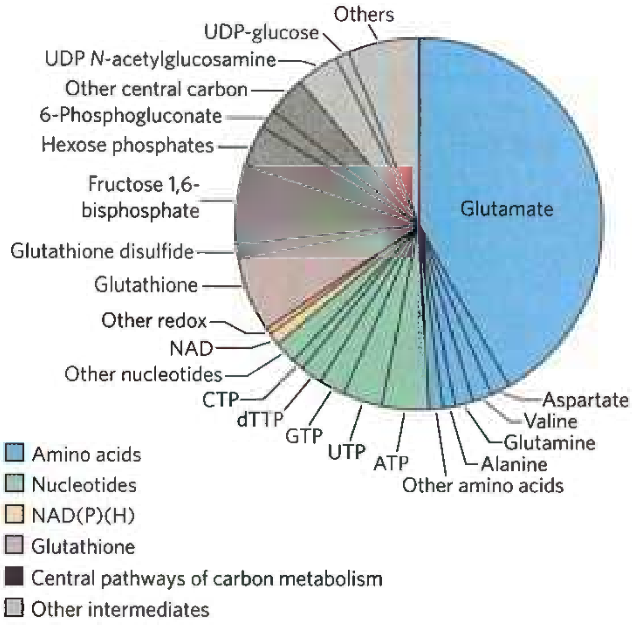


FIGURE 13-30 The metabolome of *E. coli* growing on glucose. Summary of the relative molar abundance of 103 metabolites as measured by a combination of liquid chromatography and tandem mass spectrometry (LC-MS/MS). For reference, the absolute concentration of glutamate in living cells is 9.6 mM. [Data from B. D. Bennett et al., *Nature Chem. Biol.* 5:593, 2009, Fig. 1.]

Once the regulatory mechanisms that involve protein synthesis and degradation have produced a certain number of molecules of each enzyme in a cell, the activity of those enzymes can be further regulated in several other ways: by the concentration of substrate, the presence of allosteric effectors, covalent modifications, or binding of regulatory proteins—all of which can change the activity of an individual enzyme molecule (Fig. 13-29, ❼ to ❿).

All enzymes are sensitive to the concentration of their substrate(s) (Fig. 13-29, ❼). Recall that in the simplest case (an enzyme that follows Michaelis-Menten kinetics), the initial rate of the reaction is half-maximal when the substrate is present at a concentration equal to K_m (that is, when the enzyme is half-saturated with substrate). Activity drops off at lower [S], and when $[S] << K_m$, the reaction rate is linearly dependent on [S].

The relationship between [S] and K_m is important because intracellular concentrations of substrate are often in the same range as, or lower than, K_m. The activity of hexokinase, for example, changes with [glucose], and intracellular [glucose] varies with the concentration of glucose in the blood. As we will see, the different forms (isozymes) of hexokinase have different K_m values and are therefore affected differently by changes in intracellular [glucose], in ways that make sense physiologically. For a number of phosphoryl transfers from ATP, and for redox reactions using NADPH or NAD^+, the metabolite concentration is well above the K_m (**Fig. 13-31**); these cofactors are not likely to be limiting in such reactions.

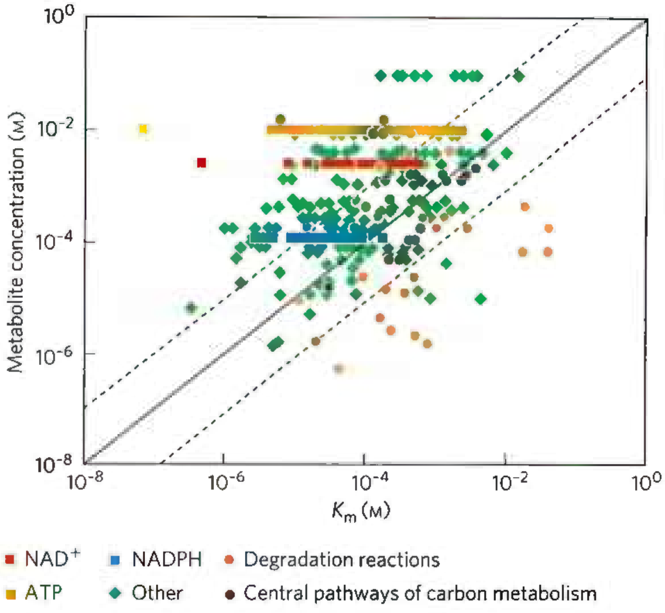


FIGURE 13-31 Comparison of K_m and substrate concentration for some metabolic enzymes. Measured metabolite concentrations for *E. coli* growing on glucose are plotted against the known K_m for enzymes that consume that metabolite. The solid line is the line of unity (where metabolite concentration = K_m), and the dashed lines each denote a 10-fold deviation from the line of unity. [Data from B. D. Bennett et al., *Nature Chem. Biol.* 5:593, 2009, Fig. 2.]

Enzyme activity can be either increased or decreased by an allosteric effector (Fig. 13-29, ❽; see Fig. 6-37). Allosteric effectors typically convert hyperbolic kinetics to sigmoid kinetics, or vice versa (see Fig. 14-24b, for example). In the steepest part of the sigmoid curve, a small change in the concentration of substrate, or of allosteric effector, can have a large impact on reaction rate. Recall from Chapter 5 (p. 157) that the cooperativity of an allosteric protein can be expressed as a Hill coefficient, with higher coefficients meaning greater cooperativity. For an allosteric enzyme with a Hill coefficient of 4, activity increases from 10% V_{max} to 90% V_{max} with only a 3-fold increase in [S], compared with the 81-fold rise in [S] needed by an enzyme with no cooperative effects (Hill coefficient of 1; **Table 13-9**).

TABLE 13-9 Relationship between Hill Coefficient and the Effect of Substrate Concentration on Reaction Rate for Allosteric Enzymes

Hill coefficient (n_H)	Required change in [S] to increase V_0 from 10% to 90% V_{max}
0.5	×6,600
1.0	×81
2.0	×9
3.0	×4.3
4.0	×3

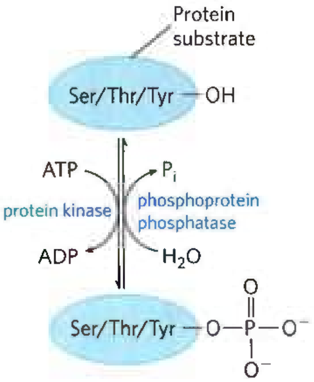


FIGURE 13-32 Protein phosphorylation and dephosphorylation. Protein kinases transfer a phosphoryl group from ATP to a Ser, Thr, or Tyr residue in an enzyme or other protein substrate. Protein phosphatases remove the phosphoryl group as P_i.

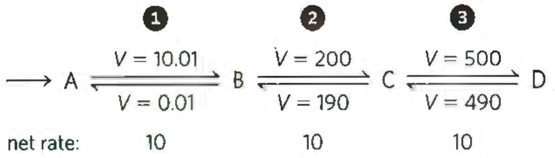


FIGURE 13-33 Near-equilibrium and nonequilibrium steps in a metabolic pathway. Steps ② and ③ of this pathway are near equilibrium in the cell; for each step, the rate (V) of the forward reaction is only slightly greater than the reverse rate, so the net forward rate (10) is relatively low and the free-energy change, ΔG, is close to zero. An increase in [C] or [D] can reverse the direction of these steps. Step ① is maintained in the cell far from equilibrium; its forward rate greatly exceeds its reverse rate. The net rate of step ① (10) is much larger than the reverse rate (0.01) and is identical to the net rates of steps ② and ③ when the pathway is operating in the steady state. Step ① has a large, negative ΔG.

Covalent modifications of enzymes or other proteins (Fig. 13-29, ⑨) occur within seconds or minutes of a regulatory signal, typically an extracellular signal. By far the most common modifications are phosphorylation and dephosphorylation (**Fig. 13-32**); up to half the proteins in a eukaryotic cell are phosphorylated under some circumstances. Phosphorylation by a specific protein kinase may alter the electrostatic features of an enzyme's active site, cause movement of an inhibitory region of the enzyme out of the active site, alter the enzyme's interaction with other proteins, or force conformational changes that translate into changes in V_{max} or K_{m}. For covalent modification to be useful in regulation, the cell must be able to restore the altered enzyme to its original activity state. A family of phosphoprotein phosphatases, at least some of which are themselves under regulation, catalyzes the dephosphorylation of proteins.

Finally, many enzymes are regulated by association with and dissociation from another, regulatory protein (Fig. 13-29, ⑩). For example, the cyclic AMP–dependent protein kinase (PKA; see Fig. 12-6) is inactive until cAMP binding separates catalytic from regulatory (inhibitory) subunits of the enzyme.

These several mechanisms for altering the flux through a step in a metabolic pathway are not mutually exclusive. P6 It is very common for a single enzyme to be regulated at the level of transcription and by both allosteric and covalent mechanisms. The combination provides fast, smooth, effective regulation in response to a very wide array of perturbations and signals.

In the discussions that follow, it is useful to think of changes in enzymatic activity as serving two distinct though complementary roles. We use the term **metabolic regulation** to refer to processes that serve to maintain homeostasis at the molecular level—to hold some cellular parameter (concentration of a metabolite, for example) at a steady level over time, even as the flow of metabolites through the pathway changes. The term **metabolic control** refers to a process that leads to a change in the output of a metabolic pathway over time, in response to some outside signal or change in circumstances. The distinction, although useful, is not always easy to make.

Reactions Far from Equilibrium in Cells Are Common Points of Regulation

For some steps in a metabolic pathway the reaction is close to equilibrium, with the cell in its dynamic steady state (**Fig. 13-33**). The net flow of metabolites through these steps is the small difference between the rates of the forward and reverse reactions, rates that are very similar when a reaction is near equilibrium. Small changes in substrate or product concentration can produce large changes in the net rate, and they can even change the direction of the net flow. We can identify these near-equilibrium reactions in a cell by comparing the **mass-action ratio**, Q, with the equilibrium constant for the reaction, K'_{eq}. Recall that for the reaction $A + B \rightarrow C + D$, $Q = [C][D]/[A][B]$. In practice, when Q and K'_{eq} are within 1 to 2 orders of magnitude of each other, the reaction is near equilibrium. This is the case for more than half of the enzymes in the glycolytic pathway, for example (**Table 13-10**).

Other reactions are far from equilibrium in the cell. For example, K'_{eq} for the phosphofructokinase-1 (PFK-1) reaction is about 1,000, but Q ([fructose 1,6-bisphosphate][ADP]/[fructose 6-phosphate][ATP]) in a hepatocyte in the steady state is about 0.1 (Table 13-10). It is *because* the reaction is so far from equilibrium in the cell that the process is exergonic under cellular conditions and tends to go in the forward direction. The reaction is held far from equilibrium because, under prevailing cellular conditions of substrate, product, and effector concentrations, the rate of conversion of fructose 6-phosphate to fructose 1,6-bisphosphate is limited by the activity of PFK-1. PFK-1 activity itself is limited by the number of PFK-1 molecules present and by the actions of allosteric effectors. Thus, the net forward rate of the enzyme-catalyzed reaction is equal to

TABLE 13-10 Equilibrium Constants, Mass-Action Ratios, and Free-Energy Changes for Enzymes of Carbohydrate Metabolism

Enzyme	K'_{eq}	Mass-action ratio, Q		Reaction near equilibrium in vivo?[a]	$\Delta G'^{\circ}$ (kJ/mol)	ΔG (kJ/mol) in heart
		Liver	Heart			
Hexokinase	1×10^{3}	2×10^{-2}	8×10^{-2}	No	−17	−27
PFK-1	1.0×10^{3}	9×10^{-2}	3×10^{-2}	No	−14	−23
Aldolase	1.0×10^{-4}	1.2×10^{-6}	9×10^{-6}	Yes	+24	−6.0
Triose phosphate isomerase	4×10^{-2}	—[b]	2.4×10^{-1}	Yes	+7.5	+3.8
Glyceraldehyde 3-phosphate dehydrogenase + phosphoglycerate kinase	2×10^{3}	6×10^{2}	9.0	Yes	−13	+3.5
Phosphoglycerate mutase	1×10^{-1}	1×10^{-1}	1.2×10^{-1}	Yes	+4.4	+0.6
Enolase	3	2.9	1.4	Yes	−3.2	−0.5
Pyruvate kinase	2×10^{4}	7×10^{-1}	40	No	−31	−17
Phosphohexose isomerase	4×10^{-1}	3.1×10^{-1}	2.4×10^{-1}	Yes	+2.2	−1.4
Pyruvate carboxylase + PEP carboxykinase	7	1×10^{-3}	—[b]	No	−5.0	−23
Glucose 6-phosphatase	8.5×10^{2}	1.2×10^{2}	—[b]	Yes	−17	−5.0

Data for K'_{eq} and Q from E. A. Newsholme and C. Start, *Regulation in Metabolism*, pp. 97, 263, Wiley Press, 1973. ΔG and $\Delta G'^{\circ}$ were calculated from these data.

[a] For simplicity, any reaction for which the absolute value of the calculated ΔG is less than 6 is considered near equilibrium.

[b] Data not available.

the net flow of glycolytic intermediates through other steps in the pathway, and the reverse flow through PFK-1 remains near zero.

The cell *cannot* allow reactions with large equilibrium constants to reach equilibrium. If [fructose 6-phosphate], [ATP], and [ADP] in the cell were held at typical levels (low millimolar concentrations) and the PFK-1 reaction were allowed to reach equilibrium by an increase in [fructose 1,6-bisphosphate], the concentration of fructose 1,6-bisphosphate would rise into the molar range, wreaking osmotic havoc on the cell. Consider another case: if the reaction ATP → ADP + P_i were allowed to approach equilibrium in the cell, the actual free-energy change (ΔG) for that reaction (ΔG_p; see Worked Example 13-2, p. 480) would approach zero, and ATP would lose the high phosphoryl group transfer potential that makes it valuable to the cell. It is therefore essential that enzymes catalyzing ATP breakdown and other highly exergonic reactions in a cell be sensitive to regulation, so that when metabolic changes are forced by external circumstances, the flow through these enzymes will be adjusted to ensure that [ATP] remains far above its equilibrium level. When such metabolic changes occur, the activities of enzymes in all interconnected pathways adjust to keep these critical steps away from equilibrium. Thus, not surprisingly, many enzymes that catalyze highly exergonic reactions are subject to a variety of subtle regulatory mechanisms. The multiplicity of these adjustments is so great that we cannot predict by examining the properties of any one enzyme in a pathway whether that enzyme has a strong influence on net flow through the entire pathway.

Adenine Nucleotides Play Special Roles in Metabolic Regulation

After the protection of its DNA from damage, perhaps nothing is more important to a cell than maintaining a constant supply and concentration of ATP. Many ATP-using enzymes have K_m values between 0.1 and 1 mM, and the ATP concentration in a typical cell is about 5 to 10 mM (Fig. 13-31). If [ATP] were to drop significantly, these enzymes would be less than fully saturated by their substrate (ATP), and the rates of hundreds of reactions that involve ATP would decrease (**Fig. 13-34**); the cell would probably not survive this kinetic effect on so many reactions.

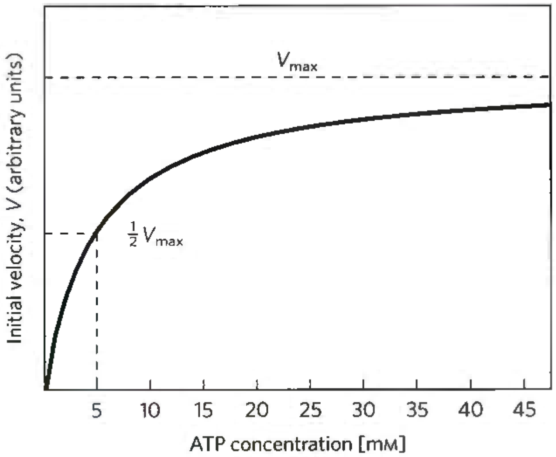


FIGURE 13-34 Effect of ATP concentration on the initial reaction velocity of a typical ATP-dependent enzyme. These experimental data yield a K_m for ATP of 5 mM. The concentration of ATP in animal tissues is ~5 mM.

There is also an important *thermodynamic* effect of lowered [ATP]. Because ATP is converted to ADP or AMP when "spent" to accomplish cellular work, the [ATP]/[ADP] ratio profoundly affects all reactions that employ these cofactors. The same is true for other important cofactors, such as NADH/NAD$^+$ and NADPH/NADP$^+$. For example, consider the reaction catalyzed by hexokinase:

$$\text{ATP} + \text{glucose} \longrightarrow \text{ADP} + \text{glucose 6-phosphate}$$

$$K'_{\text{eq}} = \frac{[\text{ADP}]_{\text{eq}}[\text{glucose 6-phosphate}]_{\text{eq}}}{[\text{ATP}]_{\text{eq}}[\text{glucose}]_{\text{eq}}} = 2 \times 10^3$$

Note that this expression holds true *only* when reactants and products are at their *equilibrium* concentrations, where $\Delta G = 0$. At any other set of concentrations, ΔG is not zero. Recall (from Section 13.1) that the ratio of products to substrates (the mass-action ratio, Q) determines the magnitude and sign of ΔG and therefore the driving force, ΔG, of the reaction:

$$\Delta G = \Delta G'^{\circ} + RT \ln \frac{[\text{ADP}][\text{glucose 6-phosphate}]}{[\text{ATP}][\text{glucose}]}$$

Because an alteration of this driving force profoundly influences every reaction that involves ATP, organisms have evolved under strong pressure to develop regulatory mechanisms responsive to the [ATP]/[ADP] ratio.

AMP concentration is an even more sensitive indicator of a cell's energetic state than is [ATP]. Normally, cells have a far higher concentration of ATP (5 to 10 mM) than of AMP (<0.1 mM). When some process (say, muscle contraction) consumes ATP, AMP is produced in two steps. First, hydrolysis of ATP produces ADP, then the reaction catalyzed by **adenylate kinase** produces AMP:

$$2\text{ADP} \longrightarrow \text{AMP} + \text{ATP}$$

If ATP is consumed such that its concentration drops 10%, the relative increase in [AMP] is much greater than that of [ADP] (**Table 13-11**). It is not surprising, therefore, that many regulatory processes are keyed to changes in [AMP]. P6 Probably the most important mediator of regulation by AMP is **AMP-activated protein kinase (AMPK)**, which responds to an increase in [AMP] by phosphorylating key proteins and thus regulating their activities. (AMPK is not to be confused with the *cyclic* AMP–dependent protein kinase PKA; see Section 12.2.) The rise in [AMP] may be caused by a reduced nutrient supply or by increased physical exercise. AMP activates AMPK allosterically, which increases glucose transport and activates glycolysis and fatty acid oxidation, while suppressing energy-requiring processes such as the synthesis of glycogen, fatty acids, cholesterol, and protein. All of the changes effected by AMPK serve to raise [ATP] and lower [AMP]. In Chapter 23, we discuss the role of AMPK in balancing anabolism and catabolism in the whole organism.

TABLE 13-11 Relative Changes in [ATP] and [AMP] When ATP Is Consumed

Adenine nucleotide	Concentration before ATP depletion (mM)	Concentration after ATP depletion (mM)	Relative change
ATP	5.0	4.5	10%
ADP	1.0	1.0	0
AMP	0.1	0.6	600%

SUMMARY 13.5 *Regulation of Metabolic Pathways*

- In a metabolically active cell in a steady state, intermediates are formed and consumed at equal rates. When a transient perturbation alters the rate of formation or consumption of a metabolite, compensating changes in enzyme activities return the system to the steady state.
- Cells regulate their metabolism by a variety of mechanisms over a time scale ranging from less than a millisecond to days, either by changing the activity of existing enzyme molecules or by changing the number of molecules of a specific enzyme.
- Various signals activate or inactivate transcription factors, which act in the nucleus to regulate gene expression. Changes in the transcriptome lead to changes in the proteome, and ultimately in the metabolome of a cell or tissue.
- In multistep processes such as glycolysis, certain reactions are essentially at equilibrium in the steady state; the rates of these reactions rise and fall with substrate concentration. Other reactions are far from equilibrium; these steps are typically the points of regulation of the overall pathway.
- Regulatory mechanisms maintain nearly constant levels of key metabolites such as ATP and NADH in cells and glucose in the blood, while matching the use or production of glucose to the organism's changing needs.
- The levels of ATP and AMP are a sensitive reflection of a cell's energy status, and when the [ATP]/[AMP] ratio decreases, the AMP-activated protein kinase (AMPK) triggers a variety of cellular responses to raise [ATP] and lower [AMP].

KEY TERMS

Terms in bold are defined in the glossary.

autotroph 461
heterotroph 461
metabolite 462
intermediary metabolism 462
catabolism 462
anabolism 462
energy transduction 466
free energy, *G* 467
exergonic 467
endergonic 467
enthalpy, *H* 467
exothermic 467
endothermic 467

entropy, S 467
standard transformed constants 468
mass-action ratio, Q 470
homolytic cleavage 472
radical 472
heterolytic cleavage 472
nucleophile 473
electrophile 473
carbanion 473
carbocation 473
aldol condensation 473
Claisen condensation 473
kinases 476
phosphorylation potential (ΔG_p) 479
thioester 482
adenylylation 485
inorganic pyrophosphatase 485
nucleoside diphosphate kinase 487
adenylate kinase 487
creatine kinase 487
phosphagens 488
electromotive force (emf) 488
conjugate redox pair 489
dehydrogenases 489
reducing equivalent 490
standard reduction potential ($E°$) 490
pyridine nucleotide 493
oxidoreductase 494
flavoprotein 495
flavin nucleotides 495
glucose 6-phosphate 496
homeostasis 498
transcription factor 498
response element 498
turnover 499
transcriptome 499
proteome 499
metabolome 499
metabolic regulation 501
metabolic control 501
adenylate kinase 503
AMP-activated protein kinase (AMPK) 503

PROBLEMS

1. Entropy Changes during Egg Development Consider a system consisting of an egg in an incubator. The white and yolk of the egg contain proteins, carbohydrates, and lipids. If fertilized, the egg transforms from a single cell to a complex organism. Discuss this irreversible process in terms of the entropy changes in the system and surroundings. Be sure that you first clearly define the system and surroundings.

2. Calculation of $\Delta G'°$ from an Equilibrium Constant Calculate the standard free-energy change for each of the three metabolically important enzyme-catalyzed reactions, using the equilibrium constants given for the reactions at 25 °C and pH 7.0.

(a) Glutamate + oxaloacetate $\xrightleftharpoons{\text{aspartate aminotransferase}}$ aspartate + α-ketoglutarate $K'_{eq} = 6.8$

(b) Dihydroxyacetone phosphate $\xrightleftharpoons{\text{triose phosphate isomerase}}$ glyceraldehyde 3-phosphate $K'_{eq} = 0.0475$

(c) Fructose 6-phosphate + ATP $\xrightleftharpoons{\text{phosphofructokinase}}$ fructose 1,6-bisphosphate + ADP $K'_{eq} = 254$

3. Calculation of the Equilibrium Constant from $\Delta G'°$ Calculate the equilibrium constant K'_{eq} for each of the three reactions at pH 7.0 and 25 °C, using the $\Delta G'°$ values in Table 13-4.

(a) Glucose 6-phosphate + H_2O $\xrightleftharpoons{\text{glucose 6-phosphatase}}$ glucose + P_i

(b) Lactose + H_2O $\xrightleftharpoons{\beta\text{-galactosidase}}$ glucose + galactose

(c) Malate $\xrightleftharpoons{\text{fumarase}}$ fumarate + H_2O

4. Experimental Determination of K'_{eq} and $\Delta G'°$ Incubating a 0.1 M solution of glucose 1-phosphate at 25 °C with a catalytic amount of phosphoglucomutase transforms some of the glucose 1-phosphate to glucose 6-phosphate. At equilibrium, the concentrations of the reaction components are

$$\underset{4.5 \times 10^{-3}\,\text{M}}{\text{Glucose 1-phosphate}} \rightleftharpoons \underset{9.6 \times 10^{-2}\,\text{M}}{\text{glucose 6-phosphate}}$$

Calculate K'_{eq} and $\Delta G'°$ for this reaction.

5. Experimental Determination of $\Delta G'°$ for ATP Hydrolysis A direct measurement of the standard free-energy change associated with the hydrolysis of ATP is technically demanding because the minute amount of ATP remaining at equilibrium is difficult to measure accurately. The value of $\Delta G'°$ can be calculated indirectly, however, from the equilibrium constants of two other enzymatic reactions having less favorable equilibrium constants:

Glucose 6-phosphate + H_2O $\longrightarrow$ glucose + P_i $K'_{eq} = 270$
ATP + glucose $\longrightarrow$ ADP + glucose 6-phosphate $K'_{eq} = 890$

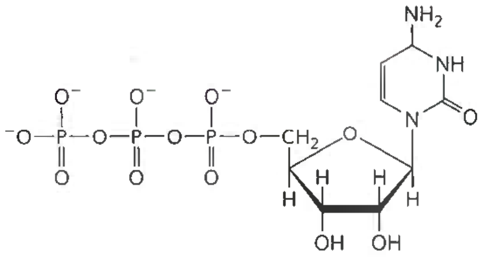
Using this information for equilibrium constants determined at 25 °C, calculate the standard free energy of hydrolysis of ATP.

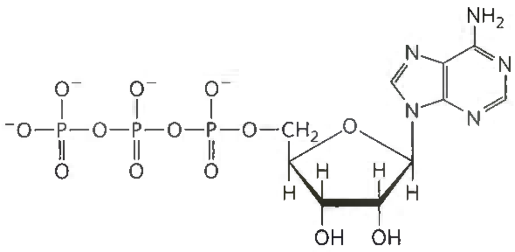
6. Difference between $\Delta G'°$ and ΔG Consider the interconversion shown, which occurs in glycolysis (Chapter 14):

Fructose 6-phosphate $\rightleftharpoons$ glucose 6-phosphate $K'_{eq} = 1.97$

(a) What is $\Delta G'°$ for the reaction (K'_{eq} measured at 25 °C)?
(b) If the concentration of fructose 6-phosphate is adjusted to 1.5 M and that of glucose 6-phosphate is adjusted to 0.50 M, what is ΔG?
(c) Why are $\Delta G'°$ and ΔG different?

7. Free Energy of Hydrolysis of CTP Compare the structure of the nucleoside triphosphate CTP with the structure of ATP.


Cytidine triphosphate (CTP)


Adenosine triphosphate (ATP)

Now predict the K'_{eq} and $\Delta G'°$ for the reaction:

$$\text{ATP} + \text{CDP} \rightarrow \text{ADP} + \text{CTP}$$

8. Dependence of ΔG on pH The free energy released by the hydrolysis of ATP under standard conditions is –30.5 kJ/mol.

If ATP is hydrolyzed under standard conditions except at pH 5.0, is more or less free energy released? Explain.

9. The $\Delta G'^\circ$ for Coupled Reactions Glucose 1-phosphate is converted into fructose 6-phosphate in two successive reactions:

$$\text{Glucose 1-phosphate} \longrightarrow \text{glucose 6-phosphate}$$
$$\text{Glucose 6-phosphate} \longrightarrow \text{fructose 6-phosphate}$$

Using the $\Delta G'^\circ$ values in Table 13-4, calculate the equilibrium constant, K'_{eq}, for the sum of the two reactions:

$$\text{Glucose 1-phosphate} \longrightarrow \text{fructose 6-phosphate}$$

10. Effect of [ATP]/[ADP] Ratio on Free Energy of Hydrolysis of ATP Using Equation 13-4, plot ΔG against ln Q (mass-action ratio) at 25 °C for the concentrations of ATP, ADP, and P_i in the table shown. $\Delta G'^\circ$ for the reaction is –30.5 kJ/mol. Use the resulting plot to explain why metabolism is regulated to keep the ratio [ATP]/[ADP] high.

	Concentration (mM)				
ATP	5	3	1	0.2	5
ADP	0.2	2.2	4.2	5.0	25
P_i	10	12.1	14.1	14.9	10

11. Strategy for Overcoming an Unfavorable Reaction: ATP-Dependent Chemical Coupling The phosphorylation of glucose to glucose 6-phosphate is the initial step in the catabolism of glucose. The direct phosphorylation of glucose by P_i is described by the equation

$$\text{Glucose} + P_i \longrightarrow \text{glucose 6-phosphate} + H_2O \qquad \Delta G'^\circ = 13.8 \text{ kJ/mol}$$

(a) Calculate the equilibrium constant for this reaction at 37 °C. In the rat hepatocyte, the physiological concentrations of glucose and P_i are maintained at approximately 4.8 mM. What is the equilibrium concentration of glucose 6-phosphate obtained by the direct phosphorylation of glucose by P_i? Does this reaction represent a reasonable metabolic step for the catabolism of glucose? Explain.

(b) In principle at least, one way to increase the concentration of glucose 6-phosphate is to drive the equilibrium reaction to the right by increasing the intracellular concentrations of glucose and P_i. Assuming a fixed concentration of P_i at 4.8 mM, how high would the intracellular concentration of glucose have to be to give an equilibrium concentration of glucose 6-phosphate of 250 μM (the normal physiological concentration)? Would this route be physiologically reasonable, given that the maximum solubility of glucose is less than 1 M?

(c) The phosphorylation of glucose in the cell is coupled to the hydrolysis of ATP; that is, part of the free energy of ATP hydrolysis is used to phosphorylate glucose:

$$(1) \quad \text{Glucose} + P_i \longrightarrow \text{glucose 6-phosphate} + H_2O \qquad \Delta G'^\circ = 13.8 \text{ kJ/mol}$$
$$(2) \quad \text{ATP} + H_2O \longrightarrow \text{ADP} + P_i \qquad \Delta G'^\circ = -30.5 \text{ kJ/mol}$$
$$\textit{Sum:} \quad \text{Glucose} + \text{ATP} \longrightarrow \text{glucose 6-phosphate} + \text{ADP}$$

Calculate K'_{eq} at 37 °C for the overall reaction. For the ATP-dependent phosphorylation of glucose, what concentration of glucose is needed to achieve a 250 μM intracellular concentration of glucose 6-phosphate when the concentrations of ATP and ADP are 3.38 mM and 1.32 mM, respectively? Does this coupling process provide a feasible route, at least in principle, for the phosphorylation of glucose in the cell? Explain.

(d) Although coupling ATP hydrolysis to glucose phosphorylation makes thermodynamic sense, we have not yet specified how this coupling is to take place. Given that coupling requires a common intermediate, one conceivable route is to use ATP hydrolysis to raise the intracellular concentration of P_i and thus drive the unfavorable phosphorylation of glucose by P_i. Is this a reasonable route? (Think about the solubility product, K_{sp}, of metabolic intermediates.)

(e) The ATP-coupled phosphorylation of glucose is catalyzed in hepatocytes by the enzyme glucokinase. This enzyme binds ATP and glucose to form a glucose-ATP-enzyme complex, and the phosphoryl group is transferred directly from ATP to glucose. Explain the advantages of this route.

12. Calculations of $\Delta G'^\circ$ for ATP-Coupled Reactions From data in Table 13-6, calculate the $\Delta G'^\circ$ value for each reaction:

(a) Phosphocreatine + ADP $\longrightarrow$ creatine + ATP

(b) ATP + fructose $\longrightarrow$ ADP + fructose 6-phosphate

13. Coupling ATP Cleavage to an Unfavorable Reaction To explore the consequences of coupling ATP hydrolysis under physiological conditions to a thermodynamically unfavorable biochemical reaction, consider the hypothetical transformation X → Y, for which $\Delta G'^\circ = 20.0$ kJ/mol.

(a) What is the ratio [Y]/[X] at equilibrium?

(b) Suppose X and Y participate in a sequence of reactions during which ATP is hydrolyzed to ADP and P_i. The overall reaction is

$$X + \text{ATP} + H_2O \longrightarrow Y + \text{ADP} + P_i$$

Calculate [Y]/[X] for this reaction at equilibrium. Assume that the temperature is 25.0 °C and the equilibrium concentrations of ATP, ADP, and P_i are 1 M.

(c) We know that [ATP], [ADP], and [P_i] are *not* 1 M under physiological conditions. Calculate [Y]/[X] for the ATP-coupled reaction when the values of [ATP], [ADP], and [P_i] are those found in rat myocytes (Table 13-5).

14. Calculations of ΔG at Physiological Concentrations Calculate the actual, physiological ΔG for the reaction

$$\text{Phosphocreatine} + \text{ADP} \longrightarrow \text{creatine} + \text{ATP}$$

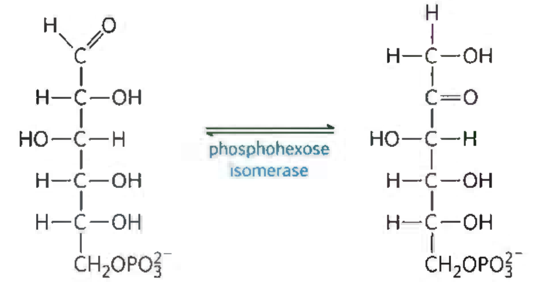
at 37 °C, as it occurs in the cytosol of neurons, with phosphocreatine at 4.7 mM, creatine at 1.0 mM, ADP at 0.73 mM, and ATP at 2.6 mM.

15. Free Energy Required for ATP Synthesis under Physiological Conditions In the cytosol of rat hepatocytes, the temperature is 37 °C and the mass-action ratio, Q, is

$$\frac{[\text{ATP}]}{[\text{ADP}][P_i]} = 5.33 \times 10^2 \text{ M}^{-1}$$

Calculate the free energy required to synthesize ATP in a rat hepatocyte.

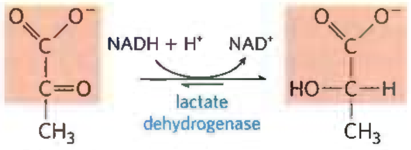
16. Chemical Logic In the glycolytic pathway, a six-carbon sugar (fructose 1,6-bisphosphate) is cleaved to form two three-carbon sugars, which undergo further metabolism. In this pathway, an isomerization of glucose 6-phosphate to fructose 6-phosphate (as shown in the diagram) occurs two steps before the cleavage reaction. The intervening step is phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate (p. 516).


phosphohexose isomerase

Glucose 6-phosphate Fructose 6-phosphate

What does the isomerization step accomplish from a chemical perspective? (Hint: Consider what might happen if the C—C bond cleavage were to proceed without the preceding isomerization.)

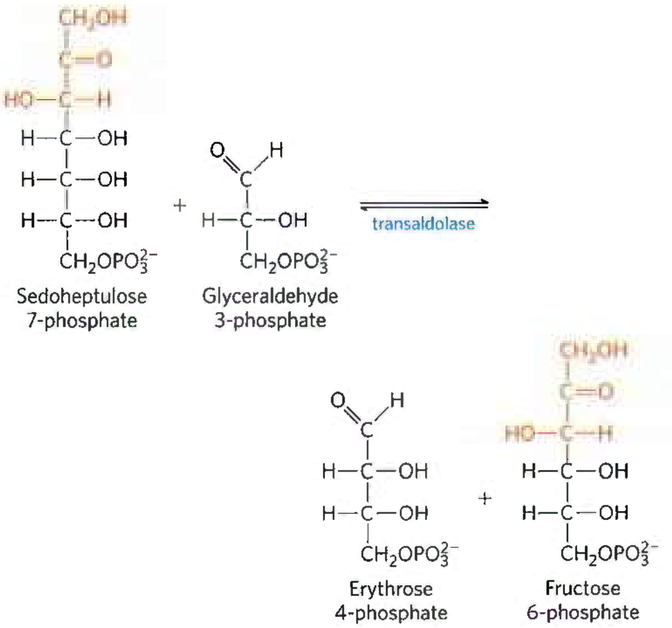
17. Enzymatic Reaction Mechanisms I Lactate dehydrogenase is one of the many enzymes that require NADH as coenzyme. It catalyzes the conversion of pyruvate to lactate:


NADH + H⁺
NAD⁺
lactate dehydrogenase

Pyruvate L-Lactate

Draw the mechanism of this reaction (show electron-pushing arrows). (Hint: This is a common reaction throughout metabolism; the mechanism is similar to that catalyzed by other dehydrogenases that use NADH, such as alcohol dehydrogenase.)

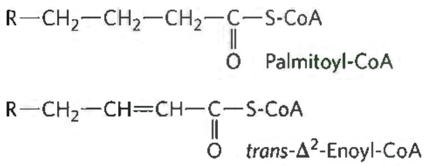
18. Enzymatic Reaction Mechanisms II Biochemical reactions often look more complex than they really are. In the pentose phosphate pathway (Chapter 14), sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate react to form erythrose 4-phosphate and fructose 6-phosphate in a reaction catalyzed by transaldolase.


Sedoheptulose 7-phosphate
Glyceraldehyde 3-phosphate
transaldolase
Erythrose 4-phosphate
Fructose 6-phosphate

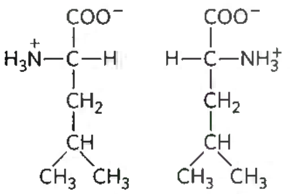
Draw a mechanism for this reaction (show electron-pushing arrows). (Hint: Take another look at aldol condensations, then consider the name of this enzyme.)

19. Recognizing Reaction Types For each pair of biomolecules, identify the type of reaction (oxidation-reduction, hydrolysis, isomerization, group transfer, or internal rearrangement) required to convert the first molecule to the second. In each case, indicate the general type of enzyme and cofactor(s) or reactants that would be required, and any other products that would result.

(a)

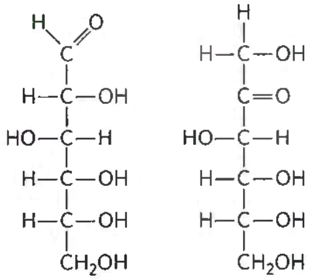

Palmitoyl-CoA
trans-Δ²-Enoyl-CoA

(b)



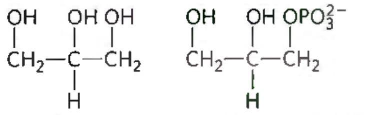
L-Leucine D-Leucine

(c)



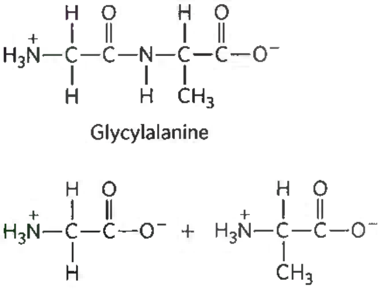
Glucose Fructose

(d)



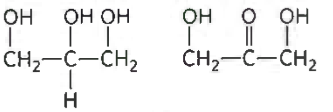
Glycerol Glycerol 3-phosphate

(e)


Glycylalanine

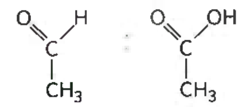
Glycine Alanine

(f)



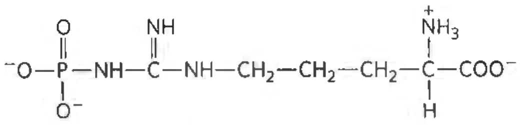
Glycerol Dihydroxyacetone

(g)



Acetaldehyde Acetic acid

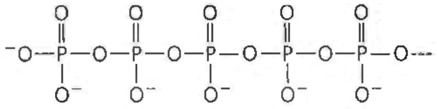
20. Effect of Structure on Group Transfer Potential Some invertebrates contain phosphoarginine. Is the standard free energy of hydrolysis of this molecule more similar to that of glucose 6-phosphate or of ATP? Explain your answer.



$${}^{-}O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-NH-\overset{\overset{NH}{\|}}{C}-NH-CH_2-CH_2-CH_2-\underset{\underset{H}{|}}{\overset{\overset{+}{NH_3}}{\overset{|}{C}}}-COO^-$$

Phosphoarginine

21. Polyphosphate as a Possible Energy Source The standard free energy of hydrolysis of inorganic polyphosphate (polyP) is about –20 kJ/mol for each P_i released. We calculated in Worked Example 13-2 that, in a cell, it takes about 50 kJ/mol of energy to synthesize ATP from ADP and P_i.



$${}^{-}O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-O\cdots$$

Inorganic polyphosphate (polyP)

Is it feasible for a cell to use polyphosphate to synthesize ATP from ADP? Explain your answer.

22. Daily ATP Utilization by Human Adults

(a) The synthesis of ATP from ADP and P_i requires a total of 30.5 kJ/mol of free energy when the reactants and products are at 1 M concentrations and the temperature is 25 °C (standard state). However, the actual physiological concentrations of ATP, ADP, and P_i are not 1 M, and the physiological temperature is 37 °C. Thus, the free energy required to synthesize ATP under physiological conditions is different from $\Delta G'^{\circ}$. Calculate the free energy required to synthesize ATP in the human hepatocyte when the physiological concentrations of ATP, ADP, and P_i are 3.5, 1.50, and 5.0 mM, respectively.

(b) A 68 kg (150 lb) adult requires a caloric intake of 2,000 kcal (8,360 kJ) of food per day (24 hours). The body metabolizes the food and uses the free energy to synthesize ATP, which then provides energy for the body's daily chemical and mechanical work. Assuming that the efficiency of converting food energy into ATP is 50%, calculate the weight of ATP used by a human adult in 24 hours. What percentage of the body weight does this represent?

(c) Although adults synthesize large amounts of ATP daily, their body weight, structure, and composition do not change significantly during this period. Explain this apparent contradiction.

23. Rates of Turnover of γ and β Phosphates of ATP After adding a small amount of ATP labeled with radioactive phosphorus in the terminal position, $[\gamma\text{-}^{32}P]$ATP, to a yeast extract, a researcher finds about half of the ^{32}P activity in P_i within a few minutes, but the concentration of ATP remains unchanged. Explain. She then carries out the same experiment using ATP labeled with ^{32}P in the central position, $[\beta\text{-}^{32}P]$ATP, but the ^{32}P does not appear in P_i within such a short time. Why?

24. Cleavage of ATP to AMP and PP_i during Metabolism Synthesis of the activated form of acetate (acetyl-CoA) is carried out in an ATP-dependent process:

$$\text{Acetate} + \text{CoA} + \text{ATP} \longrightarrow \text{acetyl-CoA} + \text{AMP} + PP_i$$

(a) The $\Delta G'^{\circ}$ for hydrolysis of acetyl-CoA to acetate and CoA is –32.2 kJ/mol. The $\Delta G'^{\circ}$ for hydrolysis of ATP to AMP and PP_i is –30.5 kJ/mol. Calculate $\Delta G'^{\circ}$ for the ATP-dependent synthesis of acetyl-CoA.

(b) Almost all cells contain the enzyme inorganic pyrophosphatase, which catalyzes the hydrolysis of PP_i to P_i. What effect does the presence of this enzyme have on the synthesis of acetyl-CoA? Explain.

25. Activation of a Fatty Acid by Reaction with Coenzyme A In the reaction sequence for fatty acid breakdown, coenzyme A (CoA), with its thiol (—SH) group, joins to the fatty acid as a thiol ester, as ATP is converted into AMP and PP_i:

$$\text{R—COO}^- + \text{ATP} + \text{CoA—SH} \longrightarrow \text{AMP} + PP_i + \text{R—CO—S—CoA}$$

The oxidation of fatty acids as fuels requires two steps. The first step transfers an activating group from ATP to the carboxyl group of the fatty acid. In the second step, CoA—SH displaces the activating group to form fatty acyl-S—CoA. Given the known products of the reaction, what is the activating group?

26. Energy for H^+ Pumping The parietal cells of the stomach lining contain membrane "pumps" that transport hydrogen ions from the cytosol (pH 7.0) into the stomach, contributing to the acidity of gastric juice (pH 1.0). Calculate the free energy required to transport 1 mol of hydrogen ions through these pumps. (Hint: See Chapter 11.) Assume a temperature of 37 °C.

27. Most-Reduced Carbon Compounds Arrange the four structures in order from most reduced to most oxidized.

(a) $R—CH_2—CH_2—OH$
(b) $R—CH_2—COO^-$
(c) $R—CH_2—CHO$
(d) $R—CH_2—CH_3$

28. Standard Reduction Potentials The standard reduction potential, E'°, of any redox pair is defined for the half-cell reaction

$$\text{Oxidizing agent} + n \text{ electrons} \longrightarrow \text{reducing agent}$$

The E'° values for the NAD^+/NADH and pyruvate/lactate conjugate redox pairs are –0.32 V and –0.19 V, respectively.

(a) Which redox pair has the greater tendency to lose electrons? Explain.

(b) Which pair is the stronger oxidizing agent? Explain.

(c) Beginning with 1 M concentrations of each reactant and product at pH 7 and 25 °C, in which direction will the following reaction proceed?

$$\text{Pyruvate} + \text{NADH} + H^+ \rightleftharpoons \text{lactate} + NAD^+$$

(d) What is the standard free-energy change ($\Delta G'^{\circ}$) for the conversion of pyruvate to lactate?

(e) What is the equilibrium constant (K'_{eq}) for this reaction?

29. Simple Biobattery Suppose you set up a simple battery using half-reactions as pictured in Figure 13-23. One electrode contains pyruvate and lactate at 1 mM, and the other electrode contains fumarate and succinate at 1 mM (see Table 13-7).

(a) In which direction will electrons initially flow?

(b) Calculate the standard reduction potential and standard free-energy change for your biological battery.

(c) When a flashlight battery "runs out," net electron movement has essentially ended. What is the equivalent situation for your biobattery?

30. Energy Span of the Respiratory Chain Electron transfer in the mitochondrial respiratory chain may be represented by the net reaction equation

$$\text{NADH} + \text{H}^+ + \frac{1}{2}\text{O}_2 \rightleftharpoons \text{H}_2\text{O} + \text{NAD}^+$$

(a) Calculate $\Delta E'^\circ$ for the net reaction of mitochondrial electron transfer. Use E'° values in Table 13-7.
(b) Calculate $\Delta G'^\circ$ for this reaction.
(c) How many ATP molecules can *theoretically* be generated by this reaction if the free energy of ATP synthesis under cellular conditions is 52 kJ/mol?

31. Dependence of Electromotive Force on Concentrations Suppose that you place an electrode into solutions of various concentrations of NAD^+ and NADH at pH 7.0 and 25 °C. Calculate the electromotive force (in volts) registered by the electrode when immersed in each solution, with reference to a half-cell of E'° 0.00 V.
(a) 1.0 mM NAD^+ and 10 mM NADH
(b) 1.0 mM NAD^+ and 1.0 mM NADH
(c) 10 mM NAD^+ and 1.0 mM NADH

32. Electron Affinity of Compounds List the four compounds or reactions in order of increasing tendency to accept electrons:
(a) α-ketoglutarate + CO_2 (yielding isocitrate) **(b)** oxaloacetate
(c) O_2 **(d)** $NADP^+$

33. Direction of Oxidation-Reduction Reactions Which of the reactions listed would you expect to proceed in the direction shown, under standard conditions, in the presence of the appropriate enzymes?
(a) Malate + $NAD^+ \longrightarrow$ oxaloacetate + NADH + H^+
(b) Acetoacetate + NADH + $H^+ \longrightarrow$ β-hydroxybutyrate + NAD^+
(c) Pyruvate + NADH + $H^+ \longrightarrow$ lactate + NAD^+
(d) Pyruvate + β-hydroxybutyrate $\longrightarrow$ lactate + acetoacetate
(e) Malate + pyruvate $\longrightarrow$ oxaloacetate + lactate
(f) Acetaldehyde + succinate $\longrightarrow$ ethanol + fumarate

34. Measurement of Intracellular Metabolite Concentrations Measuring the concentrations of metabolic intermediates in a living cell presents great experimental difficulties—usually, a cell must be destroyed before metabolite concentrations can be measured. Yet enzymes catalyze metabolic interconversions very rapidly, so a common problem associated with these types of measurements is that the findings reflect not the physiological concentrations of metabolites but the equilibrium concentrations. To prevent changes in metabolite concentrations during sample preparation, cells were quick-frozen in liquid nitrogen, then extracted under conditions that prevented enzymatic activity.

The table gives the intracellular concentrations of the substrates and products of the phosphofructokinase-1 reaction in isolated rat heart tissue.

Metabolite	Concentration (μM)[a]
Fructose 6-phosphate	87.0
Fructose 1,6-bisphosphate	22.0
ATP	11,400
ADP	1,320

Data from J. R. Williamson, *J. Biol. Chem.* 240:2308, 1965.
[a] Calculated as μmol/mL of intracellular water.

(a) Calculate Q, [fructose 1,6-bisphosphate][ADP]/[fructose 6-phosphate][ATP], for the PFK-1 reaction under physiological conditions.
(b) Given a $\Delta G'^\circ$ for the PFK-1 reaction of −14.2 kJ/mol, calculate the equilibrium constant for this reaction.
(c) Compare the values of Q and K'_{eq}. Is the physiological reaction near or far from equilibrium? Explain. What does this experiment suggest about the role of PFK-1 as a regulatory enzyme?

35. Are All Metabolic Reactions at Equilibrium?
(a) Phosphoenolpyruvate (PEP) is one of the two phosphoryl group donors in the synthesis of ATP during glycolysis. In human erythrocytes, the steady-state concentration of ATP is 2.24 mM, that of ADP is 0.25 mM, and that of pyruvate is 0.051 mM. Calculate the concentration of PEP at 25 °C, assuming that the pyruvate kinase reaction (see Fig. 13-13) is at equilibrium in the cell.
(b) The physiological concentration of PEP in human erythrocytes is 0.023 mM. Compare this with the value obtained in (a). Explain the significance of this difference.

36. Michaelis Constant K_m Compared with Substrate Concentration Malate synthase in *E. coli* catalyzes the reaction

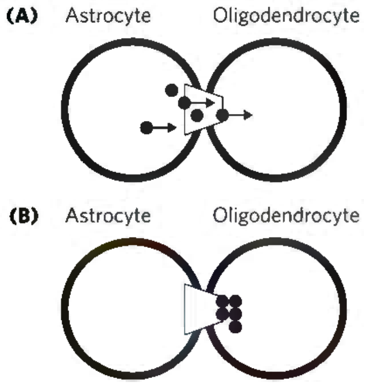
$$\text{Acetyl-CoA} + \text{glyoxylate} + \text{H}_2\text{O} \longrightarrow \text{malate} + \text{CoA-SH} + \text{H}^+$$

The experimentally measured K_m for acetyl-CoA is 9×10^6 M. In a growing culture of *E. coli*, the measured concentration of acetyl-CoA is 6×10^{-4} M. Is malate synthase operating at its V_{max} under these conditions?

DATA ANALYSIS PROBLEM

37. Thermodynamics Can Be Tricky Thermodynamics is a challenging area of study and one with many opportunities for confusion. An interesting example is found in an article by Robinson, Hampson, Munro, and Vaney, published in *Science* in 1993. Robinson and colleagues studied the movement of small molecules between neighboring cells of the nervous system through cell-to-cell channels (gap junctions). They found that the dyes Lucifer yellow (a small, negatively charged molecule) and biocytin (a small zwitterionic molecule) moved in only one direction between two particular types of glia (nonneuronal cells of the nervous system). Dye injected into astrocytes would rapidly pass into adjacent astrocytes, oligodendrocytes, or Müller cells, but dye injected into oligodendrocytes or Müller cells passed slowly if at all into astrocytes. All of these cell types are connected by gap junctions.

Although it was not a central point of their article, the authors presented a molecular model for how this unidirectional transport might occur, as shown in their Figure 3:



The figure legend reads: "Model of the unidirectional diffusion of dye between coupled oligodendrocytes and astrocytes, based on differences in connection pore diameter. Like a fish in a fish trap, dye molecules (black circles) can pass from an astrocyte to an oligodendrocyte (A) but not back in the other direction (B)."

Although this article clearly passed review at a well-respected journal, several letters to the editor (1994) followed, showing that Robinson and coauthors' model violated the second law of thermodynamics.

(a) Explain how the model violates the second law. Hint: Consider what would happen to the entropy of the system if one started with equal concentrations of dye in the astrocyte and oligodendrocyte connected by the "fish trap" type of gap junctions.

(b) Explain why this model cannot work for small molecules, although it may allow one to catch fish.

(c) Explain why a fish trap *does* work for fish.

(d) Provide two plausible mechanisms for the unidirectional transport of dye molecules between the cells that do not violate the second law of thermodynamics.

References

Letters to the editor. 1994. *Science* 265:1017–1019.

Robinson, S.R., E.C.G.M. Hampson, M.N. Munro, and D.I. Vaney. 1993. Unidirectional coupling of gap junctions between neuroglia. *Science* 262:1072–1074.

Chapter 14

GLYCOLYSIS, GLUCONEOGENESIS, AND THE PENTOSE PHOSPHATE PATHWAY

14.1 Glycolysis *511*
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14.3 Fates of Pyruvate *525*
14.4 Gluconeogenesis *533*
14.5 Coordinated Regulation of Glycolysis and Gluconeogenesis *539*
14.6 Pentose Phosphate Pathway of Glucose Oxidation *546*

We begin with the major pathways of glucose metabolism: glycolysis and fermentation, gluconeogenesis, and the pentose phosphate pathway. Glucose occupies a central position in the metabolism of plants, animals, and many microorganisms. It is relatively rich in potential energy, and thus a good fuel; the complete oxidation of glucose to carbon dioxide and water proceeds with a standard free-energy change of –2,840 kJ/mol. By storing glucose as a high molecular weight polymer such as starch or glycogen, a cell can stockpile large quantities of hexose units while maintaining a relatively low cytosolic osmolarity. When energy demands increase, glucose can be released from these intracellular storage polymers and used to produce ATP either aerobically or anaerobically.

Glucose is not only an excellent fuel, but also a remarkably versatile precursor, capable of supplying a huge array of metabolic intermediates for biosynthetic reactions. A bacterium such as *Escherichia coli* can obtain from glucose the carbon skeletons for every amino acid, nucleotide, coenzyme, fatty acid, or other metabolic intermediate it needs for growth. A comprehensive study of the metabolic fates of glucose would encompass hundreds or thousands of transformations. In animals and vascular plants, glucose has four major fates: it may be (1) used in the synthesis of complex polysaccharides destined for the extracellular space; (2) stored in cells (as a polysaccharide or as sucrose); (3) oxidized to a three-carbon compound (pyruvate) via glycolysis to provide ATP and metabolic intermediates; or (4) oxidized via the pentose phosphate (phosphogluconate) pathway to yield ribose 5-phosphate for nucleic acid synthesis and NADPH for reductive biosynthetic processes (**Fig. 14-1**).

Organisms that do not have access to glucose from other sources must make it. Photosynthetic organisms make glucose by first reducing atmospheric CO_2 to trioses, then converting the trioses to glucose. Nonphotosynthetic cells make glucose from simpler three- and four-carbon precursors by the process of gluconeogenesis, effectively reversing glycolysis in a pathway that uses many of the glycolytic enzymes.

These principles are central to understanding glucose metabolism, but many apply to all metabolic pathways:

P1 **Metabolites like glucose are often activated with a high-energy group before their catabolism.** Glycolysis is a nearly universal 10-step metabolic pathway for producing ATP by the oxidation of glucose. In this process, two

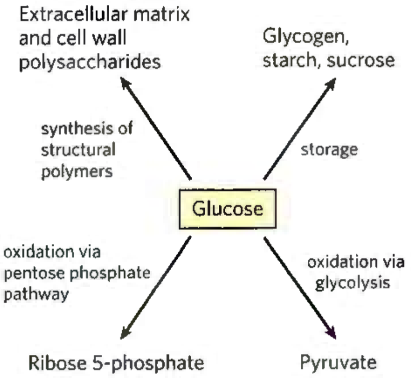
Extracellular matrix and cell wall polysaccharides
Glycogen, starch, sucrose
synthesis of structural polymers
storage
Glucose
oxidation via pentose phosphate pathway
oxidation via glycolysis
Ribose 5-phosphate
Pyruvate

FIGURE 14-1 Major pathways of glucose utilization. Although not the only possible fates for glucose, these four pathways are the most significant in most cells.

molecules of ATP are invested to activate glucose, but the products of the pathway include four ATP, as well as NADH (a form of reducing power) and the triose pyruvate, which can be metabolized further in other pathways.

P2 **Glucose and other hexoses and hexose phosphates obtained from stored polysaccharides or dietary carbohydrates feed into the glycolytic pathway.** By using a common pathway for a number of starting materials, the cell economizes on the number of enzymes that must be synthesized and simplifies the regulation of the common pathway.

P3 **Pyruvate formed under anaerobic conditions is reduced to lactate with electrons from NADH, recycling NADH to NAD^+ and allowing continued glycolysis in the processes of lactate or alcohol fermentation.** Manipulation of the fermentable material and the microorganisms present allows the synthesis of a variety of industrial products and foods.

P4 **Gluconeogenesis is the synthesis of glucose from simpler precursors like pyruvate and lactate.** Although it uses seven of the ten enzymes that also act in glycolysis, gluconeogenesis must bypass three of the most exergonic steps in glycolysis with energetically favorable reactions unique to gluconeogenesis.

P5 **Glycolysis and gluconeogenesis are reciprocally regulated so that both processes don't occur simultaneously in a futile cycle.** Most regulatory mechanisms act on reactions that are unique to each pathway.

P6 **The pentose phosphate pathway is an alternative pathway for glucose oxidation.** It yields pentoses for nucleotide synthesis and reduced cofactors for biosynthesis of fatty acids, sterols, and many other compounds.

14.1 Glycolysis

P1 In **glycolysis** (from the Greek *glykys*, "sweet" or "sugar," and *lysis*, "splitting"), a molecule of glucose is degraded in a series of enzyme-catalyzed reactions to yield two molecules of the three-carbon compound pyruvate. During the sequential reactions of glycolysis, some of the free energy released from glucose is conserved in the form of ATP and NADH. Glycolysis was the first metabolic pathway to be elucidated and is probably the best understood. From Eduard Buchner's discovery in 1897 of fermentation in cell-free extracts of yeast until the elucidation of the whole pathway in yeast and in muscle in the 1930s, the reactions of glycolysis were a major focus of biochemical research. These discoveries showed that the reactions of life could be explained chemically, without reliance on a mystical life force. This philosophical shift led physiologist Jacques Loeb to observe in 1906, "The history of this problem is instructive, as it warns us against considering problems as beyond our reach because they have not yet found their solution."[1]

The development of methods of enzyme purification, the discovery and recognition of the importance of coenzymes such as NAD, and the discovery of the pivotal metabolic role of ATP and other phosphorylated compounds all came out of studies of glycolysis. The glycolytic enzymes of many species have long since been purified and thoroughly studied.

Glycolysis is an almost universal central pathway of glucose catabolism, the pathway with the largest flux of carbon in most cells. The glycolytic breakdown of glucose is the sole source of metabolic energy in some mammalian tissues and cell types (erythrocytes, renal medulla, brain, and sperm, for example). Some plant tissues that are modified to store starch (such as potato tubers) and some aquatic plants (watercress, for example) derive most of their energy from glycolysis; many anaerobic microorganisms are entirely dependent on glycolysis.

In the course of evolution, the chemistry of the reactions of glycolysis has been completely conserved. Genome sequencing and structural studies have shown that the glycolytic enzymes of vertebrates are closely similar in amino acid sequence and three-dimensional structure to their homologs in yeast and spinach. Although some archaea and parasitic microorganisms lack one or more of the enzymes of glycolysis, they retain the core of the pathway. The glycolytic pathway, of central importance in itself, is governed by thermodynamic principles and regulatory mechanisms that are common to all pathways of cell metabolism. It serves as a model of principles we will revisit throughout Part II of this book.

An Overview: Glycolysis Has Two Phases

Before examining each step of the pathway in some detail, we take a look at glycolysis as a whole. As all sugar derivatives in glycolysis are the D isomers, we will usually omit the D designation except when emphasizing stereochemistry. The breakdown of the six-carbon glucose into two molecules of the three-carbon pyruvate occurs in 10 steps, the first 5 of which constitute the *preparatory phase* (**Fig. 14-2a**). In these reactions, glucose is first phosphorylated at the hydroxyl group on C-6 (step ❶). The glucose 6-phosphate thus formed is converted to fructose 6-phosphate (step ❷), which is again phosphorylated, this time at C-1, to yield fructose 1,6-bisphosphate (step ❸). For both phosphorylations, ATP is the phosphoryl group donor.

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[1] From J. Loeb, *The Dynamics of Living Matter*, Columbia University Press, New York, 1906.

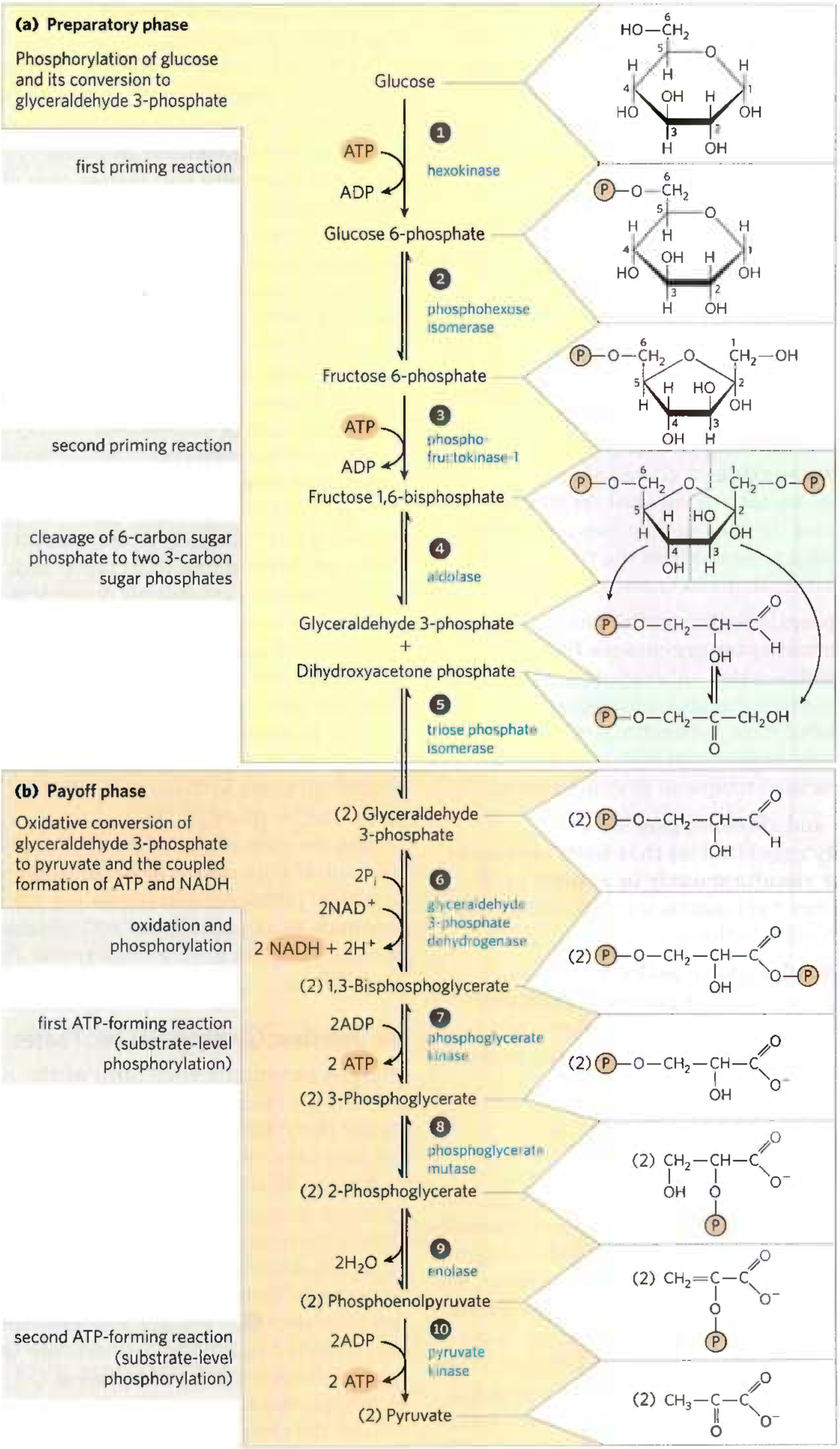

(a) Preparatory phase
Phosphorylation of glucose and its conversion to glyceraldehyde 3-phosphate
Glucose
first priming reaction
ATP
ADP
1
hexokinase
Glucose 6-phosphate
2
phosphohexose isomerase
Fructose 6-phosphate
second priming reaction
ATP
ADP
3
phospho fructokinase-1
Fructose 1,6-bisphosphate
cleavage of 6-carbon sugar phosphate to two 3-carbon sugar phosphates
4
aldolase
Glyceraldehyde 3-phosphate
+
Dihydroxyacetone phosphate
5
triose phosphate isomerase
(b) Payoff phase
Oxidative conversion of glyceraldehyde 3-phosphate to pyruvate and the coupled formation of ATP and NADH
(2) Glyceraldehyde 3-phosphate
oxidation and phosphorylation
2P$_i$
2NAD$^+$
2 NADH + 2H$^+$
6
glyceraldehyde 3-phosphate dehydrogenase
(2) 1,3-Bisphosphoglycerate
first ATP-forming reaction (substrate-level phosphorylation)
2ADP
2 ATP
7
phosphoglycerate kinase
(2) 3-Phosphoglycerate
8
phosphoglycerate mutase
(2) 2-Phosphoglycerate
2H$_2$O
9
enolase
(2) Phosphoenolpyruvate
second ATP-forming reaction (substrate-level phosphorylation)
2ADP
2 ATP
10
pyruvate kinase
(2) Pyruvate

FIGURE 14-2 The two phases of glycolysis. For each molecule of glucose that passes through the preparatory phase (a), two molecules of glyceraldehyde 3-phosphate are formed; both pass through the payoff phase (b). Pyruvate is the end product of the second phase of glycolysis. For each glucose molecule, two ATP are consumed in the preparatory phase and four ATP are produced in the payoff phase, giving a net yield of two ATP and two NADH per molecule of glucose converted to pyruvate. The numbered reaction steps correspond to the numbered headings in the text discussion. Keep in mind that each phosphoryl group, represented here as Ⓟ, has two negative charges ($-PO_3^{2-}$).

Fructose 1,6-bisphosphate is split to yield two different three-carbon molecules, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (step ④); this is the "lysis" step that gives the pathway its name. The dihydroxyacetone phosphate is isomerized to form a second molecule of glyceraldehyde 3-phosphate (step ⑤), ending the first phase of glycolysis. Note that two molecules of ATP are invested before the cleavage of glucose into two three-carbon pieces; there will be a good return on this investment. To summarize: in the preparatory phase of glycolysis the energy of ATP is invested, raising the free-energy content of the intermediates, and the carbon chains of all the metabolized hexoses are converted to a common product, glyceraldehyde 3-phosphate.

The energy gain comes in the *payoff phase* of glycolysis (Fig. 14-2b). Each molecule of glyceraldehyde 3-phosphate is oxidized and phosphorylated by inorganic phosphate (*not* by ATP) to form 1,3-bisphosphoglycerate (step ⑥). Energy is then released as the two molecules of 1,3-bisphosphoglycerate are converted to two molecules of pyruvate (steps ⑦ through ⑩). Much of this energy is conserved by the coupled phosphorylation of four molecules of ADP to ATP. The net yield is two molecules of ATP per molecule of glucose used, because two molecules of ATP were invested in the preparatory phase. Energy is also conserved in the payoff phase in the formation of two molecules of the electron carrier NADH per molecule of glucose.

P1 In the sequential reactions of glycolysis, three types of chemical transformations are particularly noteworthy: (1) degradation of the carbon skeleton of glucose to yield pyruvate; (2) phosphorylation of ADP to ATP by compounds with high phosphoryl group transfer potential, formed during glycolysis; and (3) transfer of a hydride ion to NAD^+, forming NADH. The overall chemical logic of the pathway is described in **Figure 14-3**.

ATP and NADH Formation Coupled to Glycolysis During glycolysis some of the energy of the glucose molecule is conserved in ATP, while much remains in the product, pyruvate. The overall equation for glycolysis is

$$\text{Glucose} + 2NAD^+ + 2ADP + 2P_i \longrightarrow 2\text{ pyruvate} + 2NADH + 2H^+ + 2ATP + 2H_2O \qquad (14\text{-}1)$$

For each molecule of glucose degraded to pyruvate, two molecules of ATP are generated from ADP and P_i, and two molecules of NADH are produced by the reduction

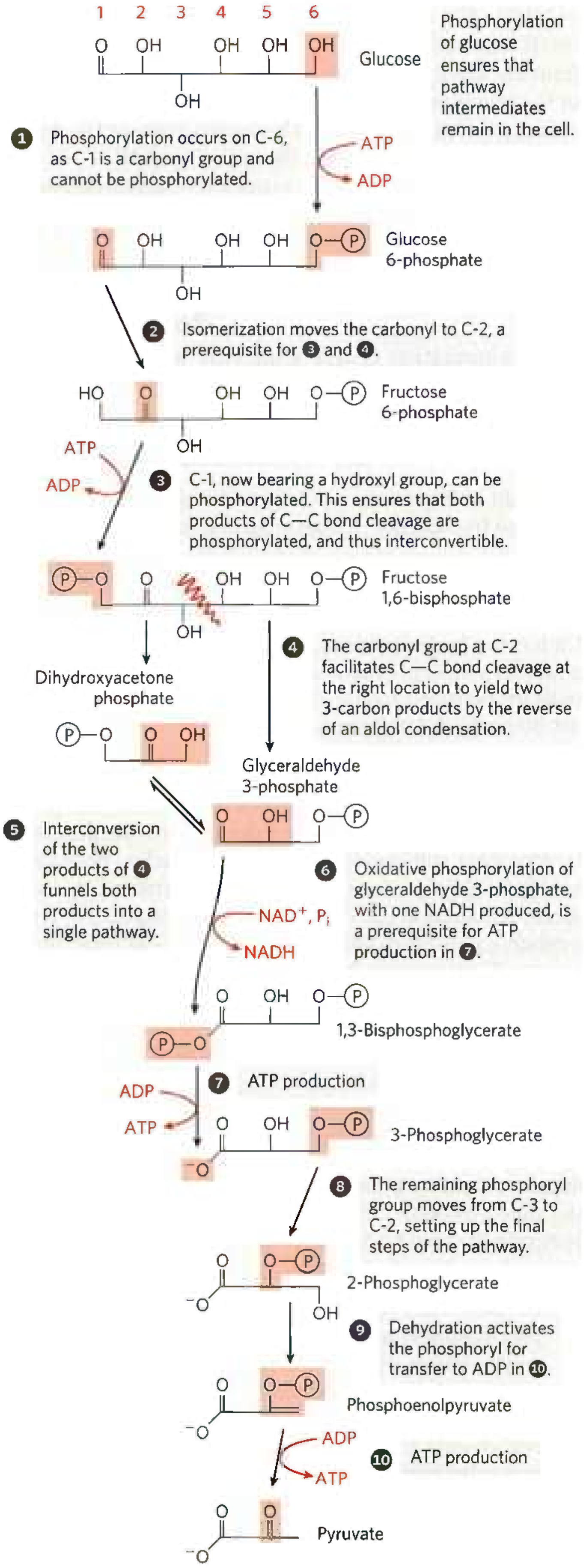

1 2 3 4 5 6
Glucose
Phosphorylation of glucose ensures that pathway intermediates remain in the cell.
① Phosphorylation occurs on C-6, as C-1 is a carbonyl group and cannot be phosphorylated.
ATP
ADP
Glucose 6-phosphate
② Isomerization moves the carbonyl to C-2, a prerequisite for ③ and ④.
Fructose 6-phosphate
ATP
ADP
③ C-1, now bearing a hydroxyl group, can be phosphorylated. This ensures that both products of C—C bond cleavage are phosphorylated, and thus interconvertible.
Fructose 1,6-bisphosphate
Dihydroxyacetone phosphate
④ The carbonyl group at C-2 facilitates C—C bond cleavage at the right location to yield two 3-carbon products by the reverse of an aldol condensation.
Glyceraldehyde 3-phosphate
⑤ Interconversion of the two products of ④ funnels both products into a single pathway.
NAD⁺, Pᵢ
NADH
⑥ Oxidative phosphorylation of glyceraldehyde 3-phosphate, with one NADH produced, is a prerequisite for ATP production in ⑦.
1,3-Bisphosphoglycerate
ADP
ATP
⑦ ATP production
3-Phosphoglycerate
⑧ The remaining phosphoryl group moves from C-3 to C-2, setting up the final steps of the pathway.
2-Phosphoglycerate
⑨ Dehydration activates the phosphoryl for transfer to ADP in ⑩.
Phosphoenolpyruvate
ADP
ATP
⑩ ATP production
Pyruvate

FIGURE 14-3 The chemical logic of the glycolytic pathway. In this simplified version of the pathway, each molecule is shown in a linear form, with carbon and hydrogen atoms not depicted, in order to highlight chemical transformations. Remember that glucose and fructose are present mostly in their cyclized forms in solution, although they are transiently present in linear form at the active sites of some of the enzymes in this pathway.

The preparatory phase, steps ① to ⑤, converts the six-carbon glucose into two three-carbon units, each of them phosphorylated. Oxidation of the three-carbon units is initiated in the payoff phase, steps ⑥ to ⑩. To produce pyruvate, the chemical steps must occur in the order shown.

of NAD^+. The reduction of NAD^+ (see Fig. 13-24) proceeds by the enzymatic transfer of a hydride ion ($:H^-$) from the aldehyde group of glyceraldehyde 3-phosphate to the nicotinamide ring of NAD^+, yielding the reduced coenzyme NADH. The other hydrogen atom of the substrate molecule is released to the solution as H^+.

We can now resolve the equation of glycolysis into two processes—the conversion of glucose to pyruvate, which is exergonic:

$$\text{Glucose} + 2NAD^+ \longrightarrow 2\ \text{pyruvate} + 2NADH + 2H^+ \quad (14\text{-}2)$$
$$\Delta G_1'^{\circ} = -146\ \text{kJ/mol}$$

and the formation of ATP from ADP and P_i, which is endergonic:

$$2ADP + 2P_i \longrightarrow 2ATP + 2H_2O \quad (14\text{-}3)$$
$$\Delta G_2'^{\circ} = 2(30.5\ \text{kJ/mol}) = 61.0\ \text{kJ/mol}$$

The sum of Equations 14-2 and 14-3 gives the overall standard free-energy change of glycolysis, $\Delta G'^{\circ}_{\text{Sum}}$:

$$\Delta G'^{\circ}_{\text{Sum}} = \Delta G_1'^{\circ} + \Delta G_2'^{\circ} = -146\ \text{kJ/mol} + 61.0\ \text{kJ/mol}$$
$$= -85\ \text{kJ/mol}$$

Under standard conditions, and under the (nonstandard) conditions that prevail in a cell, glycolysis is an essentially irreversible process, driven to completion by a large net decrease in free energy.

Energy Remaining in Pyruvate Glycolysis releases only a small fraction of the total available energy of the glucose molecule. The two molecules of pyruvate formed by glycolysis still contain most of the chemical potential energy of glucose, energy that can be extracted by oxidative reactions in the citric acid cycle (Chapter 16) and oxidative phosphorylation (Chapter 19)—aerobic processes. Under anaerobic conditions, pyruvate can be reduced to lactate or ethanol (Section 14.3). The oxidation of pyruvate is an important catabolic process, but pyruvate has anabolic fates as well. It can, for example, provide the carbon skeleton for the synthesis of the amino acid alanine or for the synthesis of fatty acids. We return to these anabolic reactions of pyruvate in later chapters.

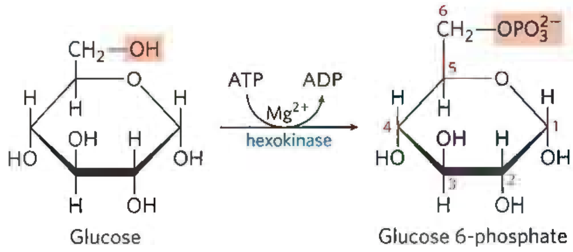
Importance of Phosphorylated Intermediates P1 Each of the nine glycolytic intermediates between glucose and pyruvate is phosphorylated (Fig. 14-2). The phosphoryl groups have three functions.

1. Because the plasma membrane lacks transporters for phosphorylated sugars, the phosphorylated glycolytic intermediates cannot leave the cell. After the initial phosphorylation, no further energy is necessary to retain phosphorylated intermediates in the cell, despite the large difference in their intracellular and extracellular concentrations.
2. Phosphoryl groups are essential components in the enzymatic conservation of metabolic energy. Energy made available with the transfer of phosphoryl groups from compounds with phosphoanhydride bonds (such as those in ATP) is partially conserved in the formation of phosphate esters such as glucose 6-phosphate. Compounds with higher group transfer potential than ATP, which are formed in glycolysis (1,3-bisphosphoglycerate and phosphoenolpyruvate), donate phosphoryl groups to ADP to form ATP.
3. Binding energy resulting from the binding of phosphate groups to the active sites of enzymes lowers the activation energy and increases the specificity of the enzymatic reactions (Chapter 6). The phosphate groups of ADP, ATP, and the glycolytic intermediates form complexes with Mg^{2+}, and the substrate binding sites of many glycolytic enzymes are specific for these Mg^{2+} complexes. Most glycolytic enzymes require Mg^{2+} for activity.

The Preparatory Phase of Glycolysis Requires ATP

In the preparatory phase of glycolysis, two molecules of ATP are invested and the hexose chain is cleaved into two triose phosphates. The realization that *phosphorylated* hexoses were intermediates in glycolysis came slowly and serendipitously. In 1906, Arthur Harden and William Young tested their hypothesis that inhibitors of proteolytic enzymes would stabilize the glucose-fermenting enzymes in yeast extract. They added blood serum (known to contain inhibitors of proteolytic enzymes) to yeast extracts and observed the predicted stimulation of glucose metabolism. However, in a control experiment intended to show that boiling the serum destroyed the stimulatory activity, they discovered that boiled serum was just as effective at stimulating glycolysis! Careful examination and testing of the contents of the boiled serum revealed that inorganic phosphate was responsible for the stimulation. Harden and Young soon discovered that glucose added to their yeast extract was converted to a hexose bisphosphate (the "Harden-Young ester," eventually identified as fructose 1,6-bisphosphate). This was the beginning of a long series of investigations on the role of organic esters and anhydrides of phosphate in biochemistry, which has led to our current understanding of the central role of phosphoryl group transfer in biology.

① Phosphorylation of Glucose In the first step of glycolysis (Fig. 14-2), glucose is activated for subsequent reactions by its phosphorylation at C-6 to yield **glucose 6-phosphate**, with ATP as the phosphoryl donor:



Glucose

Glucose 6-phosphate

$\Delta G'^{\circ} = -16.7$ kJ/mol

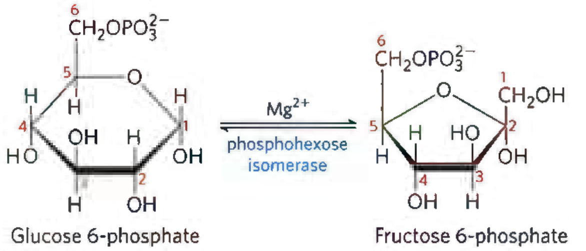
This reaction, which is irreversible under intracellular conditions, is catalyzed by **hexokinase**. Recall that kinases are enzymes that catalyze the transfer of the terminal phosphoryl group from ATP to an acceptor nucleophile (see Fig. 13-8c). Kinases are a subclass of transferases (see Table 6-3). The acceptor in the case of hexokinase is a hexose, normally glucose, although hexokinase also catalyzes the phosphorylation of other common hexoses, such as fructose and mannose, in some tissues.

Hexokinase, like many other kinases, requires Mg^{2+} for its activity, because the true substrate of the enzyme is not ATP^{4-} but the MgATP^{2-} complex (see Fig. 13-12). Mg^{2+} shields the negative charges of the phosphoryl groups in ATP, making the terminal phosphorus atom an easier target for nucleophilic attack by an —OH of glucose. Hexokinase undergoes a profound change in shape, an induced fit, when it binds glucose; two domains of the protein move about 8 Å closer to each other when ATP binds (see Fig. 6-30). This movement brings bound ATP closer to a molecule of glucose also bound to the enzyme and blocks the access of water (from the solvent), which might otherwise enter the active site and attack (hydrolyze) the phosphoanhydride bonds of ATP. Like the other nine enzymes of glycolysis, hexokinase is a soluble, cytosolic protein.

Hexokinase is present in nearly all organisms. The human genome encodes four different hexokinases (I to IV), all of which catalyze the same reaction, but differ in kinetics, regulation, and location. Two or more enzymes that catalyze the same reaction but are encoded by different genes are called **isozymes** (see Box 14-3). One of the isozymes present in hepatocytes, hexokinase IV (also called glucokinase), differs from other forms of hexokinase in kinetic and regulatory properties, with important physiological consequences that are described in Section 14.5.

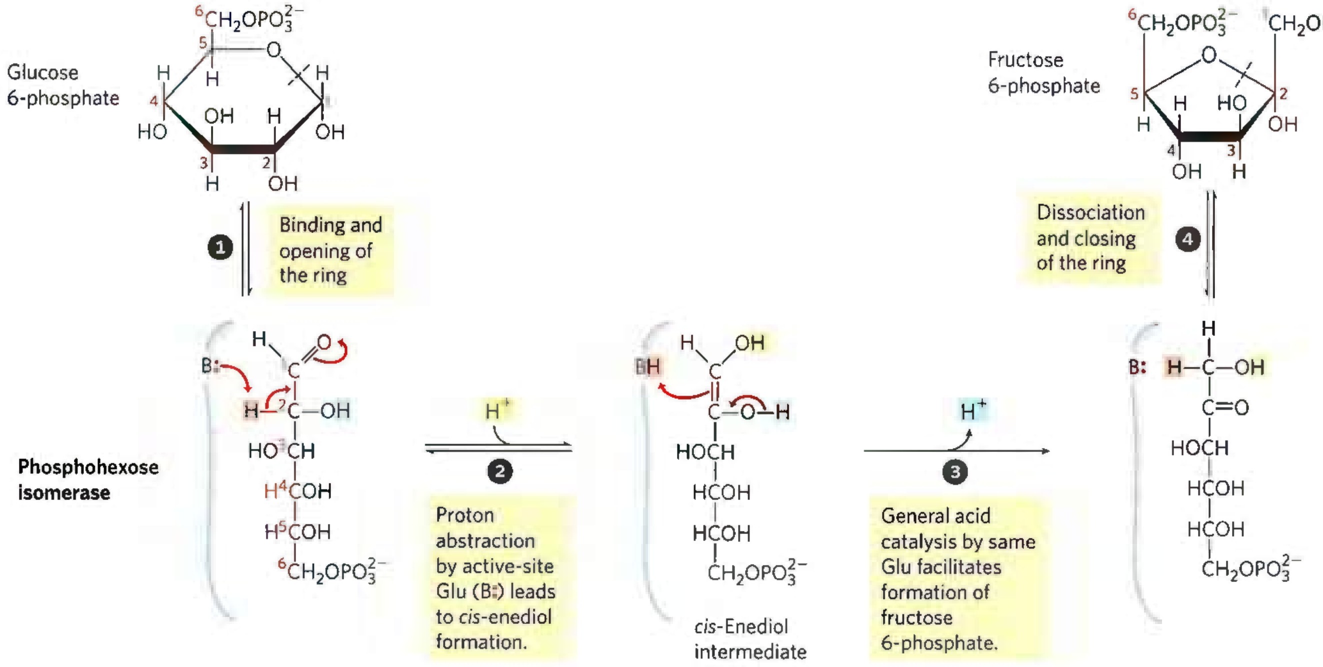
② Conversion of Glucose 6-Phosphate to Fructose 6-Phosphate

The enzyme **phosphohexose isomerase (phosphoglucose isomerase)** catalyzes the reversible isomerization of glucose 6-phosphate, an aldose, to **fructose 6-phosphate**, a ketose:



$\Delta G'^{\circ} = 1.7$ kJ/mol

The mechanism for this reaction involves an enediol intermediate (**Fig. 14-4**). The reaction proceeds readily in



① Binding and opening of the ring

② Proton abstraction by active-site Glu (B:) leads to *cis*-enediol formation.

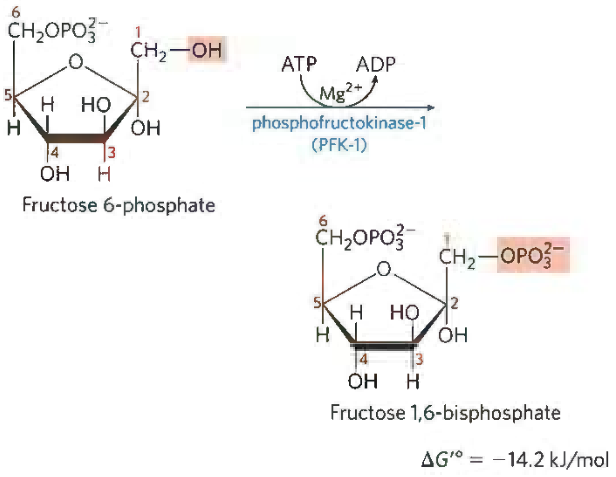
③ General acid catalysis by same Glu facilitates formation of fructose 6-phosphate.

④ Dissociation and closing of the ring

MECHANISM FIGURE 14-4 The phosphohexose isomerase reaction. The ring opening and closing reactions (steps ① and ④) are catalyzed by an active-site His residue, by mechanisms omitted here for simplicity. The proton (light red) initially at C-2 is made more easily abstractable by electron withdrawal by the adjacent carbonyl and nearby hydroxyl groups. After its transfer from C-2 to the active-site Glu residue (a weak acid), the proton is freely exchanged with the surrounding solution; that is, the proton abstracted from C-2 in step ② is not necessarily the same one that is added to C-1 in step ③.

either direction, as might be expected from the relatively small change in standard free energy.

❸ **Phosphorylation of Fructose 6-Phosphate to Fructose 1,6-Bisphosphate** In the second of the two priming reactions of glycolysis, **phosphofructokinase-1 (PFK-1)** catalyzes the transfer of a phosphoryl group from ATP to fructose 6-phosphate to yield **fructose 1,6-bisphosphate**:


ATP
ADP
Mg^{2+}
phosphofructokinase-1 (PFK-1)
Fructose 6-phosphate
Fructose 1,6-bisphosphate
$\Delta G'^\circ = -14.2$ kJ/mol

KEY CONVENTION Compounds that contain two phosphate or phosphoryl groups attached at different positions in the molecule are named *bisphosphates* (or *bisphospho* compounds); for example, fructose 1,6-bisphosphate and 1,3-bisphosphoglycerate. Compounds with two phosphates linked together as a pyrophosphoryl group are named *diphosphates*; for example, adenosine diphosphate (ADP). Similar rules apply for the naming of *trisphosphates* (such as inositol 1,4,5-trisphosphate; see p. 425) and *triphosphates* (such as adenosine triphosphate, ATP). ■

The enzyme that forms fructose 1,6-bisphosphate is called PFK-1 to distinguish it from a second enzyme (PFK-2) that catalyzes the formation of fructose 2,6-bisphosphate from fructose 6-phosphate in a separate pathway (the roles of PFK-2 and fructose 2,6-bisphosphate are discussed in Section 14.5). The PFK-1 reaction is essentially irreversible under cellular conditions, and it is the first "committed" step in the glycolytic pathway; glucose 6-phosphate and fructose 6-phosphate have other possible fates, but fructose 1,6-bisphosphate is targeted for glycolysis.

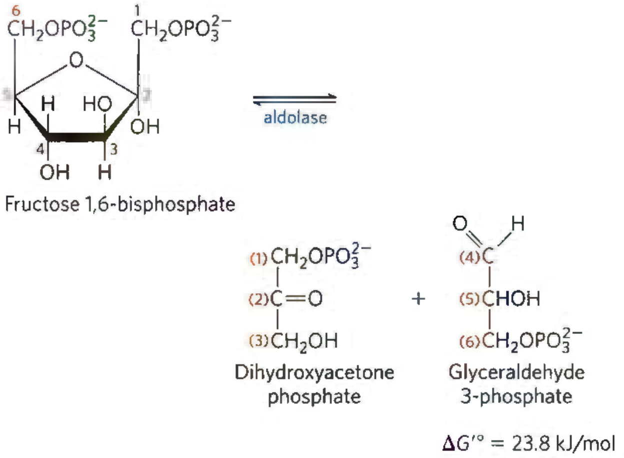
Phosphofructokinase-1 is subject to complex allosteric regulation; its activity is increased whenever the cell's ATP supply is depleted or when the ATP breakdown products, ADP and AMP (particularly the latter), accumulate. The enzyme is inhibited whenever the cell has ample ATP and is well supplied by other fuels such as fatty acids. In some organisms, fructose 2,6-bisphosphate (not to be confused with the PFK-1 reaction product, fructose 1,6-bisphosphate) is a potent allosteric activator of PFK-1. Ribulose 5-phosphate, an intermediate in the pentose phosphate pathway discussed in Section 14.6, also activates phosphofructokinase indirectly. The multiple layers of regulation of this step in glycolysis are discussed in greater detail in Section 14.5.

Some bacteria and protists and perhaps all plants have a different phosphofructokinase (PP-PFK-1) that uses pyrophosphate (PP_i), not ATP, as the phosphoryl group donor in the synthesis of fructose 1,6-bisphosphate:

$$\text{Fructose 6-phosphate} + PP_i \xrightarrow{Mg^{2+}} \text{fructose 1,6-bisphosphate} + P_i \qquad \Delta G'^\circ = -2.9 \text{ kJ/mol}$$

We will discuss this enzyme in Chapter 20.

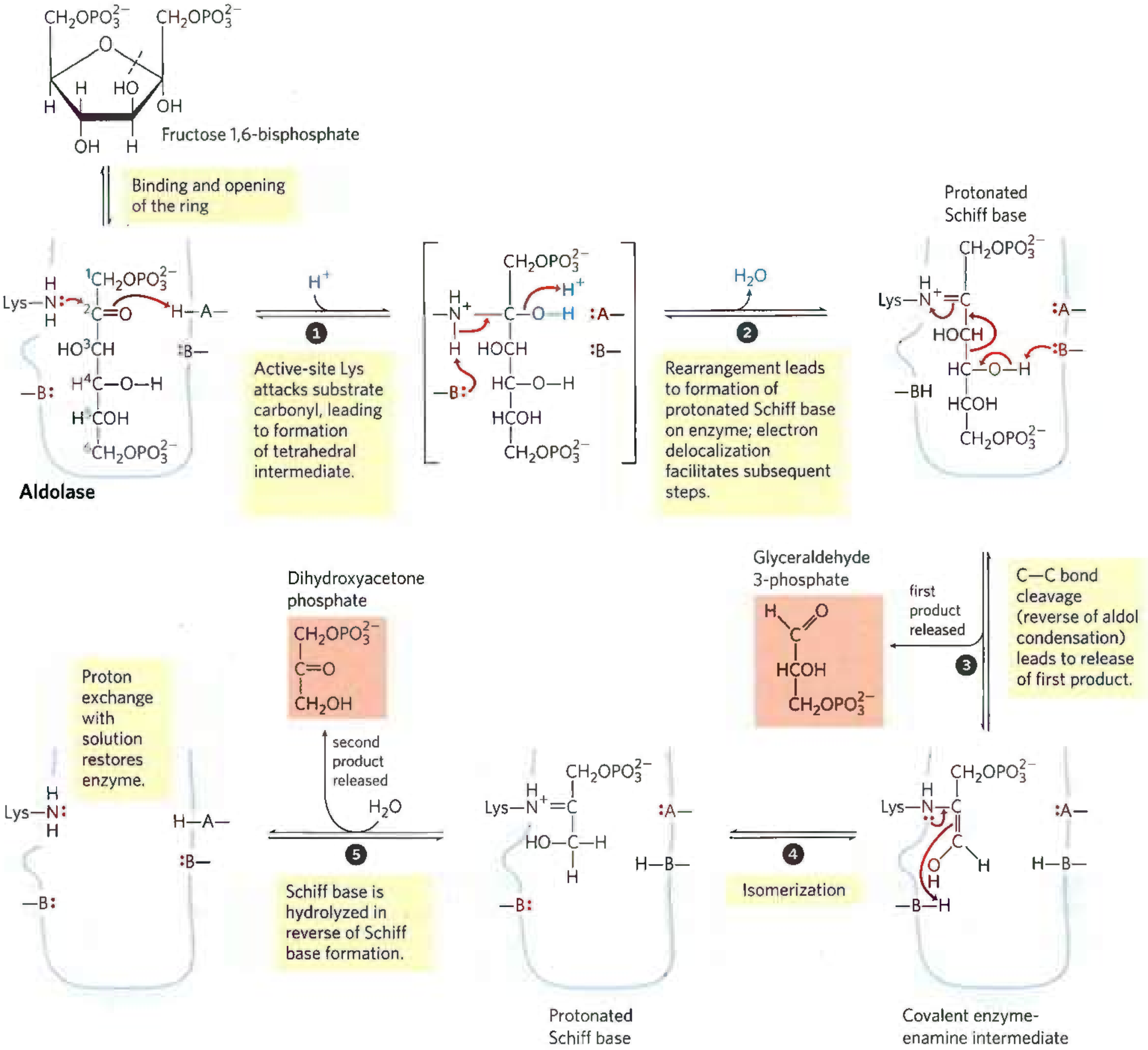
❹ **Cleavage of Fructose 1,6-Bisphosphate** The enzyme **fructose 1,6-bisphosphate aldolase**, often called simply **aldolase**, catalyzes a reverse aldol condensation (**Fig. 14-5**; see Fig. 13-4). Fructose 1,6-bisphosphate is cleaved to yield two different triose phosphates, namely, **glyceraldehyde 3-phosphate** (an aldose) and **dihydroxyacetone phosphate** (a ketose):


Fructose 1,6-bisphosphate
aldolase
Dihydroxyacetone phosphate
Glyceraldehyde 3-phosphate
$\Delta G'^\circ = 23.8$ kJ/mol

There are two classes of aldolases. Class I aldolases, found in animals and plants, use the mechanism shown in Figure 14-5. Class II enzymes, in fungi and bacteria, do not form the Schiff base intermediate. Instead, a zinc ion at the active site is coordinated with the carbonyl oxygen at C-2; the Zn^{2+} polarizes the carbonyl group and stabilizes the enolate intermediate created in the C—C bond cleavage step (see Fig. 6-23).

Although the aldolase reaction has a strongly positive standard free-energy change in the direction of fructose 1,6-bisphosphate cleavage, at the lower concentrations of reactants present in cells the actual free-energy change is small and the aldolase reaction is readily reversible. We shall see later that aldolase acts in the reverse direction during the process of gluconeogenesis.

❺ **Interconversion of the Triose Phosphates** Only one of the two triose phosphates formed by aldolase, glyceraldehyde 3-phosphate, can be directly degraded in the subsequent


Fructose 1,6-bisphosphate
Aldolase
Lys—N:
H—A—
:B—
—B:
H⁺
1
H₂O
2
Protonated Schiff base
—BH
Glyceraldehyde 3-phosphate
first product released
3
4
Isomerization
Covalent enzyme-enamine intermediate
Dihydroxyacetone phosphate
second product released
5
Proton exchange with solution restores enzyme.

Binding and opening of the ring

Active-site Lys attacks substrate carbonyl, leading to formation of tetrahedral intermediate.

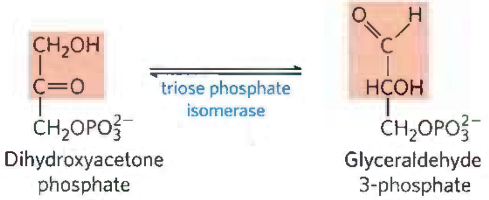
Rearrangement leads to formation of protonated Schiff base on enzyme; electron delocalization facilitates subsequent steps.

C—C bond cleavage (reverse of aldol condensation) leads to release of first product.

Schiff base is hydrolyzed in reverse of Schiff base formation.

MECHANISM FIGURE 14-5 The class I aldolase reaction. Note that cleavage between C-3 and C-4 depends on the presence of the carbonyl group at C-2, which is converted to an imine on the enzyme. A and B represent amino acid residues that serve as general acid (A) or base (B).

steps of glycolysis. The other product, dihydroxyacetone phosphate, is immediately and reversibly converted to glyceraldehyde 3-phosphate by the fifth enzyme of the glycolytic sequence, **triose phosphate isomerase**:


Dihydroxyacetone phosphate
triose phosphate isomerase
Glyceraldehyde 3-phosphate

$\Delta G'^{\circ} = 7.5\ \mathrm{kJ/mol}$

The reaction mechanism is similar to the reaction promoted by phosphohexose isomerase in step ❷ of glycolysis (Fig. 14-4). After the triose phosphate isomerase reaction, the carbon atoms derived from C-1, C-2, and C-3 of the starting glucose are chemically indistinguishable from C-6, C-5, and C-4, respectively (**Fig. 14-6**); both "halves" of glucose have yielded glyceraldehyde 3-phosphate.

This reaction completes the preparatory phase of glycolysis. The hexose molecule has been phosphorylated at C-1 and C-6 and then cleaved to form two molecules of glyceraldehyde 3-phosphate.

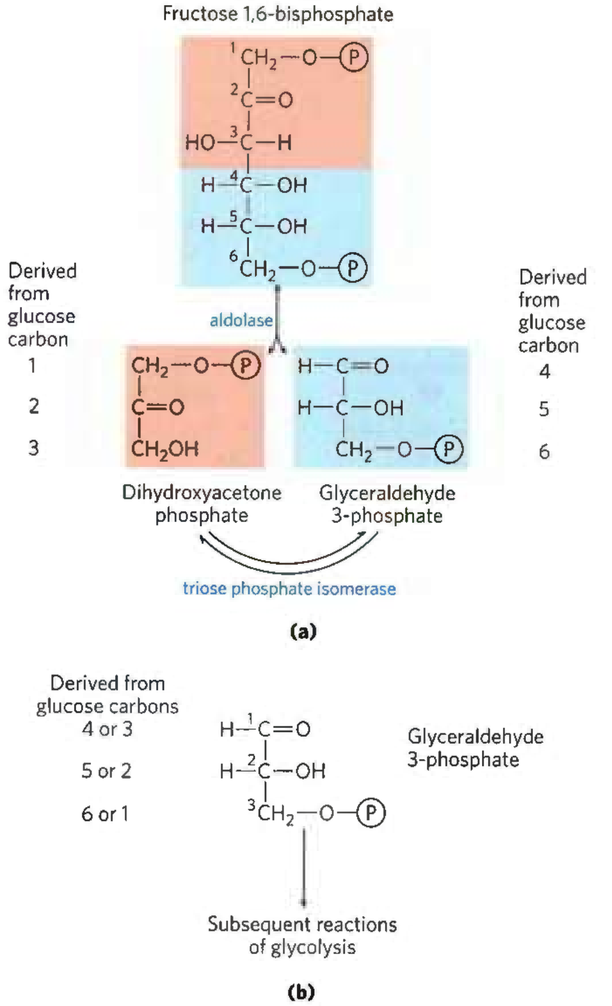
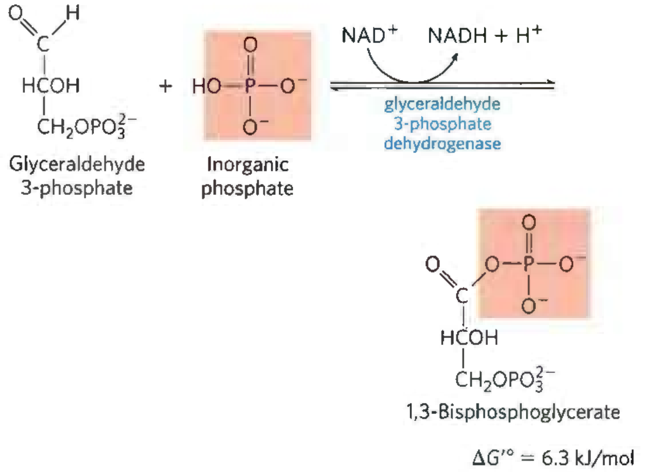

Fructose 1,6-bisphosphate
aldolase
Derived from glucose carbon
1
2
3
Derived from glucose carbon
4
5
6
Dihydroxyacetone phosphate
Glyceraldehyde 3-phosphate
triose phosphate isomerase
(a)
Derived from glucose carbons
4 or 3
5 or 2
6 or 1
Glyceraldehyde 3-phosphate
Subsequent reactions of glycolysis
(b)

FIGURE 14-6 Fate of the glucose carbons in the formation of glyceraldehyde 3-phosphate. (a) The origin of the carbons in the two three-carbon products of the aldolase and triose phosphate isomerase reactions. The end product of the two reactions is glyceraldehyde 3-phosphate (two molecules). (b) Each carbon of glyceraldehyde 3-phosphate is derived from either of two specific carbons of glucose. Note that the numbering of the carbon atoms of glyceraldehyde 3-phosphate differs from that of the glucose from which it is derived. In glyceraldehyde 3-phosphate, the most complex functional group (the carbonyl) is specified as C-1. This numbering change is important for interpreting experiments with glucose in which a single carbon is labeled with a radioisotope. (See Problems 5 and 22 at the end of this chapter.)

The Payoff Phase of Glycolysis Yields ATP and NADH

The payoff phase of glycolysis (Fig. 14-2b) includes the energy-conserving phosphorylation steps in which some of the chemical energy of the glucose molecule is conserved in the form of ATP and NADH. Remember that one molecule of glucose yields two molecules of glyceraldehyde 3-phosphate, and both halves of the glucose molecule follow the same pathway in the second phase of glycolysis. The conversion of two molecules of glyceraldehyde 3-phosphate to two molecules of pyruvate is accompanied by the formation of four molecules of ATP from ADP. However, the net yield of ATP per molecule of glucose degraded is only two, because two ATP were invested in the preparatory phase of glycolysis to phosphorylate the two ends of the hexose molecule.

❻ Oxidation of Glyceraldehyde 3-Phosphate to 1,3-Bisphosphoglycerate P1 The first step in the payoff phase is the oxidation of glyceraldehyde 3-phosphate to **1,3-bisphosphoglycerate**, catalyzed by **glyceraldehyde 3-phosphate dehydrogenase**:

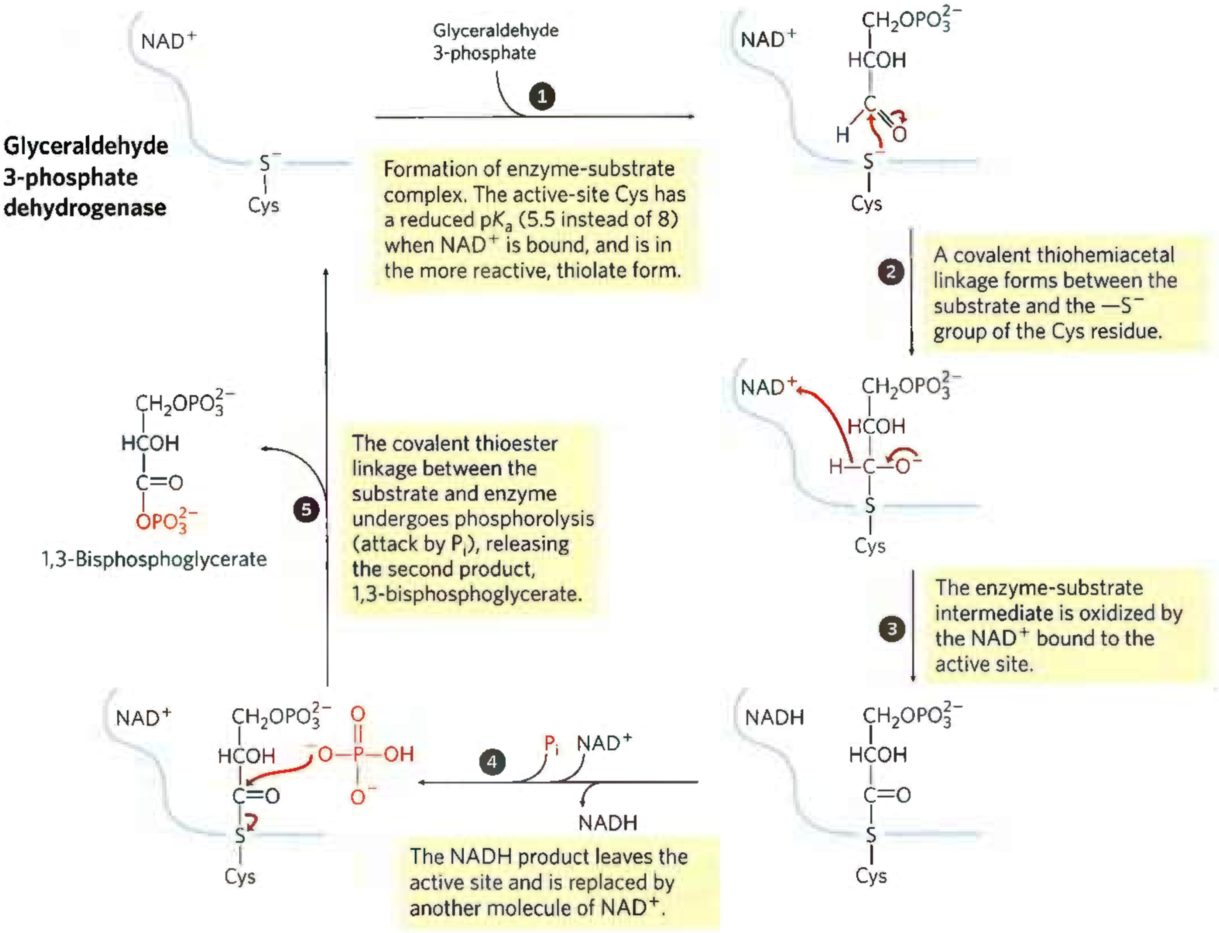

Glyceraldehyde 3-phosphate
Inorganic phosphate
NAD$^+$
NADH + H$^+$
glyceraldehyde 3-phosphate dehydrogenase
1,3-Bisphosphoglycerate
$\Delta G'^\circ = 6.3$ kJ/mol

This is the first of the two energy-conserving reactions of glycolysis that eventually lead to the formation of ATP. The aldehyde group of glyceraldehyde 3-phosphate is oxidized, not to a free carboxyl group but to a carboxylic acid anhydride with phosphoric acid. This type of anhydride, called an **acyl phosphate**, has a very high standard free energy of hydrolysis ($\Delta G'^\circ = -49.3$ kJ/mol; see Table 13-6). Much of the free energy of oxidation of the aldehyde group of glyceraldehyde 3-phosphate is conserved by formation of the acyl phosphate group at C-1 of 1,3-bisphosphoglycerate.

Glyceraldehyde 3-phosphate is covalently bound to the dehydrogenase during the reaction (**Fig. 14-7**). The aldehyde group of glyceraldehyde 3-phosphate reacts with the —SH group of an essential Cys residue in the active site, in a reaction analogous to the formation of a hemiacetal (see Fig. 7-5), in this case producing a *thio*hemiacetal. Reaction of the essential Cys residue with a heavy metal such as Hg^{2+} irreversibly inhibits the enzyme.

The amount of NAD$^+$ in a cell ($\leq 10^{-5}$ M) is far smaller than the amount of glucose metabolized in a few minutes. Glycolysis would soon come to a halt if the NADH formed in this step of glycolysis were not continuously reoxidized and recycled. We return to a discussion of this recycling of NAD$^+$ later in the chapter.

❼ Phosphoryl Transfer from 1,3-Bisphosphoglycerate to ADP The enzyme **phosphoglycerate kinase** transfers the high-energy phosphoryl group from the carboxyl


Glyceraldehyde 3-phosphate dehydrogenase
NAD+
S−
Cys
Glyceraldehyde 3-phosphate
1
CH2OPO3 2−
HCOH
C
H
O
2
3
4
5
H–C–O−
S
NADH
C=O
Pi
NAD+
O–P–OH
O−
1,3-Bisphosphoglycerate
OPO3 2−

Formation of enzyme-substrate complex. The active-site Cys has a reduced pK_a (5.5 instead of 8) when NAD^+ is bound, and is in the more reactive, thiolate form.

A covalent thiohemiacetal linkage forms between the substrate and the $—S^-$ group of the Cys residue.

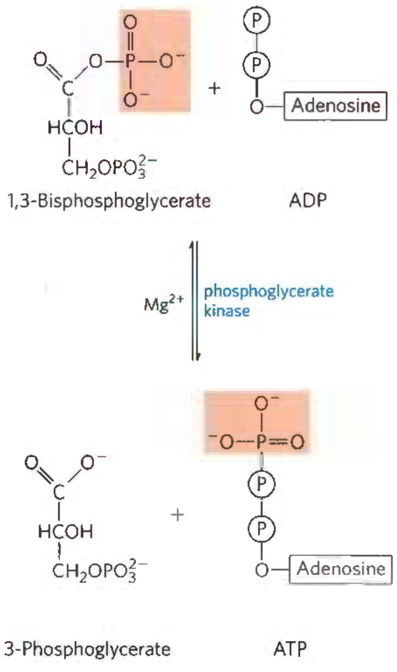
The enzyme-substrate intermediate is oxidized by the NAD^+ bound to the active site.

The NADH product leaves the active site and is replaced by another molecule of NAD^+.

The covalent thioester linkage between the substrate and enzyme undergoes phosphorolysis (attack by P_i), releasing the second product, 1,3-bisphosphoglycerate.

MECHANISM FIGURE 14-7 **The glyceraldehyde 3-phosphate dehydrogenase reaction.**

group of 1,3-bisphosphoglycerate to ADP, forming ATP and **3-phosphoglycerate**:


1,3-Bisphosphoglycerate
ADP
Adenosine
P
Mg2+
phosphoglycerate kinase
3-Phosphoglycerate
ATP
HCOH
CH2OPO3 2−

$\Delta G'^\circ = -18.8$ kJ/mol

Notice that phosphoglycerate kinase is named for the reverse reaction, in which it transfers a phosphoryl group from ATP to 3-phosphoglycerate. Like all enzymes, it catalyzes the reaction in both directions. This enzyme acts in the direction suggested by its name during gluconeogenesis (see Fig. 14-16) and during photosynthetic CO_2 assimilation (see Fig. 20-26). In glycolysis, the reaction it catalyzes proceeds as shown above, in the direction of ATP synthesis.

Steps ❻ and ❼ of glycolysis together constitute an energy-coupling process in which 1,3-bisphosphoglycerate is the common intermediate; it is formed in the first reaction (which would be endergonic in isolation), and its acyl phosphate group is transferred to ADP in the second reaction (which is strongly exergonic). The sum of these two reactions is

$$\text{Glyceraldehyde 3-phosphate} + \text{ADP} + P_i + \text{NAD}^+ \rightleftharpoons \text{3-phosphoglycerate} + \text{ATP} + \text{NADH} + \text{H}^+$$

$$\Delta G'^\circ = -12.2 \text{ kJ/mol}$$

Thus the overall reaction is exergonic.

Recall from Chapter 13 that the actual free-energy change, ΔG, is determined by the standard free-energy change, $\Delta G'^\circ$, and the mass-action ratio, Q, which is the ratio [products]/[reactants] (see Eqn 13-4). For step ⑥,

$$\Delta G = \Delta G'^\circ + RT \ln Q$$

$$= \Delta G'^\circ + RT \ln \frac{[\text{1,3-bisphosphoglycerate}][\text{NADH}]}{[\text{glyceraldehyde 3-phosphate}][\text{P}_\text{i}][\text{NAD}^+]}$$

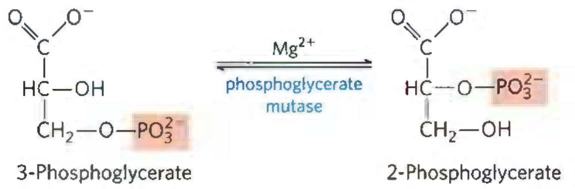
Notice that $[\text{H}^+]$ is not included in Q. In biochemical calculations, $[\text{H}^+]$ is assumed to be a constant (10^{-7} M), and this constant is included in the definition of $\Delta G'^\circ$ (p. 468).

When the mass-action ratio is less than 1.0, its natural logarithm has a negative sign. In the cytosol, where these reactions are taking place, the ratio $[\text{NADH}]/[\text{NAD}^+]$ is a small fraction, contributing to a low Q. Step ⑦, by consuming the product of step ⑥ (1,3-bisphosphoglycerate), keeps [1,3-bisphosphoglycerate] relatively low in the steady state and thereby keeps Q for the overall energy-coupling process small. When Q is small, the contribution of $\ln Q$ can make ΔG strongly negative. This is simply another way of showing how the two reactions, steps ⑥ and ⑦, are coupled through a common intermediate.

The outcome of these coupled reactions, both reversible under cellular conditions, is that the energy released on oxidation of an aldehyde to a carboxylate group is conserved by the coupled formation of ATP from ADP and P_i. The formation of ATP by phosphoryl group transfer from a substrate such as 1,3-bisphosphoglycerate is referred to as a **substrate-level phosphorylation**, to distinguish this mechanism from **respiration-linked phosphorylation**. Substrate-level phosphorylations involve soluble enzymes and chemical intermediates (1,3-bisphosphoglycerate in this case). Respiration-linked phosphorylations, on the other hand, involve membrane-bound enzymes and transmembrane gradients of protons (Chapter 19).

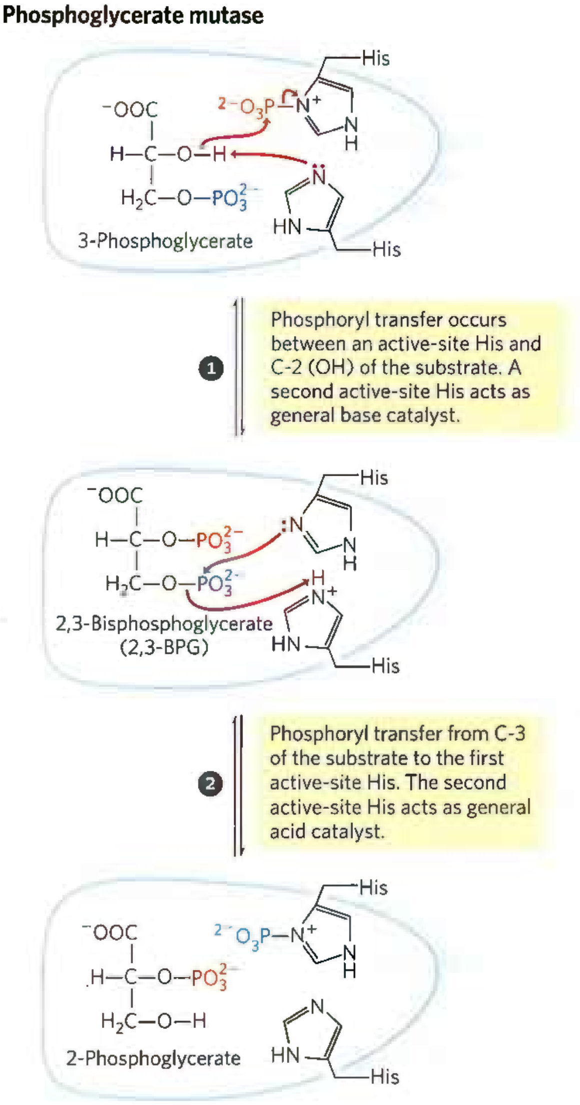
⑧ Conversion of 3-Phosphoglycerate to 2-Phosphoglycerate

The enzyme **phosphoglycerate mutase** catalyzes a reversible shift of the phosphoryl group between C-2 and C-3 of glycerate; Mg^{2+} is essential for this reaction:



$\Delta G'^\circ = 4.4$ kJ/mol

The reaction occurs in two steps (**Fig. 14-8**). A phosphoryl group initially attached to a His residue of the mutase is transferred to the hydroxyl group at C-2 of 3-phosphoglycerate, forming 2,3-bisphosphoglycerate (2,3-BPG). The phosphoryl group at C-3 of 2,3-BPG is then transferred to the same His residue, producing 2-phosphoglycerate and regenerating the phosphorylated enzyme. Phosphoglycerate mutase is initially phosphorylated by phosphoryl transfer from 2,3-BPG, which is required in small quantities to initiate the catalytic cycle and is continuously regenerated by that cycle.



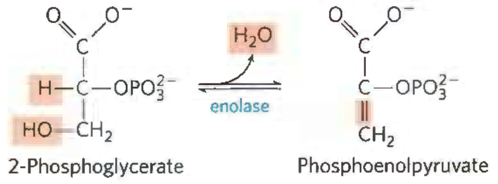
Phosphoryl transfer occurs between an active-site His and C-2 (OH) of the substrate. A second active-site His acts as general base catalyst.

Phosphoryl transfer from C-3 of the substrate to the first active-site His. The second active-site His acts as general acid catalyst.

MECHANISM FIGURE 14-8 The phosphoglycerate mutase reaction.

⑨ Dehydration of 2-Phosphoglycerate to Phosphoenolpyruvate

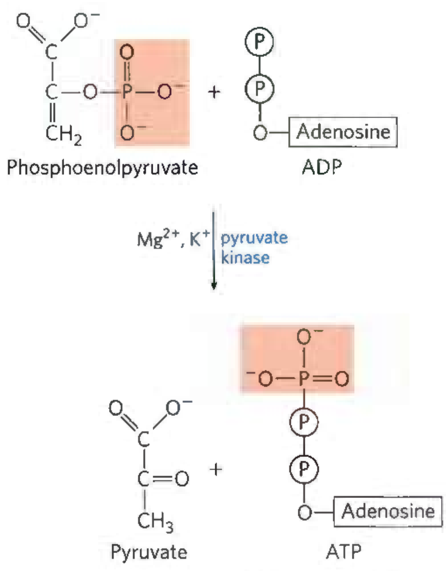
P1 In the second glycolytic reaction that generates a compound with high phosphoryl group transfer potential (the first was step ⑥), **enolase** promotes reversible removal of a molecule of water from 2-phosphoglycerate to yield **phosphoenolpyruvate (PEP)**:



$\Delta G'^\circ = 7.5$ kJ/mol

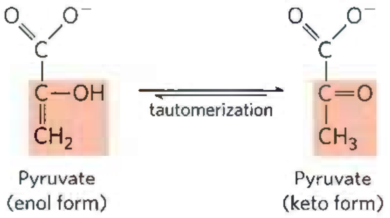
The mechanism of the enolase reaction involves an enolic intermediate stabilized by Mg^{2+} (see Fig. 6-31). The reaction converts a compound with a relatively low phosphoryl group transfer potential ($\Delta G'^\circ$ for hydrolysis of 2-phosphoglycerate is −17.6 kJ/mol) to one with high phosphoryl group transfer potential ($\Delta G'^\circ$ for PEP hydrolysis is −61.9 kJ/mol) (see Fig. 13-13).

⑩ **Transfer of the Phosphoryl Group from Phosphoenolpyruvate to ADP** The last step in glycolysis is the transfer of the phosphoryl group from phosphoenolpyruvate to ADP, catalyzed by **pyruvate kinase**, which requires K^+ and either Mg^{2+} or Mn^{2+}:



$\Delta G'^\circ = -31.4$ kJ/mol

In this substrate-level phosphorylation, the product **pyruvate** first appears in its enol form, then tautomerizes nonenzymatically to its keto form, which predominates at pH 7:



The overall reaction has a large, negative standard free-energy change, due in large part to the spontaneous conversion of the enol form of pyruvate to the keto form (see Fig. 13-13). About half of the energy released by PEP hydrolysis ($\Delta G'^\circ = -61.9$ kJ/mol) is conserved in the formation of the phosphoanhydride bond of ATP ($\Delta G'^\circ = -30.5$ kJ/mol), and the rest (−31.4 kJ/mol) constitutes a large driving force pushing the reaction toward ATP synthesis. We discuss the regulation of pyruvate kinase in Section 14.5.

The Overall Balance Sheet Shows a Net Gain of Two ATP and Two NADH Per Glucose

P1 We can now construct a balance sheet for glycolysis to account for (1) the fate of the carbon skeleton of glucose, (2) the input of P_i and ADP and output of ATP, and (3) the pathway of electrons in the oxidation-reduction reactions. The left side of the following equation shows all the inputs of ATP, NAD^+, ADP, and P_i (consult Fig. 14-2), and the right side shows all the outputs (keep in mind that each molecule of glucose yields two molecules of pyruvate):

$$\text{Glucose} + 2\text{ATP} + 2\text{NAD}^+ + 4\text{ADP} + 2\text{P}_i \longrightarrow 2\text{ pyruvate} + 2\text{ADP} + 2\text{NADH} + 2\text{H}^+ + 4\text{ATP} + 2\text{H}_2\text{O}$$

Canceling out common terms on both sides of the equation gives the overall equation for glycolysis:

$$\text{Glucose} + 2\text{NAD}^+ + 2\text{ADP} + 2\text{P}_i \longrightarrow 2\text{ pyruvate} + 2\text{NADH} + 2\text{H}^+ + 2\text{ATP} + 2\text{H}_2\text{O}$$

In the overall glycolytic process, one molecule of glucose is converted to two molecules of pyruvate (the pathway of carbon). Two molecules of ADP and two of P_i are converted to two molecules of ATP (the pathway of phosphoryl groups). Four electrons, as two hydride ions, are transferred from two molecules of glyceraldehyde 3-phosphate to two of NAD^+ (the pathway of electrons).

SUMMARY 14.1 ***Glycolysis***

- Glycolysis is a near-universal pathway by which a glucose molecule is oxidized, in two phases, to two molecules of pyruvate, with energy conserved as ATP and NADH. Ten cytosolic enzymes act sequentially in glycolysis. The overall reaction converts glucose to two molecules of pyruvate, and energy is conserved in the synthesis of two molecules of ATP and two molecules of NADH.
- In the preparatory phase of glycolysis, two molecules of ATP are invested to activate glucose to fructose 1,6-bisphosphate. The bond between C-3 and C-4 is then broken to yield two molecules of triose phosphate.
- In the payoff phase, each of the two molecules of glyceraldehyde 3-phosphate derived from glucose undergoes oxidation at C-1; some of the energy of this oxidation reaction is conserved in the form of one NADH and two ATP per triose phosphate oxidized.
- Subtracting the two ATP spent in the preparatory phase, the net equation for the overall process is

$$\text{Glucose} + 2\text{NAD}^+ + 2\text{ADP} + 2\text{P}_i \longrightarrow 2\text{ pyruvate} + 2\text{NADH} + 2\text{H}^+ + 2\text{ATP} + 2\text{H}_2\text{O}$$

14.2 Feeder Pathways for Glycolysis

Many carbohydrates besides glucose meet their catabolic fate in glycolysis, after being transformed into one of the glycolytic intermediates. The most significant are the storage polysaccharides glycogen and starch, either within cells (endogenous) or obtained in the diet; the disaccharides maltose, lactose, and sucrose; and the monosaccharides fructose, mannose, and galactose (**Fig. 14-9**).

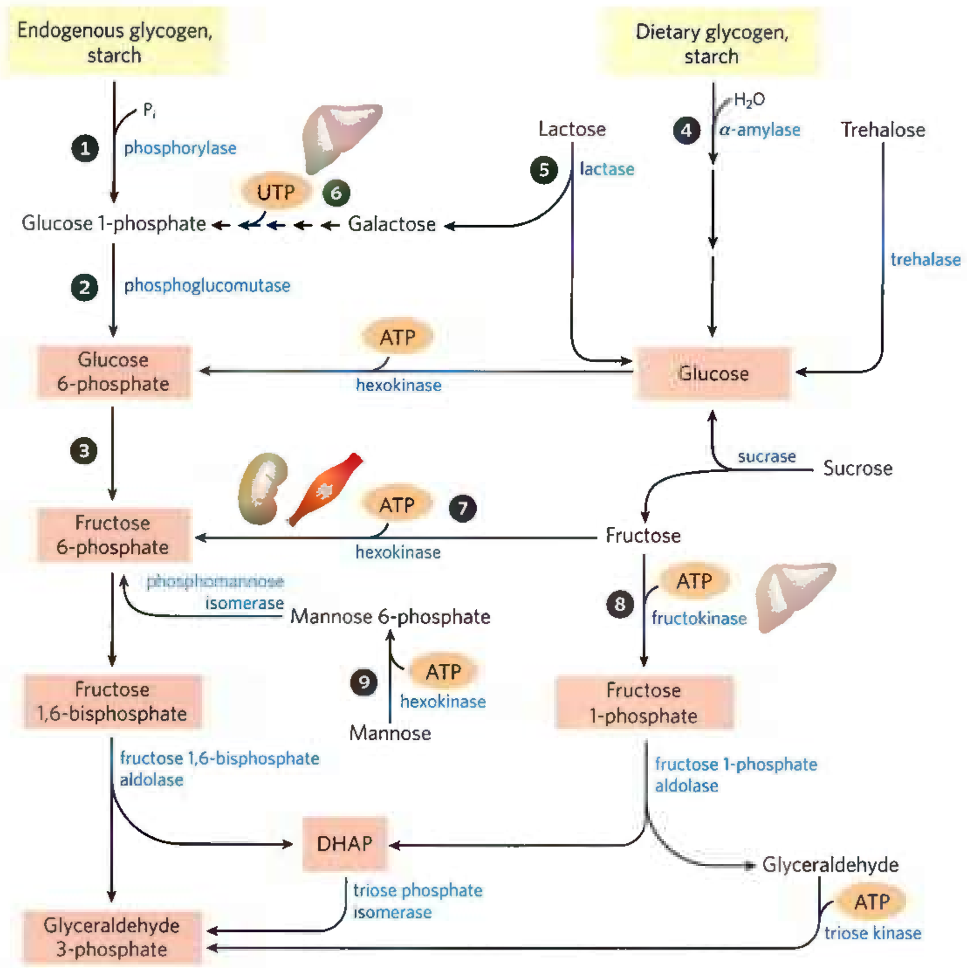

Endogenous glycogen, starch
Dietary glycogen, starch
P_i
❶ phosphorylase
H_2O
Lactose
❹ α-amylase
Trehalose
❺ lactase
UTP ❻
Glucose 1-phosphate
Galactose
trehalase
❷ phosphoglucomutase
ATP
Glucose 6-phosphate
hexokinase
Glucose
❸
sucrase
Sucrose
ATP ❼
Fructose 6-phosphate
hexokinase
Fructose
phosphomannose isomerase
Mannose 6-phosphate
ATP
❽ fructokinase
ATP
❾ hexokinase
Fructose 1,6-bisphosphate
Mannose
Fructose 1-phosphate
fructose 1,6-bisphosphate aldolase
fructose 1-phosphate aldolase
DHAP
Glyceraldehyde
triose phosphate isomerase
ATP
triose kinase
Glyceraldehyde 3-phosphate

FIGURE 14-9 **Entry of dietary glycogen, starch, disaccharides, and hexoses into the preparatory stage of glycolysis.** The numbered steps are described in the text.

Endogenous Glycogen and Starch Are Degraded by Phosphorolysis

P2 Glycogen stored in animal tissues (primarily liver and skeletal muscle) and in microorganisms is mobilized for use within the same cell by a *phosphorolytic* reaction (❶ in Fig. 14-9) catalyzed by **glycogen phosphorylase**. The product of this reaction is not free glucose, but glucose 1-phosphate. We discuss glycogen metabolism in more detail in Chapter 15. In plant tissues, starch is mobilized by a similar *phosphorolytic* reaction catalyzed by **starch phosphorylase**.

Glucose 1-phosphate produced by glycogen phosphorylase is converted to glucose 6-phosphate by **phosphoglucomutase** ❷, which catalyzes the reversible reaction

$$\text{Glucose 1-phosphate} \rightleftharpoons \text{glucose 6-phosphate}$$

Phosphoglucomutase employs essentially the same mechanism as phosphoglycerate mutase (Fig. 14-8): both entail a bisphosphate intermediate, and the enzyme is transiently phosphorylated in each catalytic cycle. The general name **mutase** is given to enzymes that catalyze the transfer of a functional group from one position to another in the same molecule. Mutases are a subclass of **isomerases**, enzymes that interconvert stereoisomers or structural or positional isomers (see Table 6-3). The glucose 6-phosphate formed in the phosphoglucomutase reaction can continue through glycolysis ❸ or enter another pathway such as the pentose phosphate pathway, described in Section 14.6.

WORKED EXAMPLE 14-1 *Energy Savings for Glycogen Breakdown by Phosphorolysis*

Calculate the energy savings (in ATP molecules per glucose monomer) achieved by breaking down glycogen by *phosphorolysis* rather than *hydrolysis* to begin the process of glycolysis.

SOLUTION: Phosphorolysis produces a phosphorylated glucose (glucose 1-phosphate), which is then converted to glucose 6-phosphate — without expenditure of the cellular energy (1 ATP) needed for formation of glucose 6-phosphate from free glucose. Thus only 1 ATP is consumed per glucose monomer in the preparatory phase, compared with 2 ATP when glycolysis starts with free glucose. The cell therefore gains 3 ATP per glucose monomer (4 ATP produced in the payoff phase minus 1 ATP used in the preparatory phase), rather than 2 — a savings of 1 ATP per glucose monomer.

Dietary Polysaccharides and Disaccharides Undergo Hydrolysis to Monosaccharides

For most humans, starch is the major source of carbohydrates in the diet (④, Fig. 14-9). Dietary starch has essentially the same structure as glycogen, and its digestion proceeds by the same pathway. Digestion begins in the mouth, where salivary **α-amylase** hydrolyzes the internal (α1→4) glycosidic linkages of starch and glycogen, producing di- and trisaccharides. These are produced by *hydrolysis* reactions, in which water, not P_i, is the attacking species. In the stomach, salivary α-amylase is inactivated by the low pH, but a second form of α-amylase, secreted by the pancreas into the small intestine, continues the digestion process.

Pancreatic α-amylase release into the small intestine yields mainly maltose and maltotriose (the di- and trisaccharides of glucose) and oligosaccharides called limit **dextrins**, fragments of amylopectin containing (α1→6) branch points, which are removed by limit **dextrinases**. Disaccharides are hydrolyzed by a family of membrane-bound hydrolases in the intestinal brush border:

$$\text{Dextrin} + n\text{H}_2\text{O} \xrightarrow[\text{dextrinase}]{} n \text{ glucose}$$

$$\text{Maltose} + \text{H}_2\text{O} \xrightarrow[\text{maltase}]{} 2 \text{ glucose}$$

$$\text{Lactose} + \text{H}_2\text{O} \xrightarrow[\text{lactose}]{} \text{galactose} + \text{glucose}$$

$$\text{Sucrose} + \text{H}_2\text{O} \xrightarrow[\text{sucrase}]{} \text{fructose} + \text{glucose}$$

$$\text{Trehalose} + \text{H}_2\text{O} \xrightarrow[\text{trehalase}]{} 2 \text{ glucose}$$

Only monosaccharides are taken up from the intestine. They are actively transported into the intestinal epithelial cells (see Fig. 11-42), then passed into the blood to be carried to various tissues, where they are catabolized via glycolysis.

As we noted in Chapter 7, most animals cannot digest cellulose for lack of the enzyme **cellulase**, which attacks the (β1→4) glycosidic bonds of cellulose. In ruminant animals, the extended stomach includes a chamber in which symbiotic microorganisms that produce cellulase break down cellulose into glucose molecules. These microorganisms use the resulting glucose in an anaerobic fermentation that produces large quantities of propionate. This propionate, after conversion to succinate (see Fig. 17-12), serves as the starting material for gluconeogenesis, which produces much of the lactose in milk.

Lactose Digestion and Lactose Intolerance The defining feature of mammals is, of course, mammary glands, which produce the disaccharide lactose for the nourishment of infants. The enzyme lactase converts lactose to glucose and galactose (⑤, Fig. 14-9), both of which are taken up from the small intestine and metabolized in the tissues by glycolysis. As infants are weaned, their lactase levels diminish, and lactase is absent in most adults—except in certain populations. About one in three adults in northern Europe and in some parts of Africa shows the **lactase persistence** phenotype. They continue to produce lactase and thus are able to digest milk into adulthood. The other two-thirds experience **lactose intolerance** due to the disappearance after childhood of most or all of the lactase activity of the intestinal epithelial cells. Without intestinal lactase, lactose cannot be completely digested and absorbed in the small intestine, and it passes into the large intestine, where bacteria convert it to toxic products that cause abdominal cramps and diarrhea. The problem is further complicated because undigested lactose and its metabolites increase the osmolarity of the intestinal contents, favoring retention of water in the intestine, and causing diarrhea. In most parts of the world where lactose intolerance is prevalent, milk is not used as a food by adults, although milk products predigested with lactase are commercially available. In certain human disorders, several or all of the intestinal disaccharidases are missing. In these cases, the digestive disturbances triggered by dietary disaccharides can sometimes be minimized by a controlled diet lacking the undigestible carbohydrates.

One way to determine whether lactase is present and active in the intestine is to compare the rise in blood glucose after the ingestion of a quantity of either glucose or lactose. When glucose is ingested, the blood glucose level increases rapidly and transiently. When lactose is ingested, lactase, if present in the intestine, will hydrolyze the lactose into glucose and galactose and the blood glucose level will rise. If lactase is absent or less active, ingesting lactose will lead to little or no transient increase in blood glucose. ■

Galactose Metabolism and Disease Galactose (⑥, Fig. 14-9), a product of the hydrolysis of lactose and therefore an important component in the diet of infants, passes in the blood from the intestine to the liver, where it is first phosphorylated at C-1, at the expense of ATP, by the enzyme **galactokinase**:

$$\text{Galactose} + \text{ATP} \xrightarrow{\text{Mg}^{2+}} \text{galactose 1-phosphate} + \text{ADP}$$

The galactose 1-phosphate is then transferred to a uridine nucleotide by a transferase. The resulting UDP-galactose is epimerized at C-4, forming UDP-glucose by a set of reactions in which UDP functions as an activator of hexose groups (**Fig. 14-10**) and a "tag" that these hexoses are in a separate pool from those destined for another process such as glycolysis. The epimerization, catalyzed by UDP-glucose 4-epimerase, involves first the oxidation of the C-4 —OH group to a ketone, then reduction of the ketone to an —OH, with inversion of the configuration at C-4. NAD is the cofactor for both the oxidation and the reduction. The glucose 1-phosphate made this way is converted to glucose 6-phosphate by phosphoglucomutase.

A defect in any of the enzymes in this pathway has serious medical consequences. In galactokinase-deficiency **galactosemia**, caused by a defect in the

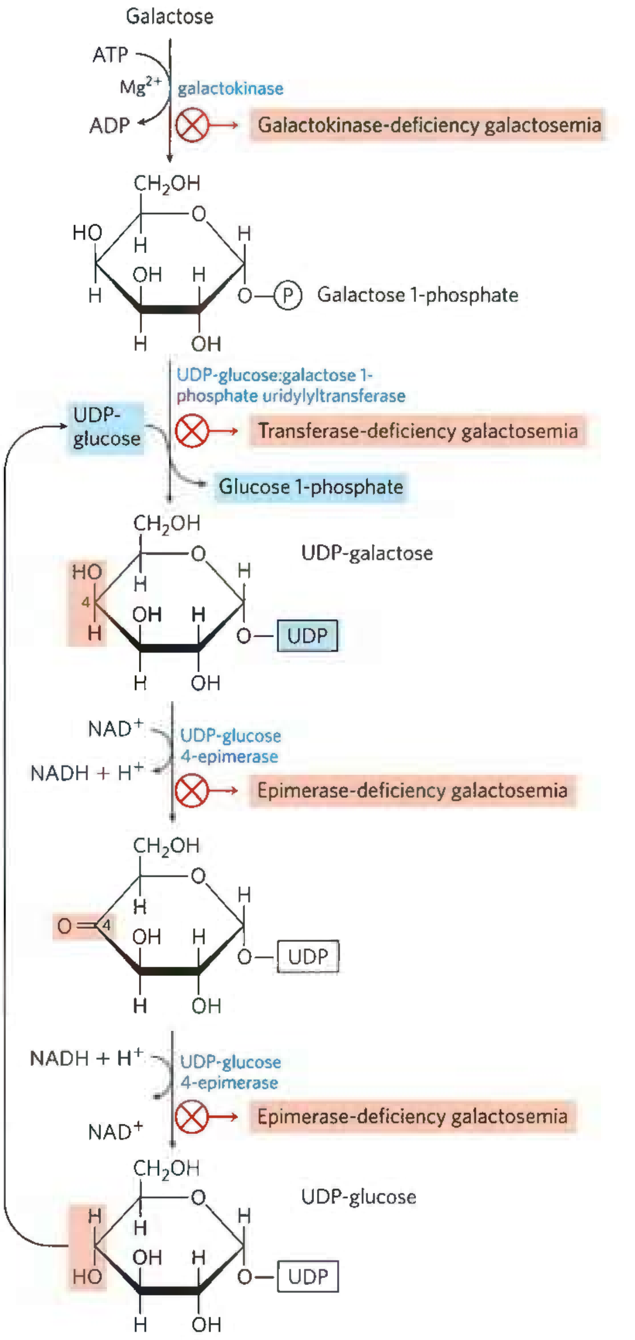
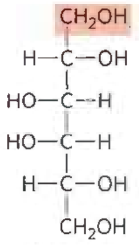

Galactose
ATP
Mg2+
galactokinase
ADP
Galactokinase-deficiency galactosemia
CH2OH
HO
H
OH
Galactose 1-phosphate
UDP-glucose:galactose 1-phosphate uridylyltransferase
UDP-glucose
Transferase-deficiency galactosemia
Glucose 1-phosphate
UDP-galactose
UDP
4
NAD+
NADH + H+
UDP-glucose 4-epimerase
Epimerase-deficiency galactosemia
O
UDP-glucose

FIGURE 14-10 Conversion of galactose to glucose 1-phosphate. The conversion proceeds through a sugar-nucleotide derivative, UDP-galactose, which is formed when galactose 1-phosphate displaces glucose 1-phosphate from UDP-glucose. UDP-galactose is then converted by UDP-glucose 4-epimerase to UDP-glucose, in a reaction that involves oxidation of C-4 (light red) by NAD$^+$, then reduction of C-4 by NADH; the result is inversion of the configuration at C-4. The UDP-glucose is recycled through another round of the same reaction. The net effect of this cycle is the conversion of galactose 1-phosphate to glucose 1-phosphate; there is no net production or consumption of UDP-galactose or UDP-glucose. Defects in the enzymes that catalyze each of these steps result in the various galactosemias shown.

GALK gene, high galactose concentrations are found in blood and urine. Affected individuals develop cataracts in infancy, caused by deposition of the galactose metabolite galactitol in the lens.



D-Galactitol

The other symptoms in this disorder are relatively mild, and strict limitation of galactose in the diet greatly diminishes their severity. Transferase-deficiency galactosemia, caused by a defect in the *GALT* gene, is more serious; it is characterized by poor growth in childhood, speech abnormality, mental deficiency, and liver damage that may be fatal, even when galactose is withheld from the diet. Epimerase-deficiency galactosemia, caused by a defect in the *GALE* gene, leads to similar symptoms, but they are less severe when dietary galactose is carefully controlled. ■

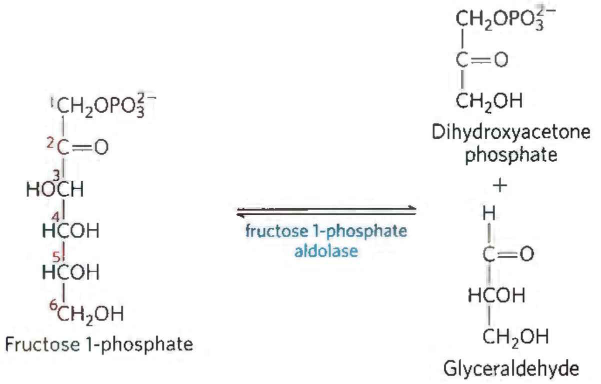
Fructose and Mannose P2 In most organisms, hexoses other than glucose can undergo glycolysis after conversion to a phosphorylated derivative. Fructose, present in free form in many fruits and formed by hydrolysis of sucrose in the small intestine of vertebrates, is phosphorylated by hexokinase:

$$\text{Fructose} + \text{ATP} \xrightarrow{\text{Mg}^{2+}} \text{fructose 6-phosphate} + \text{ADP}$$

This is a major pathway of fructose entry into glycolysis in the muscles and kidney (❼, Fig. 14-9). In the liver, fructose enters by a different pathway. The liver enzyme **fructokinase** catalyzes the phosphorylation of fructose at C-1 rather than C-6 (❽, Fig. 14-9):

$$\text{Fructose} + \text{ATP} \xrightarrow{\text{Mg}^{2+}} \text{fructose 1-phosphate} + \text{ADP}$$

The fructose 1-phosphate is then cleaved to glyceraldehyde and dihydroxyacetone phosphate by **fructose 1-phosphate aldolase**:


Fructose 1-phosphate
fructose 1-phosphate aldolase
Dihydroxyacetone phosphate
+
Glyceraldehyde

Dihydroxyacetone phosphate is converted to glyceraldehyde 3-phosphate by the glycolytic enzyme triose phosphate isomerase. Glyceraldehyde is phosphorylated by ATP and **triose kinase** to glyceraldehyde 3-phosphate:

$$\text{Glyceraldehyde} + \text{ATP} \xrightarrow{\text{Mg}^{2+}} \text{glyceraldehyde 3-phosphate} + \text{ADP}$$

Thus, both products of fructose 1-phosphate hydrolysis enter the glycolytic pathway as glyceraldehyde 3-phosphate.

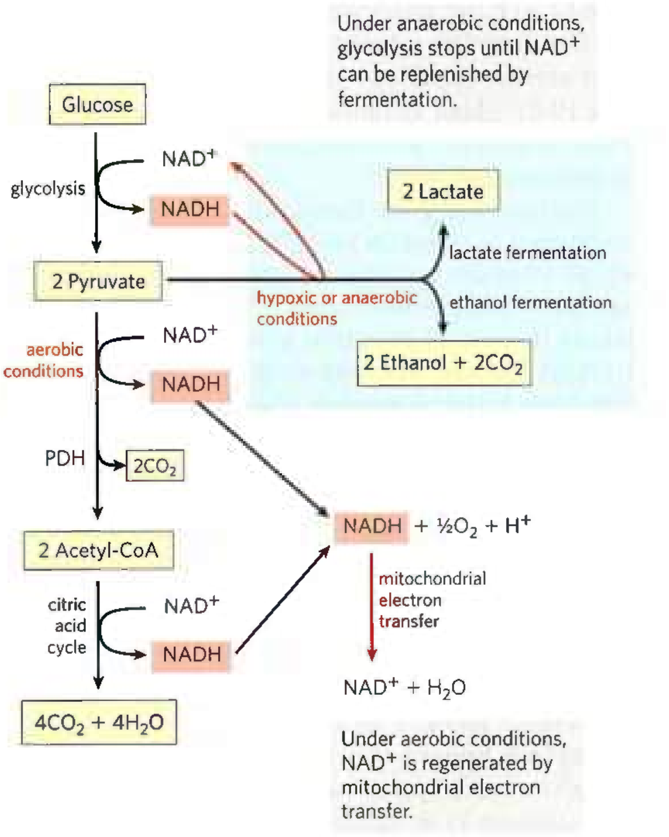
Mannose, released in the digestion of various polysaccharides and glycoproteins of foods, is phosphorylated at C-6 by hexokinase (❾, Fig. 14-9):

$$\text{Mannose} + \text{ATP} \xrightarrow{Mg^{2+}} \text{mannose 6-phosphate} + \text{ADP}$$

Phosphohexose isomerase converts mannose 6-phosphate to fructose 6-phosphate, which enters glycolysis.

SUMMARY 14.2 ***Feeder Pathways for Glycolysis***

- Endogenous glycogen and starch, polymeric storage forms of glucose, undergo sequential phosphorolysis of glucose residues, forming glucose 1-phosphate. Phosphoglucomutase converts the glucose 1-phosphate to glucose 6-phosphate, which can enter glycolysis at a point in the preparatory phase that requires the investment of only one more ATP.
- Ingested polysaccharides and disaccharides are converted to monosaccharides by hydrolytic enzymes in saliva and in the small intestine. The monosaccharides pass through intestinal cells to the bloodstream, which transports them to the liver or other tissues.
- Lactase is present in infants but often absent in adults, producing lactose intolerance. D-Hexoses, including galactose, fructose, and mannose, can be phosphorylated and funneled into glycolysis. Galactose is converted to glucose 1-phosphate through UDP-galactose and UDP-glucose intermediates. A genetic defect in enzymes of this pathway results in one of several galactosemias of varying severity.



Under anaerobic conditions, glycolysis stops until NAD^+ can be replenished by fermentation.

Under aerobic conditions, NAD^+ is regenerated by mitochondrial electron transfer.

FIGURE 14-11 Three possible catabolic fates of the pyruvate formed in glycolysis and the recycling of NADH. Red arrows follow the regeneration of NAD^+ from NADH. Under *aerobic* conditions, pyruvate is activated to acetyl-CoA and is completely oxidized to CO_2 and water through the citric acid cycle and mitochondrial oxidative phosphorylation. NADH produced in this pathway is oxidized to NAD^+ through mitochondrial electron transfer. Under *anaerobic* conditions, pyruvate reduction to lactate or to ethanol is required to produce the NAD^+ needed for glycolysis to continue. Pyruvate also serves as a precursor in many anabolic reactions, not shown here.

14.3 Fates of Pyruvate

With the exception of some interesting variations in the bacterial realm, the pyruvate formed by glycolysis is further metabolized via one of three catabolic routes (**Fig. 14-11**). P3 Under aerobic conditions, glycolysis is only the first stage in the *complete* degradation of glucose. The pyruvate formed in the final step of glycolysis is oxidized to acetate (acetyl-CoA), which enters the citric acid cycle and is oxidized completely to CO_2 and H_2O (Chapter 16). The electrons from these oxidations are carried by NADH and $FADH_2$, which are ultimately reoxidized to NAD^+ and FAD by passage of electrons to O_2 through a chain of carriers in mitochondrial respiration, to form H_2O. The energy from the electron-transfer reactions drives the synthesis of ATP (Chapter 19).

The earliest cells lived in an atmosphere almost devoid of oxygen and evolved deriving energy from fuel molecules under anaerobic conditions. Under anaerobic or low-oxygen conditions (**hypoxia**), NADH cannot be reoxidized to NAD^+ by passing its electrons to O_2. But for glycolysis to continue, NAD^+ must be regenerated. Under these conditions, glucose is degraded by **fermentation** (defined below), which leads to one of two different fates for the pyruvate formed by glycolysis. In **lactic acid fermentation**, pyruvate accepts electrons from NADH and is reduced to lactate while regenerating the NAD^+ necessary for glycolysis. In **ethanol (alcohol) fermentation**, pyruvate is further catabolized to ethanol (Fig. 14-11).

The Pasteur and Warburg Effects Are Due to Dependence on Glycolysis Alone for ATP Production

During his studies on the fermentation of glucose by yeast, Louis Pasteur discovered that both the rate and the total amount of glucose consumption under anaerobic conditions were many times greater than under aerobic conditions. Later studies of muscle showed the same large difference in the rates of glycolysis under anaerobic and aerobic conditions. The biochemical basis of this "Pasteur effect" is now clear. The ATP yield from glycolysis alone (2 ATP per molecule of glucose) is much smaller than that from the complete oxidation of glucose to CO_2 under aerobic conditions (30 or 32 ATP per glucose; see Table 19-5). About 15 times as much glucose must therefore be consumed anaerobically as aerobically to yield the same amount of ATP.

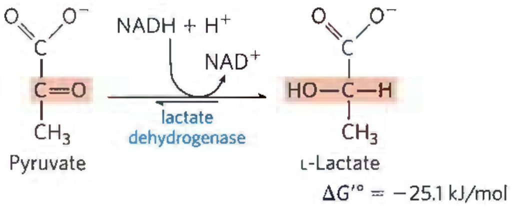
The flux of glucose through the glycolytic pathway is regulated to maintain nearly constant ATP levels (as well as adequate supplies of glycolytic intermediates that serve biosynthetic roles). The required adjustment in the rate of glycolysis is achieved by a complex interplay among ATP consumption, NAD^+ regeneration from NADH formed in glycolysis, and allosteric regulation of several glycolytic enzymes—including hexokinase, PFK-1, and pyruvate kinase—and by second-to-second fluctuations in the concentration of key metabolites that reflect the cellular balance between ATP production and consumption. On a slightly longer time scale, glycolysis is regulated by the hormones glucagon, epinephrine, and insulin, and by changes in the expression of the genes for several glycolytic enzymes. An especially interesting case is glycolysis in tumors. The German biochemist Otto Warburg first observed in 1928 that tumors of nearly all types carry out glycolysis at a much higher rate than normal tissue, *even when oxygen is available*. This "Warburg effect" is the basis for several methods of detecting and treating cancer (**Box 14-1**).

Otto Warburg, 1883–1970
[Science Photo Library/Science Source]

Warburg is generally considered the preeminent biochemist of the first half of the twentieth century. He made seminal contributions to many other areas of biochemistry, including respiration, photosynthesis, and the enzymology of intermediary metabolism. Beginning in 1930, Warburg and his associates purified and crystallized seven of the enzymes of glycolysis. They developed an experimental tool that revolutionized biochemical studies of oxidative metabolism: the Warburg manometer, which directly measured the oxygen consumption of tissues by monitoring changes in gas volume, and thus allowed quantitative measurement of any enzyme with oxidase activity.

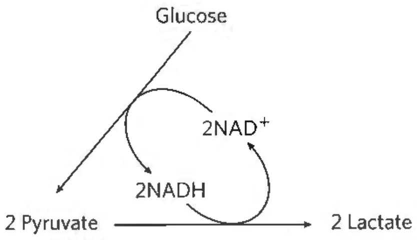
Pyruvate Is the Terminal Electron Acceptor in Lactic Acid Fermentation

P3 When animal tissues cannot be supplied with sufficient oxygen to support aerobic oxidation of the pyruvate and NADH produced in glycolysis, NAD^+ is regenerated from NADH by the reduction of pyruvate to **lactate**. Some tissues and cell types (such as erythrocytes, which have no mitochondria and thus cannot oxidize pyruvate to CO_2) produce lactate from glucose even under aerobic conditions. The reduction of pyruvate in this pathway is catalyzed by **lactate dehydrogenase**, which forms the L isomer of lactate at pH 7:



The overall equilibrium of the reaction strongly favors lactate formation, as shown by the large negative standard free-energy change.

In glycolysis, dehydrogenation of the two molecules of glyceraldehyde 3-phosphate derived from each molecule of glucose converts two molecules of NAD^+ to two of NADH. Because the reduction of two molecules of pyruvate to two of lactate regenerates two molecules of NAD^+, there is no net change in NAD^+ or NADH:



The lactate formed by active skeletal muscles (or by erythrocytes or retinal cells) can be recycled; it is carried in the blood to the liver, where it is converted to glucose during the recovery from strenuous muscular activity. When lactate is produced in large quantities during vigorous muscle contraction (during a sprint, for example), the acidification that results from ionization of lactic acid in muscle and blood limits the period of vigorous activity. The best-conditioned athletes can sprint at top speed for no more than a minute (**Box 14-2**).

Although conversion of glucose to lactate includes two oxidation-reduction steps, there is no net change in the oxidation state of carbon; in glucose ($C_6H_{12}O_6$) and lactic acid ($C_3H_6O_3$), the H:C ratio is the same. Nevertheless, some of the energy of the glucose molecule has been extracted by its conversion to lactate—enough to give a net yield of two molecules of ATP for every glucose molecule consumed. **Fermentation** is the general term for such processes, which extract energy (as ATP) but do not consume oxygen or change the concentrations of NAD^+ or NADH.

BOX 14-1 **MEDICINE**

High Rate of Glycolysis in Tumors Suggests Targets for Chemotherapy and Facilitates Diagnosis

In many types of tumors found in humans and other animals, glucose uptake and glycolysis proceed about 10 times faster than in normal, noncancerous tissues. Most tumor cells grow under hypoxic conditions (i.e., with limited oxygen supply) because, at least initially, they lack the capillary network to supply sufficient oxygen. Cancer cells located more than 100 to 200 μm from the nearest capillaries must depend on glycolysis alone (without further oxidation of pyruvate) for much of their ATP production. The energy yield (2 ATP per glucose) is far lower than can be obtained by the complete oxidation of pyruvate to CO_2 in mitochondria (about 30 ATP per glucose; Chapter 19). So, to make the same amount of ATP, tumor cells must take up much more glucose than do normal cells, converting it to pyruvate and then to lactate as they recycle NADH. It is likely that two early steps in the transformation of a normal cell into a tumor cell are (1) the change to dependence on glycolysis alone for ATP production and (2) the development of tolerance to a low pH in the extracellular fluid (caused by release of lactic acid). In general, the more aggressive the tumor, the greater its rate of glycolysis.

This increase in glycolysis is achieved, at least in part, by increased synthesis of the glycolytic enzymes and of the plasma membrane transporters GLUT1 and GLUT3 (see Table 11-1) that carry glucose into cells. (GLUT1 and GLUT3 are not dependent on insulin.) The **hypoxia-inducible transcription factor (HIF-1)** is a protein that acts at the level of mRNA synthesis to stimulate the production of at least eight glycolytic enzymes and the glucose transporters when oxygen supply is limited (Fig. 1). With the resulting high rate of glycolysis, the tumor cell can survive anaerobic conditions until the supply of blood vessels has caught up with tumor growth. Another protein induced by HIF-1 is the peptide hormone VEGF (vascular endothelial growth factor), which stimulates the outgrowth of blood vessels (angiogenesis) toward the tumor.

There is also evidence that the tumor suppressor protein p53, which is mutated in most types of cancer (see Section 12.9), controls the synthesis and assembly of mitochondrial proteins essential to the passage of electrons to O_2. Cells with mutant p53 are defective in mitochondrial electron transfer and are forced to rely more heavily on glycolysis for ATP production (Fig. 1).

This heavier reliance of tumors than of normal tissue on glycolysis suggests a possibility for anticancer therapy: inhibitors of glycolysis might target and kill tumors by depleting their supply of ATP. Three inhibitors of hexokinase have shown promise as chemotherapeutic agents: 2-deoxyglucose, lonidamine, and 3-bromopyruvate. By preventing the formation of glucose 6-phosphate, these compounds not only deprive tumor cells of glycolytically produced ATP but also prevent the formation of pentose phosphates via the pentose phosphate

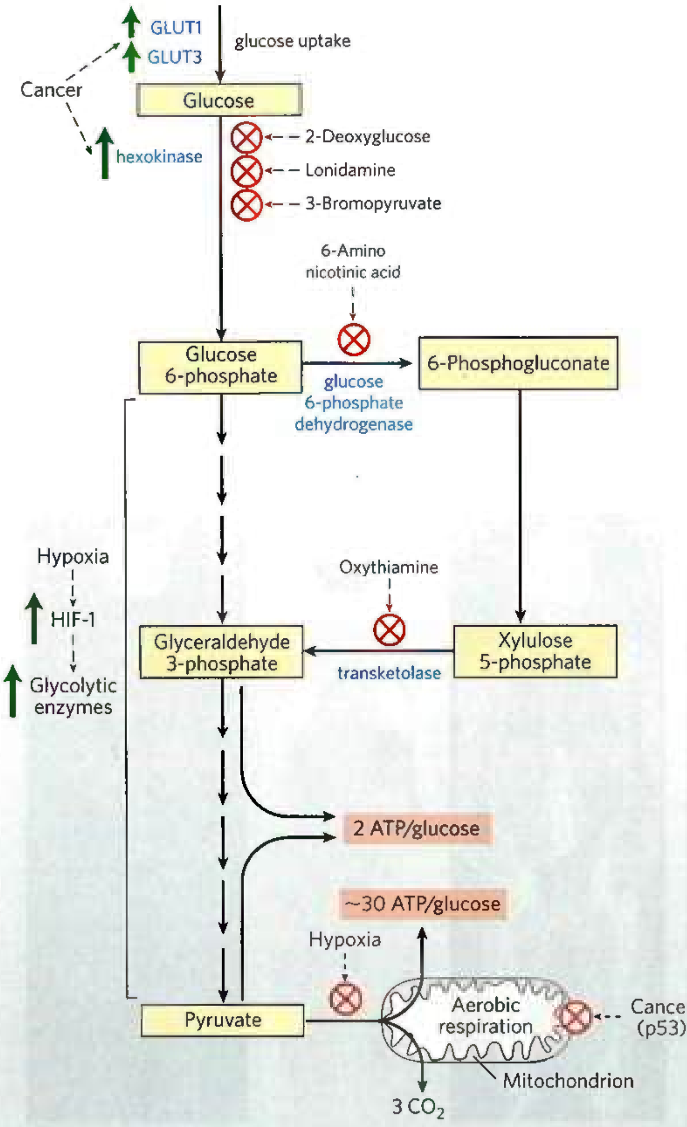

GLUT1
GLUT3
glucose uptake
Cancer
Glucose
hexokinase
2-Deoxyglucose
Lonidamine
3-Bromopyruvate
6-Amino nicotinic acid
Glucose 6-phosphate
glucose 6-phosphate dehydrogenase
6-Phosphogluconate
Hypoxia
HIF-1
Glycolytic enzymes
Oxythiamine
Glyceraldehyde 3-phosphate
transketolase
Xylulose 5-phosphate
2 ATP/glucose
~30 ATP/glucose
Hypoxia
Pyruvate
Aerobic respiration
Cancer (p53)
Mitochondrion
3 CO_2

FIGURE 1 The anaerobic metabolism of glucose in tumor cells yields far less ATP (2 per glucose) than the complete oxidation to CO_2 that takes place in healthy cells under aerobic conditions (~30 ATP per glucose), so a tumor cell must consume much more glucose to produce the same amount of ATP. Glucose transporters and most of the glycolytic enzymes are overproduced in tumors. Compounds that inhibit hexokinase, glucose 6-phosphate dehydrogenase, or transketolase block ATP production by glycolysis, thus depriving the cancer cell of energy and killing it.

(Continued on next page)

High Rate of Glycolysis in Tumors Suggests Targets for Chemotherapy and Facilitates Diagnosis (*Continued*)

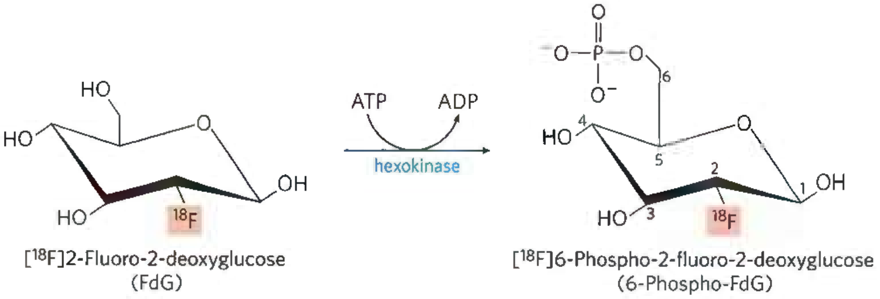

HO
HO
HO
^{18}F
OH
O
[^{18}F]2-Fluoro-2-deoxyglucose (FdG)
ATP
ADP
hexokinase
O
$^{-}$O—P—O
O^{-}
6
4
5
2
1
3
HO
HO
^{18}F
OH
O
[^{18}F]6-Phospho-2-fluoro-2-deoxyglucose (6-Phospho-FdG)

FIGURE 2 Phosphorylation of ^{18}F-labeled 2-fluoro-2-deoxyglucose by hexokinase traps the FdG in cells (as 6-phospho-FdG), where its presence can be detected by positron emission from ^{18}F.

pathway, which also begins with glucose 6-phosphate. Without pentose phosphates, a cell cannot synthesize the nucleotides essential to DNA and RNA synthesis and thus cannot grow or divide. Another anticancer drug already approved for clinical use is imatinib (Gleevec), described in Box 12-4. It inhibits a specific tyrosine kinase, preventing the increased synthesis of hexokinase normally triggered by that kinase. The thiamine analog oxythiamine, which blocks the action of a transketolase-like enzyme that converts xylulose 5-phosphate to glyceraldehyde 3-phosphate (Fig. 1), is in preclinical trials as an antitumor drug.

The high glycolytic rate in tumor cells also has diagnostic usefulness. The relative rates at which tissues take up glucose can be used in some cases to pinpoint the location of tumors. In positron emission tomography (PET), individuals are injected with a harmless, isotopically labeled glucose analog that is taken up but not metabolized by tissues. The labeled compound is 2-fluoro-2-deoxyglucose (FdG), in which the hydroxyl group at the C-2 of glucose is replaced with ^{18}F (Fig. 2). This compound is taken up via GLUT transporters and is a good substrate for hexokinase, but it cannot be converted to the enediol intermediate in the phosphohexose isomerase reaction (see Fig. 14-4) and therefore accumulates as 6-phospho-FdG. The extent of its accumulation depends on its rate of uptake and phosphorylation, which, as noted above, is typically 10 or more times higher in tumors than in normal tissue. Decay of ^{18}F yields positrons (two per ^{18}F atom) that can be detected by a series of sensitive detectors positioned around the body, which allows accurate localization of accumulated 6-phospho-FdG (Fig. 3).

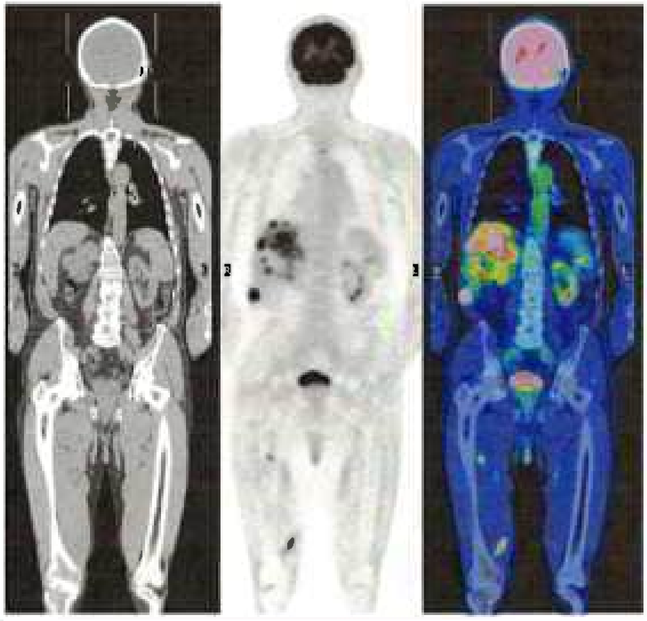


FIGURE 3 Detection of cancerous tissue by positron emission tomography (PET). The adult male patient had undergone surgical removal of a primary skin cancer (malignant melanoma). The image on the left, obtained by whole-body computed tomography (CT scan), shows the location of the soft tissues and bones. The central panel is a PET scan after the patient had ingested ^{18}F-labeled 2-fluoro-2-deoxyglucose (FdG). Dark spots indicate regions of high glucose utilization. As expected, the brain and bladder are heavily labeled—the brain because it uses most of the glucose consumed in the body, and the bladder because the ^{18}F-labeled 6-phospho-FdG is excreted in the urine. When the intensity of the label in the PET scan is translated into false color (the intensity increases from green to yellow to red) and the image is superimposed on the CT scan, the fused image (right) reveals cancer in the bones of the upper spine, in the liver, and in some regions of muscle, all the result of cancer spreading from the primary malignant melanoma. [Diomedia/ISM/Centre Jean Perrin.]

BOX 14-2

Glucose Catabolism at Limiting Concentrations of Oxygen

Most vertebrates are essentially aerobic organisms; they convert glucose to pyruvate by glycolysis, then use molecular oxygen to oxidize the pyruvate completely to CO_2 and H_2O. Anaerobic catabolism of glucose to lactate occurs during short bursts of extreme muscular activity — for example, in a sprint — during which oxygen cannot be carried to the muscles fast enough to oxidize pyruvate. Instead, the muscles use their stored glucose (glycogen) as fuel to generate ATP by fermentation, with lactate as the end product. In a sprint, lactate in the blood builds up to high concentrations. It is slowly converted back to glucose by gluconeogenesis in the liver in the subsequent rest or recovery period, during which oxygen is consumed at a gradually diminishing rate until the breathing rate returns to normal. The excess oxygen consumed in the recovery period represents a repayment of the oxygen debt. This is the amount of oxygen required to supply ATP for gluconeogenesis during recovery respiration, in order to regenerate the glycogen "borrowed" from liver and muscle to carry out intense muscular activity in the sprint. The cycle of reactions that includes glucose conversion to lactate in muscle and lactate conversion to glucose in liver is called the Cori cycle, for Carl and Gerty Cori, whose studies in the 1930s and 1940s clarified the pathway and its role (see Box 15-1).

The circulatory systems of most small vertebrates can carry oxygen to their muscles fast enough to avoid having to use muscle glycogen anaerobically. For example, migrating birds often fly great distances at high speeds without rest and without incurring an oxygen debt. Many running animals of moderate size also maintain an essentially aerobic metabolism in their skeletal muscle. However, the circulatory systems of larger animals, including humans, cannot completely sustain aerobic metabolism in skeletal muscles over long periods of intense muscular activity. These animals generally are slow-moving under normal circumstances and engage in intense muscular activity only in the gravest emergencies, because such bursts of activity require long recovery periods to repay the oxygen debt.

Alligators and crocodiles, for example, are normally sluggish animals. Yet, when provoked, they are capable of lightning-fast charges and dangerous lashings of their powerful tails. Such intense bursts of activity are short and must be followed by long periods of recovery. The fast emergency movements require lactic acid fermentation to generate ATP in skeletal muscles. The stores of muscle glycogen are rapidly expended in intense muscular activity, and lactate reaches very high concentrations in myocytes and extracellular fluid. Whereas a trained athlete can recover from a 100 m sprint in 30 min or less, an alligator may require many hours of rest and extra oxygen consumption to clear the excess lactate from its blood and regenerate muscle glycogen after a burst of activity.

Other large animals, such as the elephant and the rhinoceros, have similar metabolic characteristics, as do diving mammals such as whales and seals. Dinosaurs and other huge, now-extinct animals probably had to depend on lactic acid fermentation to supply energy for muscular activity, followed by very long recovery periods during which they were vulnerable to attack by smaller predators that were better able to use oxygen and thus better adapted to continuous, sustained muscular activity.

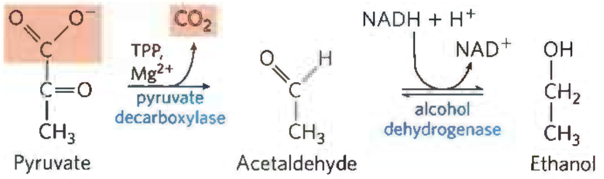
Deep-sea explorations have revealed many species of marine life at great ocean depths, where the oxygen concentration is near zero. For example, the primitive coelacanth, a large fish recovered from depths of 4,000 m or more off the coast of South Africa, has an essentially anaerobic metabolism in virtually all its tissues. It converts carbohydrates to lactate and other products, most of which must be excreted. Some marine vertebrates ferment glucose to ethanol and CO_2 in order to generate ATP.

Francena
McCorory

Francena McCorory, Olympic sprinter [Ezra Shaw/Getty Images.]

Ethanol Is the Reduced Product in Ethanol Fermentation

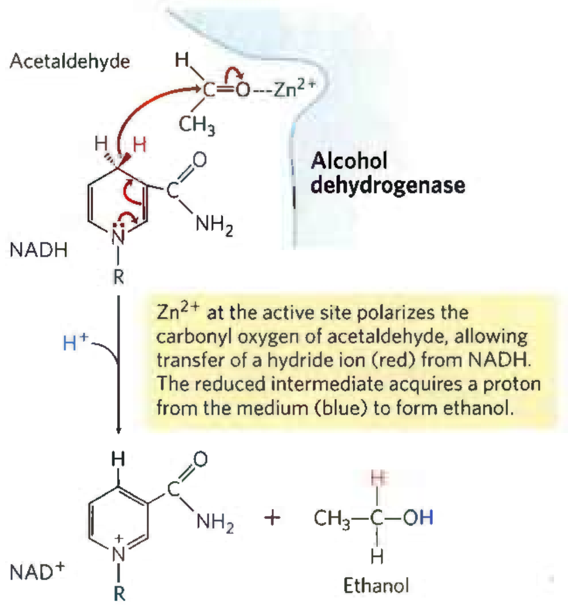
P3 Yeast and other microorganisms ferment glucose to ethanol and CO_2, rather than to lactate. Glucose is metabolized to pyruvate by glycolysis, and the pyruvate is converted to ethanol and CO_2 in a two-step process:



In the first step, pyruvate is decarboxylated to form acetaldehyde in an irreversible reaction catalyzed by **pyruvate decarboxylase**. This reaction is a simple decarboxylation and does not involve the net oxidation of pyruvate. Pyruvate decarboxylase requires Mg^{2+} and has a tightly bound coenzyme, thiamine pyrophosphate, which is discussed below. In the second step, acetaldehyde is reduced to ethanol through the action of **alcohol dehydrogenase**, with the reducing power furnished by NADH derived from the dehydrogenation of glyceraldehyde 3-phosphate. This reaction is a well-studied case of hydride transfer from NADH (**Fig. 14-12**). Ethanol and CO_2 are thus the end products of ethanol fermentation, and the overall equation is

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i \longrightarrow 2\ \text{ethanol} + 2\text{CO}_2 + 2\text{ATP} + 2\text{H}_2\text{O}$$

As in lactic acid fermentation, there is no net change in the ratio of hydrogen to carbon atoms when glucose (H:C ratio = 12/6 = 2) is fermented to two ethanol and two CO_2 (combined H:C ratio = 12/6 = 2). In all fermentations, the H:C ratio of the reactants and products remains the same.



Zn^{2+} at the active site polarizes the carbonyl oxygen of acetaldehyde, allowing transfer of a hydride ion (red) from NADH. The reduced intermediate acquires a proton from the medium (blue) to form ethanol.

MECHANISM FIGURE 14-12 The alcohol dehydrogenase reaction.

Pyruvate decarboxylase is present in brewer's and baker's yeast (different strains of the species *Saccharomyces cerevisiae*) and in all other organisms that ferment glucose to ethanol, including some plants. The CO_2 produced by pyruvate decarboxylation in brewer's yeast is responsible for the characteristic carbonation of champagne. The ancient art of brewing beer involves several enzymatic processes in addition to the reactions of ethanol fermentation. In baking, CO_2 released by pyruvate decarboxylase when yeast is mixed with a fermentable sugar causes dough to rise. The enzyme is absent in vertebrate tissues and in other organisms that carry out lactic acid fermentation.

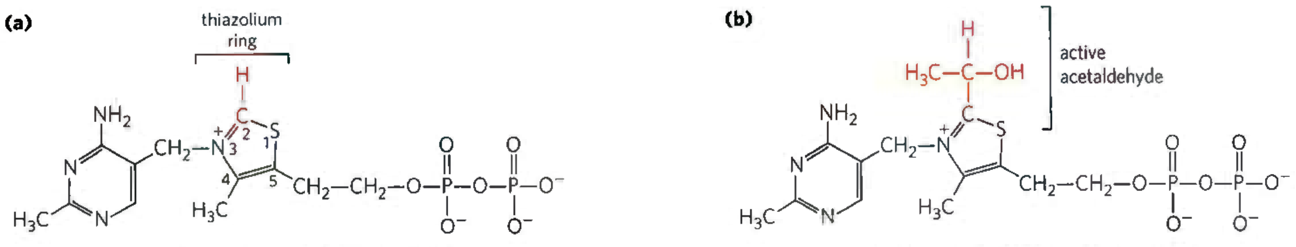
Alcohol dehydrogenase is present in many organisms that metabolize ethanol, including humans. In the liver it catalyzes the oxidation of ethanol, either ingested or produced by intestinal microorganisms, with the concomitant reduction of NAD^+ to NADH. In this case, the reaction proceeds in the direction opposite to that involved in the production of ethanol by fermentation.

The pyruvate decarboxylase reaction provides our first encounter with **thiamine pyrophosphate (TPP)** (**Fig. 14-13**), a coenzyme derived from vitamin B_1. TPP plays an important role in the cleavage of bonds adjacent to a carbonyl group, such as the decarboxylation of α-keto acids, and in chemical rearrangements in which an activated acetaldehyde group is transferred from one carbon atom to another (**Table 14-1**). The functional part of TPP, the thiazolium ring, has a relatively acidic proton at C-2. Loss of this proton produces a carbanion that is the active species in TPP-dependent reactions. The carbanion readily adds to carbonyl groups, and the thiazolium ring is thereby positioned to act as an "electron sink" that greatly facilitates reactions such as the decarboxylation catalyzed by pyruvate decarboxylase.

Fermentations Produce Some Common Foods and Industrial Chemicals

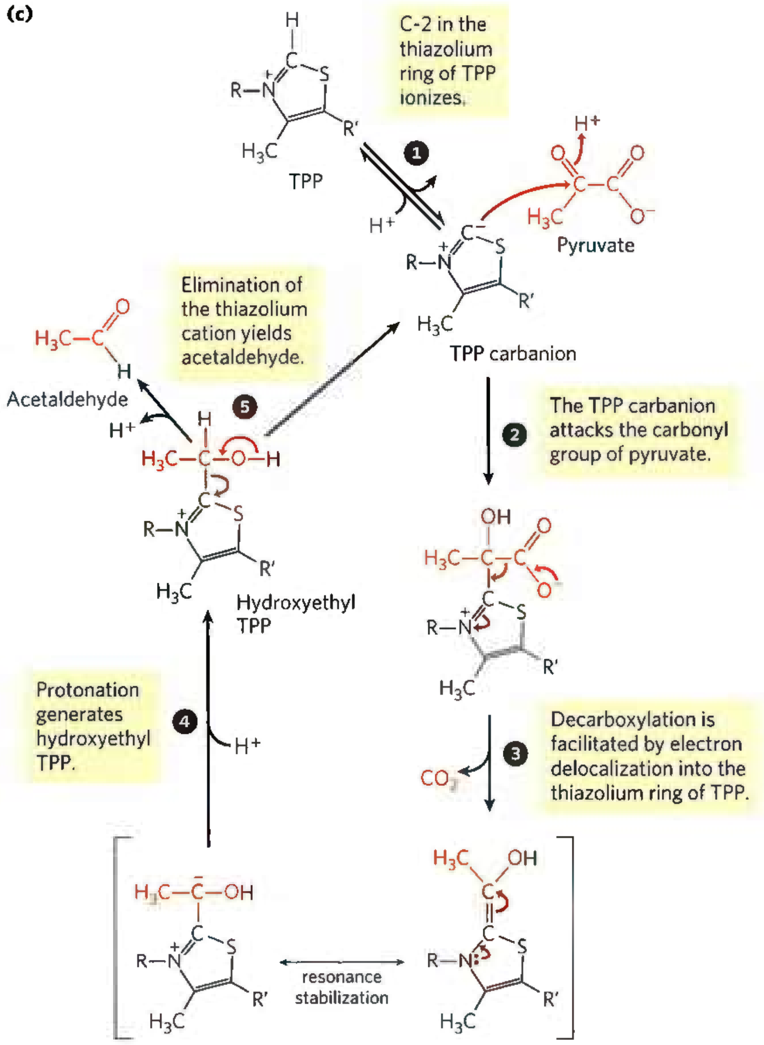
Our progenitors learned millennia ago to use fermentation in the production and preservation of foods and beverages. P3 Certain microorganisms present in raw food products ferment the carbohydrates and yield metabolic products that give the foods their characteristic forms, textures, and tastes. In modern times, industrial fermentation produces organic chemicals and fuels.

Fermented Foods Yogurt, already known in biblical times, is produced when the bacterium *Lactobacillus bulgaricus* ferments the carbohydrate in milk, producing lactic acid; the resulting drop in pH causes the milk proteins to precipitate, producing the thick texture and sour taste of unsweetened yogurt. Another



Thiamine pyrophosphate (TPP)

Hydroxyethyl thiamine pyrophosphate



C-2 in the thiazolium ring of TPP ionizes.

The TPP carbanion attacks the carbonyl group of pyruvate.

Decarboxylation is facilitated by electron delocalization into the thiazolium ring of TPP.

Protonation generates hydroxyethyl TPP.

Elimination of the thiazolium cation yields acetaldehyde.

MECHANISM FIGURE 14-13 Thiamine pyrophosphate (TPP) and its role in pyruvate decarboxylation. (a) TPP is the coenzyme form of vitamin B_1 (thiamine). The reactive carbon atom in the thiazolium ring of TPP is shown in red. In the reaction catalyzed by pyruvate decarboxylase, two of the three carbons of pyruvate are carried transiently on TPP in the form of a hydroxyethyl, or "active acetaldehyde," group (b), which is subsequently released as acetaldehyde. (c) The thiazolium ring of TPP stabilizes carbanion intermediates by providing an electrophilic (electron-deficient) structure into which the carbanion electrons can be delocalized by resonance. Structures with this property, often called "electron sinks," play a role in many biochemical reactions—here, facilitating carbon–carbon bond cleavage. Dietary insufficiency of thiamine causes the serious disease beriberi and the Wernicke-Korsakoff syndrome.

bacterium, *Propionibacterium freudenreichii*, ferments milk to produce propionic acid and CO_2; the propionic acid precipitates milk proteins, and bubbles of CO_2 cause the holes characteristic of Swiss cheese. Many other food products are the result of fermentations: pickles, sauerkraut, sausage, soy sauce, kimchi, kefir, dahi, and kombucha. The drop in pH associated with fermentation also helps to preserve foods, because most of the microorganisms that cause food spoilage cannot grow at low pH. In agriculture, plant byproducts such as corn stalks are preserved for use as animal feed by packing them into a large container (a silo) with limited access to air; microbial fermentation produces acids that lower the pH. The silage that results from this fermentation process can be kept as animal feed for long periods without spoilage.

Fermented Beverages P3 Beer brewing was a science learned early in human history, and later refined for larger-scale production (**Fig. 14-14**). Brewers prepare beer by ethanol fermentation of the carbohydrates in cereal grains (seeds) such as barley, carried out by yeast

TABLE 14-1 Some TPP-Dependent Reactions

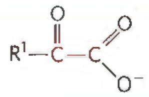
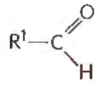
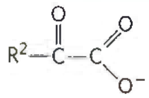
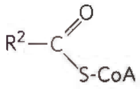
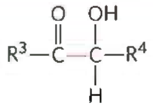
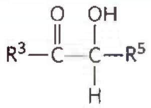
Enzyme	Pathway(s)	Bond cleaved	Bond formed
Pyruvate decarboxylase	Ethanol fermentation		
Pyruvate dehydrogenase α-Ketoglutarate dehydrogenase	Synthesis of acetyl-CoA Citric acid cycle		
Transketolase	Carbon-assimilation reactions Pentose phosphate pathway	 $R^3—\overset{O}{\overset{\Vert}{C}}—\underset{H}{\overset{OH}{C}}—R^4$	 $R^3—\overset{O}{\overset{\Vert}{C}}—\underset{H}{\overset{OH}{C}}—R^5$

FIGURE 14-14 Beer brewing. Large breweries and microbreweries produce beers with a wide variety of flavors, the result of differences in materials and fermentation conditions. [vgajic/Getty Images.]

glycolytic enzymes. The carbohydrates, largely polysaccharides, must first be degraded to disaccharides and monosaccharides. In the malting process, the barley seeds are allowed to germinate until they form the hydrolytic enzymes required to break down their polysaccharides, at which point germination is stopped by controlled heating. The product is malt, which contains enzymes that catalyze the hydrolysis of the β linkages of cellulose and other cell wall polysaccharides of the barley husks, and enzymes such as α-amylase and maltase. The malt is mixed with water, mashed, and boiled with hops to add flavor. Yeast cells added to this mixture grow and reproduce rapidly, using energy obtained from available sugars. No ethanol forms during this stage, because the yeast, amply supplied with oxygen, oxidizes the pyruvate formed by glycolysis to CO_2 and H_2O via the citric acid cycle.

When all the dissolved oxygen in the vat has been consumed, the yeast cells switch to anaerobic metabolism and ferment the sugars into ethanol and CO_2. The fermentation process is controlled in part by the concentration of the ethanol formed, by the pH, and by the amount of remaining sugar. After fermentation has been stopped, the cells are removed and the "raw" beer is ready for final processing.

Chemical Production by Fermentation In 1910, Chaim Weizmann (later to become the first president of Israel) discovered that the bacterium *Clostridium acetobutyricum* ferments starch to butanol and acetone. P3 This discovery opened the field of industrial fermentations, in which some readily available material rich in carbohydrate is supplied to a pure culture of a specific microorganism, which ferments it into a product of greater commercial value. Microbial fermentations produce formic, acetic, propionic, butyric, and succinic acids, and ethanol, glycerol, methanol, isopropanol, butanol, and butanediol. Industrial fermentations are also used to produce certain antibiotics, including penicillin, streptomycin, and chloramphenicol. These fermentations are generally carried out in huge closed vats in which temperature and access to air are controlled to favor the multiplication of the desired microorganism and to exclude contaminating organisms. The beauty of industrial fermentations is that complicated, multistep chemical transformations are carried out in high yields and with few side products by chemical factories that reproduce themselves—microbial cells.

Fuel Production by Fermentation Much of the technology developed for large-scale production of alcoholic beverages can be applied to the production of ethanol as a renewable fuel. The principal advantage of ethanol as a fuel is that it can be produced from relatively *inexpensive* and *renewable* resources rich in sucrose, starch, or cellulose—starch from corn or wheat; sucrose from beets or cane; and cellulose from straw, forest industry waste, or municipal solid waste. Typically, the raw material (feedstock) is converted chemically to monosaccharides, then fed to a hardy strain of yeast in an industrial-scale fermenter. The fermentation can yield not only ethanol for fuel but also side products such as proteins that can be used as animal feed.

SUMMARY 14.3 *Fates of Pyruvate*

- The NADH formed in glycolysis must be recycled to regenerate NAD^+, which is required as an electron acceptor in the first step of the payoff phase. Under aerobic conditions, electrons pass from NADH to O_2 in mitochondrial respiration. The Warburg effect is the observation that tumor cells have high rates of glycolysis, with fermentation of glucose to lactate, even in the presence of oxygen. It is the basis of PET scanning used to diagnose tumors.
- Under anaerobic or hypoxic conditions, many organisms regenerate NAD^+ by transferring electrons from NADH to pyruvate, forming lactate. Other organisms, such as yeast, regenerate NAD^+ by reducing pyruvate to ethanol and CO_2.
- A variety of microorganisms can ferment sugar in fresh foods, resulting in changes in pH, taste, and texture, and preserving food from spoilage. Fermentations are used in industry to produce many commercially valuable organic compounds from inexpensive starting materials.

14.4 Gluconeogenesis

The central role of glucose in metabolism arose early in evolution, and this sugar remains the nearly universal fuel and building block in modern organisms, from microbes to humans. In mammals, some tissues depend almost completely on glucose for their metabolic energy. For the human brain and nervous system, as well as the erythrocytes, testes, renal medulla, and embryonic tissues, glucose from the blood is the sole or major fuel source. The brain alone requires about 120 g of glucose each day—more than half of all the glucose stored as glycogen in muscle and liver. However, the supply of glucose from these stores is not always sufficient; between meals and during longer fasts, or after vigorous exercise, glycogen is depleted. For these times, organisms need a method for synthesizing glucose from noncarbohydrate precursors. This is accomplished by a pathway called **gluconeogenesis** ("new formation of sugar"), which converts pyruvate and related three- and four-carbon compounds to glucose.

P4 Gluconeogenesis occurs in all animals, plants, fungi, and microorganisms. The reactions are essentially the same in all tissues and all species. The important precursors of glucose in animals are three-carbon compounds such as lactate, pyruvate, and glycerol, as well as certain amino acids (**Fig. 14-15**). In mammals, gluconeogenesis takes place mainly in the liver, and to a lesser extent in the renal cortex and in the epithelial cells that line the small intestine. The glucose produced passes into the blood to supply other tissues. After vigorous exercise, lactate produced by anaerobic glycolysis in skeletal muscle returns to the liver and is converted to glucose, which moves back to muscle and is converted to glycogen—a circuit called the Cori cycle (see Fig. 23-17). In plant seedlings, stored fats and proteins are converted, via paths that include gluconeogenesis, to the disaccharide sucrose for transport throughout the developing plant. Glucose and its derivatives are precursors for the synthesis of plant cell walls, nucleotides and coenzymes, and a variety of other essential metabolites. In many microorganisms, gluconeogenesis starts from simple organic compounds of two or three carbons, such as acetate, lactate, and propionate, in their growth medium.

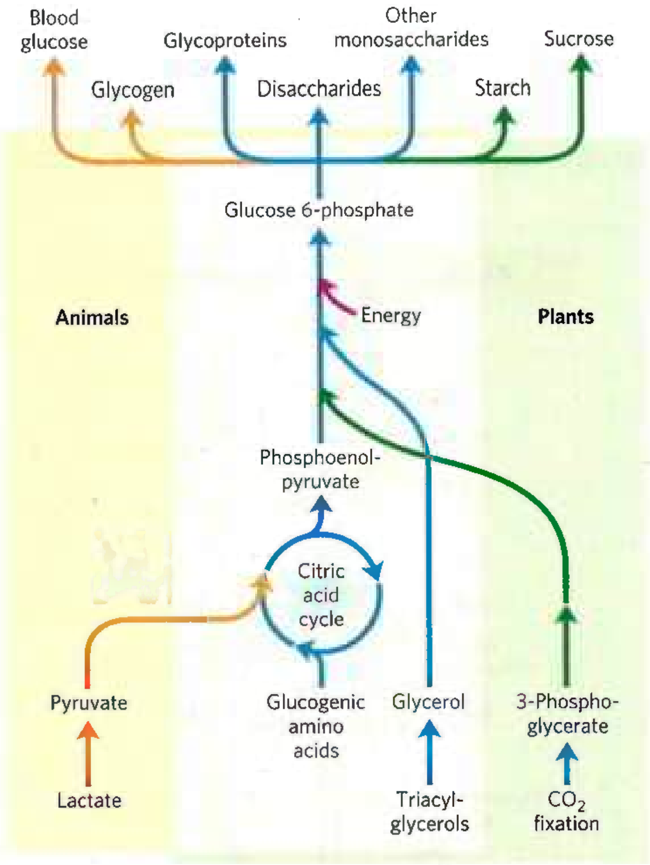


FIGURE 14-15 Carbohydrate synthesis from simple precursors. The pathway from phosphoenolpyruvate to glucose 6-phosphate is common to the biosynthetic conversion of many different precursors of carbohydrates in animals and plants. The path from pyruvate to phosphoenolpyruvate leads through oxaloacetate, an intermediate of the citric acid cycle, which we discuss in Chapter 16. Any compound that can be converted to either pyruvate or oxaloacetate can therefore serve as starting material for gluconeogenesis. This includes alanine and aspartate, which are convertible to pyruvate and oxaloacetate, respectively, and other amino acids that can also yield three- or four-carbon fragments, the so-called glucogenic amino acids. Plants and photosynthetic bacteria are uniquely able to convert CO_2 to carbohydrates, using the Calvin cycle, as we shall see in Section 20.4.

Although the reactions of gluconeogenesis are the same in all organisms, the metabolic context and the regulation of the pathway differ from one species to another and from tissue to tissue. In this section we focus on gluconeogenesis as it occurs in the mammalian liver. In Chapter 20 we show how photosynthetic organisms use this pathway to convert the primary products of photosynthesis into glucose, to be stored as sucrose or starch.

P4 Gluconeogenesis and glycolysis are not identical pathways running in opposite directions, although they do share several steps (**Fig. 14-16**); 7 of the 10 enzymatic reactions of gluconeogenesis are the reverse of glycolytic reactions. However, three reactions of glycolysis are essentially irreversible in vivo and cannot be used in gluconeogenesis: the conversion of glucose to glucose 6-phosphate by hexokinase, the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate by phosphofructokinase-1, and the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase. In cells, these three reactions are characterized by a large negative free-energy change, whereas other glycolytic reactions have a ΔG near 0 (**Table 14-2**). In gluconeogenesis, the three irreversible steps are bypassed by a separate set of enzymes, catalyzing reactions that are sufficiently exergonic to be effectively irreversible in the direction of glucose synthesis. Thus, both glycolysis and gluconeogenesis are irreversible processes in cells. In animals, both pathways occur largely in the cytosol, necessitating their reciprocal and coordinated regulation, described in Section 14.5.

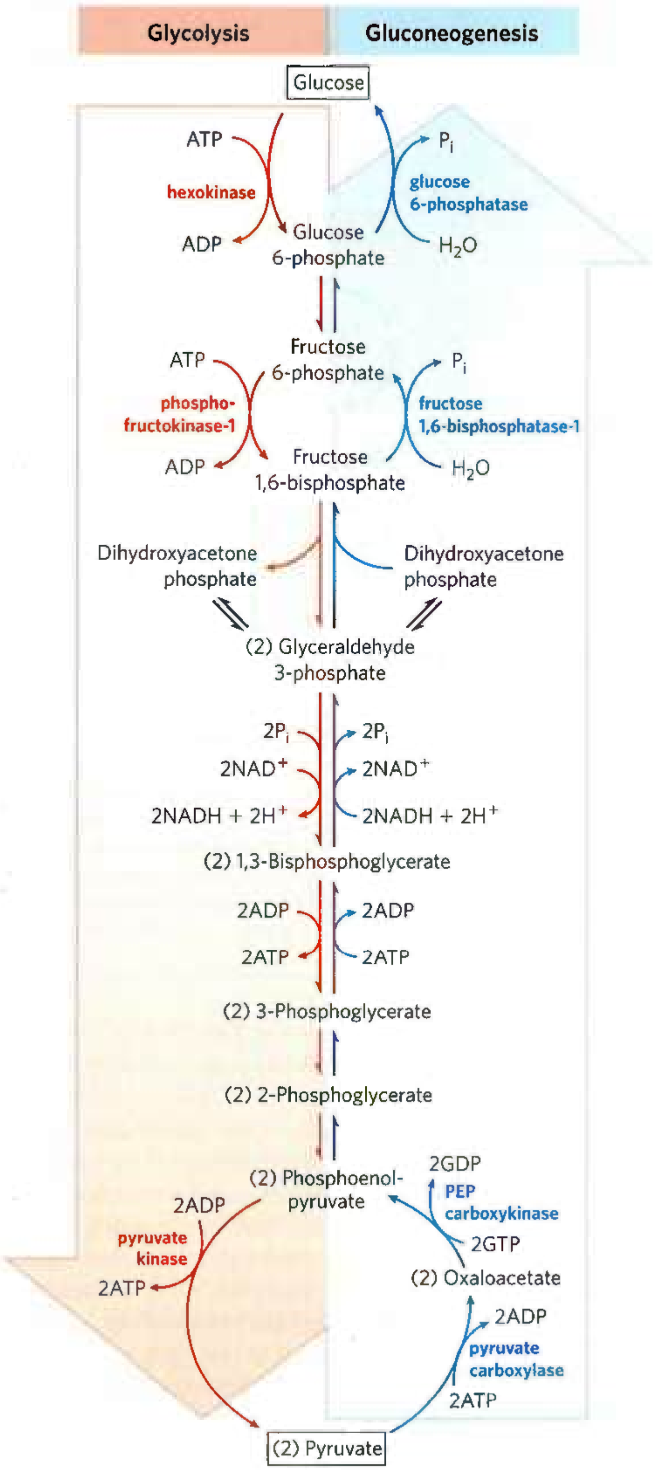

Glycolysis
Gluconeogenesis
Glucose
ATP
hexokinase
ADP
Glucose 6-phosphate
P_i
glucose 6-phosphatase
H_2O
Fructose 6-phosphate
ATP
phospho-fructokinase-1
ADP
Fructose 1,6-bisphosphate
P_i
fructose 1,6-bisphosphatase-1
H_2O
Dihydroxyacetone phosphate
Dihydroxyacetone phosphate
(2) Glyceraldehyde 3-phosphate
$2P_i$
$2NAD^+$
$2NADH + 2H^+$
$2P_i$
$2NAD^+$
$2NADH + 2H^+$
(2) 1,3-Bisphosphoglycerate
2ADP
2ATP
2ADP
2ATP
(2) 3-Phosphoglycerate
(2) 2-Phosphoglycerate
(2) Phosphoenol-pyruvate
2ADP
pyruvate kinase
2ATP
2GDP
PEP carboxykinase
2GTP
(2) Oxaloacetate
2ADP
pyruvate carboxylase
2ATP
(2) Pyruvate

FIGURE 14-16 Opposing pathways of glycolysis and gluconeogenesis in liver. The reactions of glycolysis are on the left side, in red; the opposing pathway of gluconeogenesis is on the right, in blue. The major sites of regulation of gluconeogenesis shown here are discussed in Section 14.5.

We begin by considering the three bypass reactions of gluconeogenesis. (Keep in mind that "bypass" refers throughout to the bypass of irreversible glycolytic reactions.)

The First Bypass: Conversion of Pyruvate to Phosphoenolpyruvate Requires Two Exergonic Reactions

P4 The first of the bypass reactions in gluconeogenesis is the conversion of pyruvate to phosphoenolpyruvate (PEP). This reaction cannot occur by simple reversal of the pyruvate kinase reaction of glycolysis (p. 521), which has a large, negative free-energy change and is therefore irreversible under the conditions prevailing in intact cells (Table 14-2, step ⑩). Instead, the phosphorylation of pyruvate is achieved by a roundabout sequence of reactions that in eukaryotes requires enzymes in both the cytosol and mitochondria. As we shall see, the pathway shown in Figure 14-16 and described in detail here is one of two routes from pyruvate to PEP; it is the predominant path when pyruvate or alanine is the glucogenic precursor. A second pathway, described later, predominates when lactate is the glucogenic precursor.

Pyruvate is first transported from the cytosol into mitochondria or is generated from alanine within mitochondria by transamination, in which the α-amino group is transferred from alanine (leaving pyruvate) to an α-keto carboxylic acid (transamination reactions are discussed in detail in Chapter 18). Then **pyruvate carboxylase**, a mitochondrial enzyme that requires the coenzyme **biotin**, converts the pyruvate to oxaloacetate:

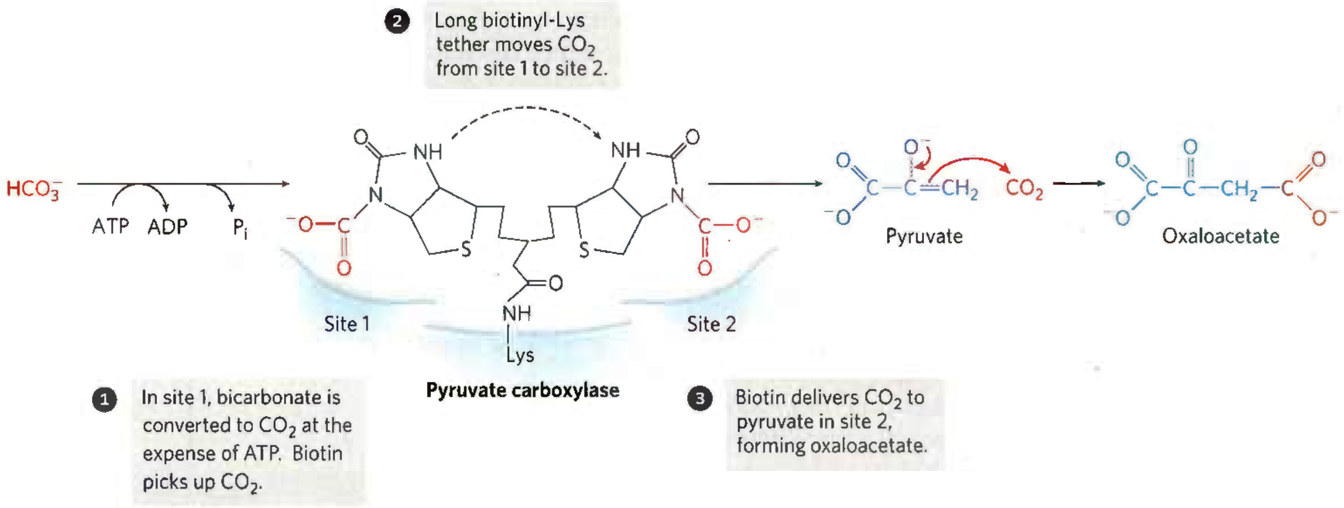
$$\text{Pyruvate} + HCO_3^- + \text{ATP} \longrightarrow \text{oxaloacetate} + \text{ADP} + P_i \qquad (14\text{-}4)$$

The carboxylation reaction involves biotin as a carrier of activated bicarbonate, as shown in **Figure 14-17**; the reaction mechanism is shown in Figure 16-16. (Note that HCO_3^- is formed by ionization of carbonic acid formed from $CO_2 + H_2O$.) HCO_3^- is phosphorylated by ATP to form a mixed anhydride (a carboxyphosphate); then biotin displaces the phosphate in the formation of carboxybiotin.

TABLE 14-2 Free-Energy Changes of Glycolytic Reactions in Erythrocytes

Glycolytic reaction step	$\Delta G'^{\circ}$ (kJ/mol)	ΔG (kJ/mol)
① Glucose + ATP ⟶ glucose 6-phosphate + ADP	−16.7	−33.4
② Glucose 6-phosphate ⇌ fructose 6-phosphate	1.7	0 to 25
③ Fructose 6-phosphate + ATP ⟶ fructose1,6-bisphosphate + ADP	−14.2	−22.2
④ Fructose1,6-bisphosphate ⇌ dihydroxyacetone phosphate + glyceraldehyde 3-phosphate	23.8	−6 to 0
⑤ Dihydroxyacetone phosphate ⇌ glyceraldehyde 3-phosphate	7.5	0 to 4
⑥ Glyceraldehyde 3-phosphate + P_i + NAD^+ ⇌ 1,3-bisphosphoglycerate + NADH + H^+	6.3	−2 to 2
⑦ 1,3-Bisphosphoglycerate + ADP ⇌ 3-phosphoglycerate + ATP	−18.8	0 to 2
⑧ 3-Phosphoglycerate ⇌ 2-phosphoglycerate	4.4	0 to 0.8
⑨ 3-Phosphoglycerate ⇌ phosphoenolpyruvate + H_2O	7.5	0 to 3.3
⑩ Phosphoenolpyruvate + ADP ⟶ pyruvate + ATP	−31.4	−16.7

Note: $\Delta G'^{\circ}$ is the standard free-energy change, as defined in Chapter 13 (p. 468). ΔG is the free-energy change calculated from the actual concentrations of glycolytic intermediates present under physiological conditions in erythrocytes, at pH 7. The glycolytic reactions bypassed in gluconeogenesis are shown in red. Biochemical equations are not necessarily balanced for H or charge (p. 478).



② Long biotinyl-Lys tether moves CO_2 from site 1 to site 2.

① In site 1, bicarbonate is converted to CO_2 at the expense of ATP. Biotin picks up CO_2.

③ Biotin delivers CO_2 to pyruvate in site 2, forming oxaloacetate.

FIGURE 14-17 Role of biotin in the pyruvate carboxylase reaction. The cofactor biotin is covalently attached to pyruvate carboxylase through an amide linkage to the ε-amino group of a Lys residue, forming a biotinyl-enzyme. The reaction takes place in two phases, which occur at two different sites in the enzyme. The long biotinyl-Lys arm carries the substrate from one site to the other.

Pyruvate carboxylase is the first regulatory enzyme in the gluconeogenic pathway, requiring acetyl-CoA as a positive effector. Acetyl-CoA is produced by fatty acid oxidation (Chapter 17), and its accumulation signals the availability of fatty acids as fuel. As we shall see in Chapter 16, the pyruvate carboxylase reaction can replenish intermediates in another central metabolic pathway, the citric acid cycle.

Because the mitochondrial membrane has no transporter for oxaloacetate, before export to the cytosol the oxaloacetate formed from pyruvate must be reduced to malate by mitochondrial **malate dehydrogenase**, at the expense of NADH:

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{L-malate} + \text{NAD}^+ \quad (14\text{-}5)$$

The standard free-energy change for this reaction is quite high, but under physiological conditions (including a very low concentration of oxaloacetate) $\Delta G \approx 0$ and the reaction is readily reversible. Mitochondrial malate dehydrogenase functions in both gluconeogenesis and the citric acid cycle, but the overall flow of metabolites in the two processes is in opposite directions.

Malate leaves the mitochondrion through a specific transporter in the inner mitochondrial membrane (see Fig. 19-31), and in the cytosol it is reoxidized to oxaloacetate, with the production of cytosolic NADH:

$$\text{Malate} + \text{NAD}^+ \longrightarrow \text{oxaloacetate} + \text{NADH} + \text{H}^+ \quad (14\text{-}6)$$

The oxaloacetate is then converted to PEP by **phosphoenolpyruvate carboxykinase** (**Fig. 14-18**). This Mg^{2+}-dependent reaction requires GTP as the phosphoryl group donor:

$$\text{Oxaloacetate} + \text{GTP} \rightleftharpoons \text{PEP} + \text{CO}_2 + \text{GDP} \quad (14\text{-}7)$$

The reaction is reversible under intracellular conditions; the formation of one high-energy phosphate compound

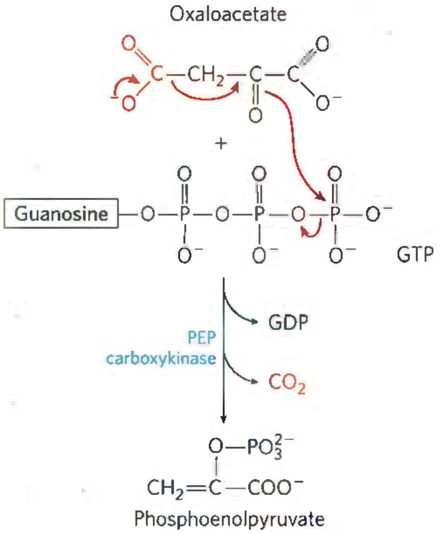

Oxaloacetate
Guanosine
GTP
PEP carboxykinase
GDP
CO_2
Phosphoenolpyruvate

FIGURE 14-18 Synthesis of phosphoenolpyruvate from oxaloacetate. In the cytosol, oxaloacetate is converted to phosphoenolpyruvate by PEP carboxykinase. The CO_2 incorporated in the pyruvate carboxylase reaction is lost here as CO_2. The decarboxylation leads to a rearrangement of electrons that facilitates attack of the carbonyl oxygen of the pyruvate moiety on the γ phosphate of GTP.

(PEP) is balanced by the hydrolysis of another (GTP). The overall equation for this set of bypass reactions, the sum of Equations 14-4 through 14-7, is

$$\text{Pyruvate} + \text{ATP} + \text{GTP} + HCO_3^- \longrightarrow$$
$$\text{PEP} + \text{ADP} + \text{GDP} + P_i + CO_2 \qquad (14\text{-}8)$$
$$\Delta G'^\circ = 0.9 \text{ kJ/mol}$$

Two high-energy phosphate equivalents (one from ATP and one from GTP), each yielding about 50 kJ/mol under cellular conditions, must be expended to phosphorylate one molecule of pyruvate to PEP. In contrast, when PEP is converted to pyruvate during glycolysis, only one ATP is generated from ADP. Although the standard free-energy change ($\Delta G'^\circ$) of the two-step path from pyruvate to PEP is 0.9 kJ/mol, the actual free-energy change (ΔG), calculated from measured cellular concentrations of intermediates, is very strongly negative (−25 kJ/mol); this results from the ready consumption of PEP in other reactions such that its concentration remains relatively low. The reaction is thus effectively irreversible in the cell.

Note that the CO_2 added to pyruvate in the pyruvate carboxylase step (Fig. 14-17) is the same molecule that is lost in the PEP carboxykinase reaction (Fig. 14-18). This carboxylation-decarboxylation sequence represents a way of "activating" pyruvate, in that the decarboxylation of oxaloacetate facilitates PEP formation. In Chapter 21 we shall see how a similar carboxylation-decarboxylation sequence is used to activate acetyl-CoA for fatty acid biosynthesis (see Fig. 21-1).

There is a logic to the route of these reactions through the mitochondrion. The [NADH]/[NAD^+] ratio in the cytosol is several orders of magnitude lower than in mitochondria. Because cytosolic NADH is consumed in gluconeogenesis (in the conversion of 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate; Fig. 14-16), glucose biosynthesis cannot proceed unless NADH is available. The transport of malate from the mitochondrion to the cytosol and its reconversion there to oxaloacetate effectively moves reducing equivalents to the cytosol, where they are scarce. This path from pyruvate to PEP therefore provides an important balance between NADH produced and consumed in the cytosol during gluconeogenesis.

P4 A second pyruvate → PEP bypass predominates when lactate is the glucogenic precursor (**Fig. 14-19**). This pathway makes use of lactate produced by glycolysis in erythrocytes or anaerobic muscle, for example, and it is particularly important in large vertebrates after vigorous exercise (Box 14-2). The conversion of lactate to pyruvate in the cytosol of hepatocytes yields NADH, and the export of reducing equivalents (as malate) from mitochondria is therefore unnecessary. After the pyruvate produced by the lactate dehydrogenase reaction is transported into the mitochondrion (by a transporter in the inner mitochondrial membrane specific for pyruvate),

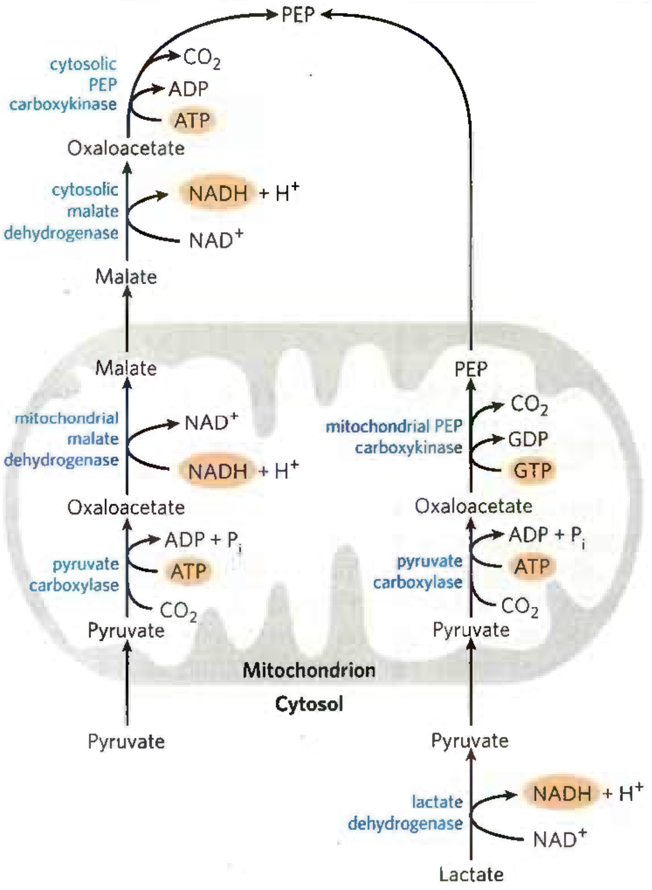

PEP
cytosolic PEP carboxykinase
CO_2
ADP
ATP
Oxaloacetate
cytosolic malate dehydrogenase
NADH + H^+
NAD^+
Malate
Malate
mitochondrial malate dehydrogenase
NAD^+
NADH + H^+
Oxaloacetate
pyruvate carboxylase
ADP + P_i
ATP
CO_2
Pyruvate
PEP
mitochondrial PEP carboxykinase
CO_2
GDP
GTP
Oxaloacetate
pyruvate carboxylase
ADP + P_i
ATP
CO_2
Pyruvate
Mitochondrion
Cytosol
Pyruvate
Pyruvate
lactate dehydrogenase
NADH + H^+
NAD^+
Lactate

FIGURE 14-19 Alternative paths from pyruvate to phosphoenolpyruvate. The relative importance of the two pathways depends on the availability of lactate or pyruvate and the cytosolic requirements for NADH for gluconeogenesis. The path on the right predominates when lactate is the precursor, because cytosolic NADH is generated in the lactate dehydrogenase reaction and does not have to be shuttled out of the mitochondrion (see text).

it is converted to oxaloacetate by pyruvate carboxylase, as described above. This oxaloacetate, however, is converted directly to PEP by a mitochondrial isozyme of PEP carboxykinase, and the PEP is transported out of the mitochondrion to continue on the gluconeogenic path. The mitochondrial and cytosolic isozymes of PEP carboxykinase are encoded by separate genes in the nuclear chromosomes, providing another example of two distinct enzymes catalyzing the same reaction but having different cellular locations or metabolic roles (recall the isozymes of hexokinase).

The Second and Third Bypasses Are Simple Dephosphorylations by Phosphatases

The second glycolytic reaction that cannot participate in gluconeogenesis is the phosphorylation of fructose 6-phosphate by PFK-1 (Table 14-2, step ❸). Because this reaction is highly exergonic and therefore irreversible in intact cells, the generation of fructose 6-phosphate from fructose 1,6-bisphosphate (Fig. 14-16) is catalyzed by a different enzyme, Mg^{2+}-dependent **fructose 1,6-bisphosphatase (FBPase-1)**, which promotes the essentially irreversible *hydrolysis* of the C-1 phosphate (*not* phosphoryl group transfer to ADP):

$$\text{Fructose 1,6-phosphate} + H_2O \longrightarrow \text{fructose 6-phosphate} + P_i \qquad \Delta G'^{\circ} = -16.3 \text{ kJ/mol}$$

FBPase-1 is so named to distinguish it from another, similar enzyme (FBPase-2) with a regulatory role, which we discuss in Section 14.5.

The third bypass is the final reaction of gluconeogenesis, the dephosphorylation of glucose 6-phosphate to yield glucose (Fig. 14-16). Reversal of the hexokinase reaction (p. 514) would require phosphoryl group transfer from glucose 6-phosphate to ADP, forming ATP, an energetically unfavorable reaction (Table 14-2, step ❶). The reaction catalyzed by **glucose 6-phosphatase** does not require synthesis of ATP; it is a simple hydrolysis of a phosphate ester:

$$\text{Glucose 6-phosphate} + H_2O \longrightarrow \text{glucose} + P_i \qquad \Delta G'^{\circ} = -13.8 \text{ kJ/mol}$$

This Mg^{2+}-activated enzyme is a membrane protein in the lumen of the endoplasmic reticulum of hepatocytes, renal cells, and epithelial cells of the small intestine (see Fig. 15-6), but not in other tissues, which are therefore unable to supply glucose to the blood. If other tissues had glucose 6-phosphatase, this enzyme's activity would hydrolyze the glucose 6-phosphate needed within those tissues for glycolysis. Glucose produced by gluconeogenesis in the liver or kidney or ingested in the diet is delivered to these other tissues, including brain and muscle, through the bloodstream.

Gluconeogenesis Is Energetically Expensive, But Essential

P4 The sum of the biosynthetic reactions leading from pyruvate to free blood glucose (**Table 14-3**) is

$$2\,\text{Pyruvate} + 4\text{ATP} + 2\text{GTP} + 2\text{NADH} + 2H^+ + 4H_2O \longrightarrow \text{glucose} + 4\text{ADP} + 2\text{GDP} + 6P_i + 2\text{NAD}^+ \qquad (14\text{-}9)$$

For each molecule of glucose formed from pyruvate, six high-energy phosphate groups are required: four from ATP and two from GTP. In addition, two molecules of NADH are required for the reduction of two molecules of 1,3-bisphosphoglycerate. Clearly, Equation 14-9 is not simply the reverse of the equation for conversion of

TABLE 14-3 Sequential Reactions in Gluconeogenesis Starting from Pyruvate

$\text{Pyruvate} + HCO_3^- + \text{ATP} \longrightarrow \text{oxaloacetate} + \text{ADP} + P_i$	×2
$\text{Oxaloacetate} + \text{GTP} \rightleftharpoons \text{phosphoenolpyruvate} + CO_2 + \text{GDP}$	×2
$\text{Phosphoenolpyruvate} + H_2O \rightleftharpoons \text{2-phosphoglycerate}$	×2
$\text{2-Phosphoglycerate} \rightleftharpoons \text{3-phosphoglycerate}$	×2
$\text{3-Phosphoglycerate} + \text{ATP} \rightleftharpoons \text{1,3-bisphosphoglycerate} + \text{ADP}$	×2
$\text{1,3-Bisphosphoglycerate} + \text{NADH} + H^+ \rightleftharpoons \text{glyceraldehyde 3-phosphate} + \text{NAD}^+ + P_i$	×2
$\text{Glyceraldehyde 3-phosphate} \rightleftharpoons \text{dihydroxyacetone phosphate}$	
$\text{Glyceraldehyde 3-phosphate} + \text{dihydroxyacetone phosphate} \rightleftharpoons \text{fructose 1,6-bisphosphate}$	
$\text{Fructose 1,6-bisphosphate} \longrightarrow \text{fructose 6-phosphate} + P_i$	
$\text{Fructose 6-phosphate} \rightleftharpoons \text{glucose 6-phosphate}$	
$\text{Glucose 6-phosphate} + H_2O \longrightarrow \text{glucose} + P_i$	
Sum: $2\,\text{Pyruvate} + 4\text{ATP} + 2\text{GTP} + 2\text{NADH} + 2H^+ + 4H_2O \longrightarrow \text{glucose} + 4\text{ADP} + 2\text{GDP} + 6P_i + 2\text{NAD}^+$	

Note: The bypass reactions are in red; all other reactions are reversible steps of glycolysis. The figures at the right indicate that the reaction is to be counted twice, because two three-carbon precursors are required to make a molecule of glucose. The reactions required to replace the cytosolic NADH consumed in the glyceraldehyde 3-phosphate dehydrogenase reaction (the conversion of lactate to pyruvate in the cytosol or the transport of reducing equivalents from mitochondria to the cytosol in the form of malate) are not considered in this summary. Biochemical equations are not necessarily balanced for H and charge (p. 478).

glucose to pyruvate by glycolysis, which would require only two molecules of ATP:

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i + \text{NAD}^+ \longrightarrow 2\ \text{pyruvate} + 2\text{ATP} + 2\text{NADH} + 2\text{H}^+ + 2\text{H}_2\text{O}$$

This makes the synthesis of glucose from pyruvate a relatively expensive process. Much of this high energy cost is necessary to ensure the irreversibility of gluconeogenesis. Under intracellular conditions, the overall free-energy change of glycolysis is at least −63 kJ/mol. Under the same conditions the overall ΔG of gluconeogenesis is −16 kJ/mol. Thus both glycolysis and gluconeogenesis are essentially irreversible processes in cells. A second advantage to investing energy to convert pyruvate to glucose is that if pyruvate were instead excreted, its considerable potential for ATP production by complete, aerobic oxidation would be lost (more than 10 ATP are produced per pyruvate, as we shall see in Chapter 16).

The biosynthetic pathway to glucose described above allows the net synthesis of glucose not only from pyruvate but also from the four-, five-, and six-carbon intermediates of the citric acid cycle (Chapter 16). The citric acid cycle intermediates can undergo oxidation to oxaloacetate (see Fig. 16-7). Some or all of the carbon atoms of most amino acids derived from proteins are ultimately catabolized to pyruvate or to intermediates of the citric acid cycle. Such amino acids can therefore undergo net conversion to glucose and are said to be **glucogenic** (Table 14-4). Alanine and glutamine, the principal molecules that transport amino groups from extrahepatic tissues to the liver (see Fig. 18-9), are particularly important glucogenic amino acids in mammals. After removal of their amino groups in liver mitochondria, the carbon skeletons remaining (pyruvate and α-ketoglutarate, respectively) are readily funneled into gluconeogenesis.

TABLE 14-4 Glucogenic Amino Acids, Grouped by Site of Entry

Pyruvate	**Succinyl-CoA**
Alanine	Isoleucine[a]
Cysteine	Methionine
Glycine	Threonine
Serine	Valine
Threonine	**Fumarate**
Tryptophan[a]	Phenylalanine[a]
α-Ketoglutarate	Tyrosine[a]
Arginine	**Oxaloacetate**
Glutamate	Asparagine
Glutamine	Aspartate
Histidine	
Proline	

Note: All these amino acids are precursors of blood glucose or liver glycogen, because they can be converted to pyruvate or citric acid cycle intermediates. Of the 20 common amino acids, only leucine and lysine are unable to furnish carbon for net glucose synthesis.

[a] These amino acids are also ketogenic (see Fig. 18-15).

Mammals Cannot Convert Fatty Acids to Glucose; Plants and Microorganisms Can

No net conversion of fatty acids to glucose occurs in mammals. As we shall see in Chapter 17, the catabolism of most fatty acids yields only acetyl-CoA. Mammals cannot use acetyl-CoA as a precursor of glucose, because the pyruvate dehydrogenase reaction is irreversible and cells have no other pathway to convert acetyl-CoA to pyruvate. Plants, yeast, and many bacteria do have a pathway (the glyoxylate cycle; see Fig. 20-45) for converting acetyl-CoA to oxaloacetate, so these organisms can use fatty acids as the starting material for gluconeogenesis. This is important during the germination of seedlings, for example; before leaves develop and photosynthesis can provide energy and carbohydrates, the seedling relies on stored seed oils for energy production and cell wall biosynthesis.

Although mammals cannot convert fatty acids to carbohydrate, they can use the small amount of glycerol produced from the breakdown of fats (triacyl*glycerols*) for gluconeogenesis. Phosphorylation of glycerol by glycerol kinase, followed by oxidation of the central carbon, yields dihydroxyacetone phosphate, an intermediate in gluconeogenesis in liver.

As we shall see in Chapter 21, glycerol phosphate is an essential intermediate in triacylglycerol synthesis in adipocytes, but these cells lack glycerol kinase and so cannot simply phosphorylate glycerol. Instead, adipocytes carry out a truncated version of gluconeogenesis, known as **glyceroneogenesis**: the conversion of pyruvate to dihydroxyacetone phosphate via the early reactions of gluconeogenesis, followed by reduction of the dihydroxyacetone phosphate to glycerol 3-phosphate (see Fig. 21-21).

SUMMARY 14.4 *Gluconeogenesis*

- Gluconeogenesis is a multistep process in which glucose is produced from lactate, pyruvate, or oxaloacetate, or any compound (including citric acid cycle intermediates) that can be converted to one of these intermediates. Seven steps are the reversal of glycolytic reactions; three differ and must be bypassed with exergonic reactions.
- In the first bypass, pyruvate is converted to PEP via oxaloacetate in two steps catalyzed by pyruvate carboxylase (which uses ATP) and PEP carboxykinase (which uses GTP).
- In the second bypass, FBPase-1 removes a phosphate group from fructose 1,6-bisphosphate, producing fructose 6-phosphate. In the third bypass, glucose 6-phosphatase converts glucose 6-phosphate to glucose.
- In mammals, gluconeogenesis in the liver, kidney, and small intestine provides glucose for use by the brain, muscles, and erythrocytes. Formation of one molecule of glucose from pyruvate requires four ATP, two GTP, and two NADH; it is expensive.

- Animals cannot convert acetyl-CoA derived from fatty acids into glucose; they lack the enzymatic machinery to convert acetyl-CoA to pyruvate. Plants and microorganisms have the glyoxylate pathway, which allows them to make glucose from fatty acids.

14.5 Coordinated Regulation of Glycolysis and Gluconeogenesis

P5 Glycolysis (the conversion of glucose to pyruvate) and gluconeogenesis (the conversion of pyruvate to glucose) generally do not both occur at the same time in the same tissues. In mammals, gluconeogenesis occurs primarily in the liver, where its role is to provide glucose for export to other tissues when glycogen stores are exhausted and when no dietary glucose is available. Glycolysis occurs in most tissues, including, brain, kidney, muscle and liver. Glycolysis provides ATP to support all of the energy-requiring activities of cells: active transport of ions; synthesis of macromolecules and of their precursors; synthesis of lipids and storage compounds like glycogen; and muscle contraction.

At each of the three points where glycolytic reactions are bypassed by alternative, gluconeogenic reactions (Fig. 14-16), simultaneous operation of both pathways would consume ATP without accomplishing any chemical or biological work. For example, PFK-1 and FBPase-1 catalyze opposing reactions:

$$\text{ATP} + \text{fructose 6-phosphate} \xrightarrow{\text{PFK-1}} \text{ADP} + \text{fructose 1,6-bisphosphate}$$

$$\text{Fructose 1,6-bisphosphate} + \text{H}_2\text{O} \xrightarrow{\text{FBPase-1}} \text{fructose 6-phosphate} + \text{P}_\text{i}$$

The sum of these two reactions is

$$\text{ATP} + \text{H}_2\text{O} \longrightarrow \text{ADP} + \text{P}_\text{i} + \text{heat}$$

that is, hydrolysis of ATP without any useful metabolic work being done. Clearly, if these two reactions were allowed to proceed simultaneously at a high rate in the same cell, a large amount of chemical energy would be dissipated as heat.

We look now in some detail at the mechanisms that regulate glycolysis and gluconeogenesis at the three points where these pathways diverge.

Hexokinase Isozymes Are Affected Differently by Their Product, Glucose 6-Phosphate

Hexokinase, which catalyzes the entry of glucose into the glycolytic pathway, is a regulatory enzyme. As noted in Section 14.1, humans have four isozymes of hexokinase (designated I to IV), encoded by four different genes (**Box 14-3**). **P5** The different hexokinase isozymes of liver and muscle reflect the different roles of these organs in carbohydrate metabolism: muscle consumes glucose, using it for energy production; liver maintains blood glucose homeostasis by consuming or producing glucose, depending on the prevailing blood glucose concentration.

The predominant hexokinase isozyme of myocytes (**hexokinase II**) has a high affinity for glucose—it is half-saturated at about 0.1 mM. Because glucose entering myocytes from the blood (where the glucose concentration is 4 to 5 mM) produces an intracellular glucose concentration high enough to saturate hexokinase II, the muscle enzyme normally acts at or near its V_{max}. Muscle **hexokinase I** and hexokinase II are allosterically inhibited by their product, glucose 6-phosphate, so whenever the cellular concentration of glucose 6-phosphate rises above its normal level, these isozymes are temporarily and reversibly inhibited, bringing the rate of glucose 6-phosphate formation into balance with the rate of its utilization and reestablishing the steady state.

The predominant hexokinase isozyme of liver is hexokinase IV (also called glucokinase), which differs in three important respects from hexokinases I, II, and III of muscle. First, the glucose concentration at which hexokinase IV is half-saturated (about 10 mM) is higher than the usual concentration of glucose in the blood. Because an efficient glucose transporter in hepatocytes (GLUT2) rapidly equilibrates the glucose concentrations in cytosol and blood, the high K_m of hexokinase IV allows its direct regulation by the blood glucose concentration (**Fig. 14-20**). When blood glucose is high, as it is after a meal rich in carbohydrates, excess glucose is transported into hepatocytes, where hexokinase IV converts it to glucose 6-phosphate. Because hexokinase IV is not saturated at 10 mM glucose, its activity continues to increase as the glucose concentration rises to 10 mM or more. Under conditions of low blood glucose, the glucose concentration in a hepatocyte is low relative to the K_m of hexokinase IV, and the glucose generated by gluconeogenesis leaves the cell before being trapped by phosphorylation.

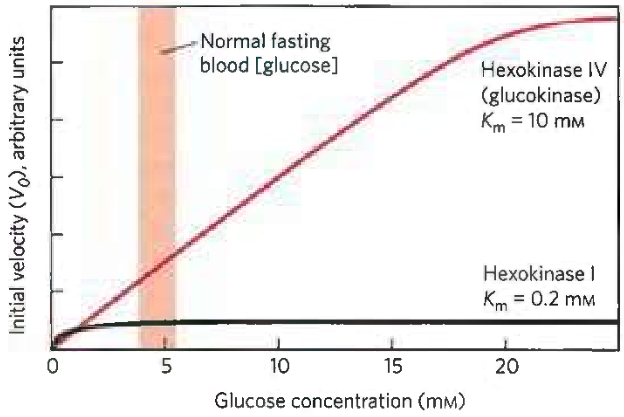


FIGURE 14-20 Comparison of the kinetic properties of hexokinase IV (glucokinase) and hexokinase I. Note the much lower K_m for hexokinase I. When blood glucose rises above 5 mM, hexokinase IV activity increases, but hexokinase I is already operating near V_{max} and cannot respond to an increase in glucose concentration. Hexokinases I, II, and III have similar kinetic properties.

BOX 14-3

Isozymes: Different Proteins That Catalyze the Same Reaction

The four forms of hexokinase found in mammalian tissues are but one example of a common biological situation: the same reaction catalyzed by two or more different molecular forms of an enzyme. These multiple forms, called isozymes or isoenzymes, may occur in the same species, in the same tissue, even in the same cell. The different forms (isoforms) of the enzyme generally differ in kinetic or regulatory properties, in the cofactor they use (NADH or NADPH for dehydrogenase isozymes, for example), or in their subcellular distribution (soluble or membrane-bound). Isozymes may have similar, but not identical, amino acid sequences, and in many cases they clearly share a common evolutionary origin.

One of the first enzymes found to have isozymes was lactate dehydrogenase (LDH; p. 526), which in vertebrate tissues exists as at least five different isozymes separable by electrophoresis. All LDH isozymes contain four polypeptide chains (each of M_r 33,500), each type containing a different ratio of two kinds of polypeptides. The M (for muscle) chain and the H (for heart) chain are encoded by two different genes.

In skeletal muscle the predominant isozyme contains four M chains, and in heart the predominant isozyme contains four H chains. Other tissues have some combination of the five possible types of LDH isozymes:

Type	Composition	Location
LDH_1	HHHH	Heart and erythrocyte
LDH_2	HHHM	Heart and erythrocyte
LDH_3	HHMM	Brain and kidney
LDH_4	HMMM	Skeletal muscle and liver
LDH_5	MMMM	Skeletal muscle and liver

Differences in the isozyme content of tissues can be used to assess the timing and extent of heart damage due to myocardial infarction (heart attack). Damage to heart tissue results in the release of heart LDH into the blood. Shortly after a heart attack, the blood level of total LDH increases, and there is more LDH_2 than LDH_1. After 12 hours the amounts of LDH_1 and LDH_2 are very similar, and after 24 hours there is more LDH_1 than LDH_2. This switch in the $[LDH_1]/[LDH_2]$ ratio, combined with increased concentrations in the blood of another heart enzyme, creatine kinase, is very strong evidence of a recent myocardial infarction. ■

The different LDH isozymes have significantly different values of V_{max} and K_m, particularly for pyruvate. The properties of LDH_4 favor rapid reduction of very low concentrations of pyruvate to lactate in skeletal muscle, whereas those of isozyme LDH_1 favor rapid oxidation of lactate to pyruvate in the heart.

In general, the distribution of different isozymes of a given enzyme reflects at least four factors:

1. *Different metabolic patterns in different organs.* For glycogen phosphorylase, the isozymes in skeletal muscle and liver have different regulatory properties, reflecting the different roles of glycogen breakdown in these two tissues.
2. *Different locations and metabolic roles for isozymes in the same cell.* The isocitrate dehydrogenase isozymes of the cytosol and the mitochondrion are an example (Chapter 16).
3. *Different stages of development in embryonic or fetal tissues and in adult tissues.* For example, the fetal liver has a characteristic isozyme distribution of LDH, which changes as the organ develops into its adult form. Some enzymes of glucose catabolism in malignant (cancer) cells occur as their fetal, not adult, isozymes.
4. *Different responses of isozymes to allosteric modulators.* This difference is useful in fine-tuning metabolic rates. Hexokinase IV (glucokinase) of liver and the hexokinase isozymes of other tissues differ in their sensitivity to inhibition by glucose 6-phosphate.

Second, hexokinase IV is not inhibited by glucose 6-phosphate, and it can therefore continue to operate when the accumulation of glucose 6-phosphate completely inhibits hexokinases I, II, and III. Third, hexokinase IV is subject to inhibition by the reversible binding of a regulatory protein specific to liver (**Fig. 14-21**). The binding is much tighter in the presence of the allosteric effector fructose 6-phosphate. Glucose competes with fructose 6-phosphate for binding and causes dissociation of the regulatory protein from the hexokinase, relieving the inhibition. Immediately after a carbohydrate-rich meal, when blood glucose is high, glucose enters the hepatocyte via GLUT2 and activates hexokinase IV by this mechanism. During a fast, when blood glucose drops below 5 mM, fructose 6-phosphate triggers the inhibition of hexokinase IV by the regulatory protein, so the liver does not compete with other organs for the scarce glucose. The mechanism of inhibition by the regulatory protein is interesting: the protein anchors hexokinase IV inside the nucleus, where it is segregated from the other enzymes of glycolysis in the cytosol. When the glucose concentration in the cytosol rises, it equilibrates with glucose in the nucleus by transport through the nuclear pores. Glucose causes dissociation of the regulatory protein, and hexokinase IV enters the cytosol and begins to phosphorylate glucose.

Hexokinase IV is also regulated at the level of protein synthesis. Circumstances that call for greater energy production (low [ATP], high [AMP], vigorous muscle contraction) or for greater glucose consumption (high blood [glucose], for example) cause increased transcription of the hexokinase IV gene. Glucose 6-phosphatase,

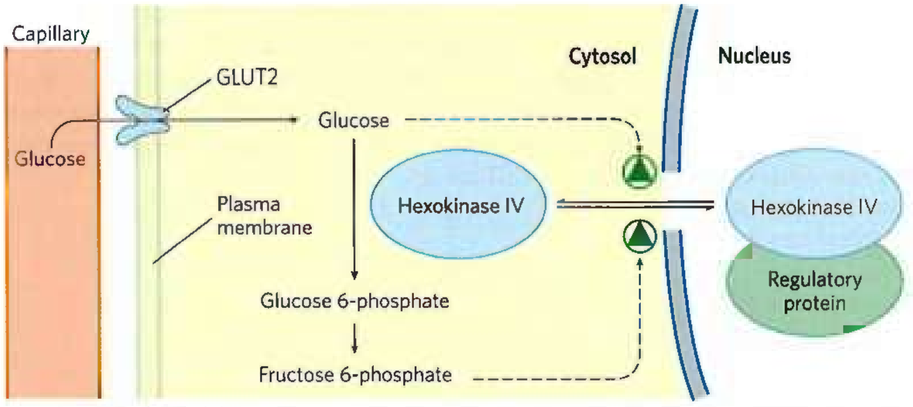

Capillary
Glucose
GLUT2
Glucose
Cytosol
Nucleus
Plasma membrane
Hexokinase IV
Hexokinase IV
Regulatory protein
Glucose 6-phosphate
Fructose 6-phosphate

FIGURE 14-21 Regulation of hexokinase IV (glucokinase) by sequestration in the nucleus. The protein inhibitor of hexokinase IV is a nuclear binding protein that draws hexokinase IV into the nucleus when the fructose 6-phosphate concentration in liver is high and releases it to the cytosol when the glucose concentration is high.

the gluconeogenic enzyme that bypasses the hexokinase step of glycolysis, is transcriptionally regulated by factors that call for increased production of glucose (low blood glucose, glucagon signaling). The transcriptional regulation of these two enzymes (along with other enzymes of glycolysis and gluconeogenesis) is described below.

Phosphofructokinase-1 and Fructose 1,6-Bisphosphatase Are Reciprocally Regulated

Glucose 6-phosphate can flow either into glycolysis or through any of several other pathways, including glycogen synthesis and the pentose phosphate pathway. The metabolically irreversible reaction catalyzed by PFK-1 is the step that commits glucose to glycolysis. In addition to its substrate-binding sites, this complex enzyme has several regulatory sites at which allosteric activators or inhibitors bind.

ATP is not only a substrate for PFK-1 but also an end product of the glycolytic pathway. When high cellular [ATP] signals that ATP is being produced faster than it is being consumed, ATP inhibits PFK-1 by binding to an allosteric site and lowering the affinity of the enzyme for its substrate fructose 6-phosphate (**Fig. 14-22**). ADP and AMP, which increase in concentration as consumption of ATP outpaces production, act allosterically to relieve this inhibition by ATP. These effects combine to produce higher enzyme activity when ADP or AMP accumulates and lower activity when ATP accumulates.

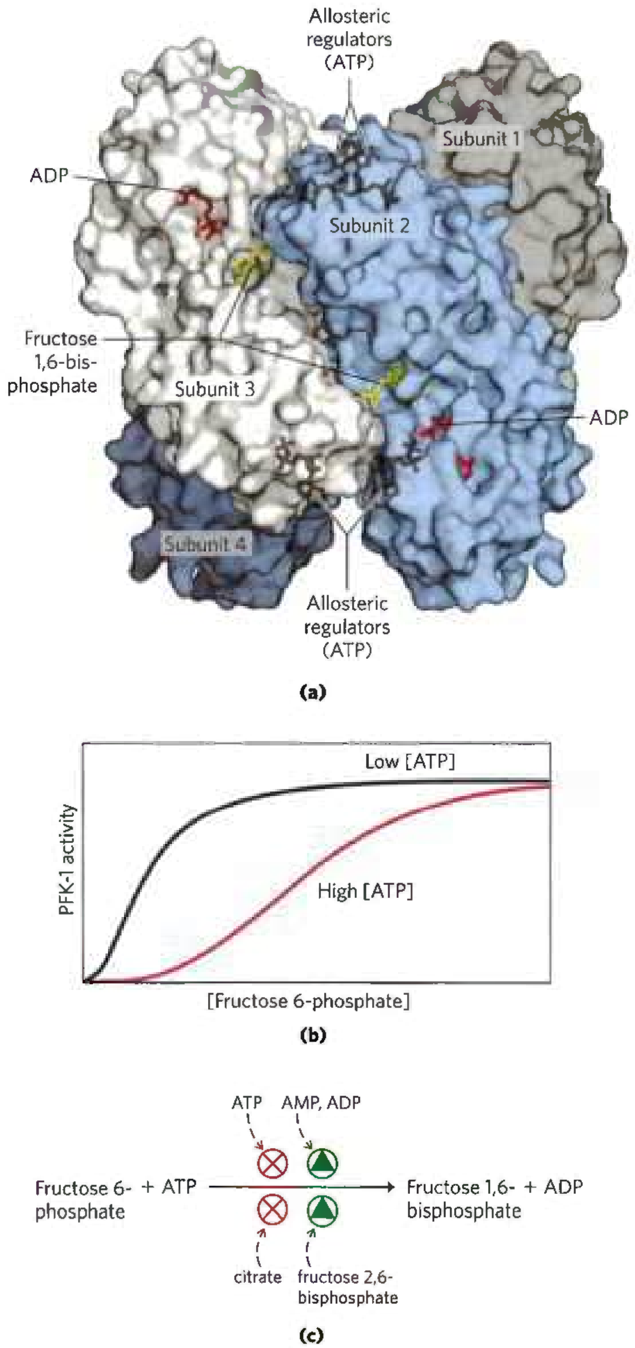

Allosteric regulators (ATP)
Subunit 1
ADP
Subunit 2
Fructose 1,6-bis-phosphate
Subunit 3
ADP
Subunit 4
Allosteric regulators (ATP)
(a)
Low [ATP]
PFK-1 activity
High [ATP]
[Fructose 6-phosphate]
(b)
ATP
AMP, ADP
Fructose 6-phosphate + ATP
Fructose 1,6-bisphosphate + ADP
citrate
fructose 2,6-bisphosphate
(c)

FIGURE 14-22 Phosphofructokinase-1 (PFK-1) and its regulation. (a) Surface contour image of *E. coli* PFK-1, showing portions of its four identical subunits. Each subunit has its own catalytic site, where the products ADP and fructose 1,6-bisphosphate (red and yellow stick structures, respectively) are almost in contact, and its own binding sites for the allosteric regulator ATP, buried in the protein in the positions indicated. (b) Allosteric regulation of muscle PFK-1 by ATP, shown by a substrate-activity curve. At low [ATP], the $K_{0.5}$ for fructose 6-phosphate is relatively low, enabling the enzyme to function at a high rate at relatively low [fructose 6-phosphate]. (Recall from Chapter 6 that $K_{0.5}$ is the K_m term for regulatory enzymes; when $K_{0.5}$ is larger, the binding is weaker.) When [ATP] is high, $K_{0.5}$ for fructose 6-phosphate is greatly increased, as indicated by the sigmoid relationship between substrate concentration and enzyme activity. (c) Summary of the regulators affecting PFK-1 activity. [(a) Data from PDB ID 1PFK, Y. Shirakihara and P. R. Evans, *J. Mol. Biol.* 204:973, 1988.]

Citrate (the ionized form of citric acid), a key intermediate in the aerobic oxidation of pyruvate, fatty acids, and amino acids, is also an allosteric regulator of PFK-1. High citrate concentration increases the inhibitory effect of ATP, further reducing the flow of glucose through glycolysis. In this case, as in several others encountered later, citrate serves as an intracellular signal that the cell is meeting its current needs for energy-yielding metabolism by the oxidation of fats and proteins.

The corresponding step in gluconeogenesis is the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate (**Fig. 14-23**). The enzyme that catalyzes this reaction, FBPase-1, is strongly inhibited (allosterically) by AMP; when the cell's supply of ATP is low (corresponding to high [AMP]), the ATP-requiring synthesis of glucose slows.

Thus, these opposing steps in the glycolytic and gluconeogenic pathways—those catalyzed by PFK-1 and FBPase-1—are regulated in a coordinated and reciprocal manner. In general, when sufficient concentrations of acetyl-CoA or citrate (the product of acetyl-CoA condensation with oxaloacetate) are present, or when a high proportion of the cell's adenylate is in the form of ATP, gluconeogenesis is favored. When the concentration of AMP increases, it promotes glycolysis by stimulating PFK-1 (and, as we shall see in Section 15.3, promotes glycogen degradation by activating glycogen phosphorylase).

Fructose 2,6-Bisphosphate Is a Potent Allosteric Regulator of PFK-1 and FBPase-1

The special role of the liver in maintaining a constant blood glucose level requires additional regulatory mechanisms to coordinate glucose production and consumption. When the blood glucose level decreases, the hormone **glucagon** signals the liver to produce and release more glucose and to stop consuming it for its own needs. One source of glucose is glycogen stored in the liver; another source is gluconeogenesis, using pyruvate, lactate, glycerol, or certain amino acids as starting material. When blood glucose is high, insulin signals the liver to use glucose as a fuel and as a precursor for the synthesis and storage of glycogen and triacylglycerol.

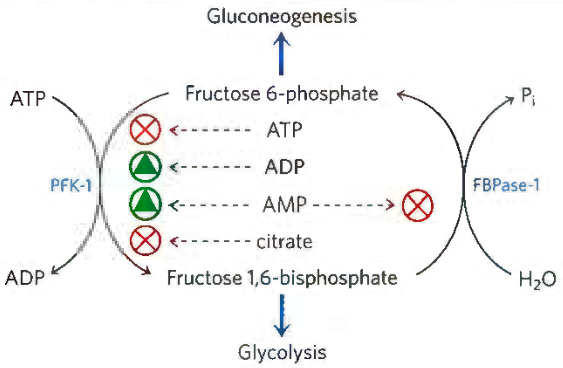
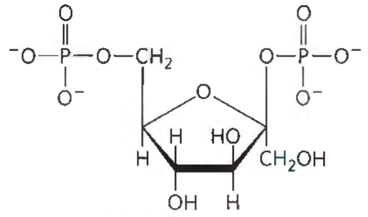


FIGURE 14-23 **Regulation of phosphofructokinase-1 (PFK-1) and fructose 1,6-bisphosphatase (FBPase-1).**

P5 The rapid hormonal regulation of glycolysis and gluconeogenesis is mediated by **fructose 2,6-bisphosphate**, an allosteric effector for the enzymes PFK-1 and FBPase-1:



Fructose 2,6-bisphosphate

When fructose 2,6-bisphosphate binds to its allosteric site on PFK-1, it increases the enzyme's affinity for its substrate fructose 6-phosphate (**Fig. 14-24a**) and reduces its affinity for the allosteric inhibitors ATP and citrate. At the physiological concentrations of its substrates, ATP and fructose 6-phosphate, and of its other positive and negative effectors (ATP, AMP, citrate), PFK-1 is virtually inactive in the absence of fructose 2,6-bisphosphate. Fructose 2,6-bisphosphate has the opposite effect on FBPase-1: it reduces its affinity for its substrate (Fig. 14-24b), thereby slowing gluconeogenesis.

The cellular concentration of the allosteric regulator fructose 2,6-bisphosphate is set by the relative rates of its formation and breakdown (**Fig. 14-25a**). It is formed by phosphorylation of fructose 6-phosphate, catalyzed by **phosphofructokinase-2 (PFK-2)**, and broken down by **fructose 2,6-bisphosphatase (FBPase-2)**. (Note that these enzymes are distinct from PFK-1 and FBPase-1, which catalyze the formation and breakdown, respectively, of fructose 1,6-bisphosphate.) PFK-2 and FBPase-2 are two separate enzymatic activities of a single, bifunctional protein. The balance of these two activities in the liver, which determines the cellular level of fructose 2,6-bisphosphate, is set by glucagon and insulin (Fig. 14-25b).

As we saw in Chapter 12, glucagon stimulates the adenylyl cyclase of liver to synthesize 3′,5′-cyclic AMP (cAMP) from ATP. Cyclic AMP then activates cAMP-dependent protein kinase, which transfers a phosphoryl group from ATP to the bifunctional protein PFK-2/FBPase-2. Phosphorylation of this protein enhances its FBPase-2 activity and inhibits its PFK-2 activity. Glucagon thereby lowers the cellular level of fructose 2,6-bisphosphate, inhibiting glycolysis and stimulating gluconeogenesis. The resulting production of more glucose enables the liver to replenish blood glucose in response to glucagon. Insulin has the opposite effect, stimulating the activity of a phosphoprotein phosphatase that catalyzes removal of the phosphoryl group from the bifunctional protein PFK-2/FBPase-2, activating its PFK-2 activity, increasing the level of fructose 2,6-bisphosphate, stimulating glycolysis, and inhibiting gluconeogenesis.

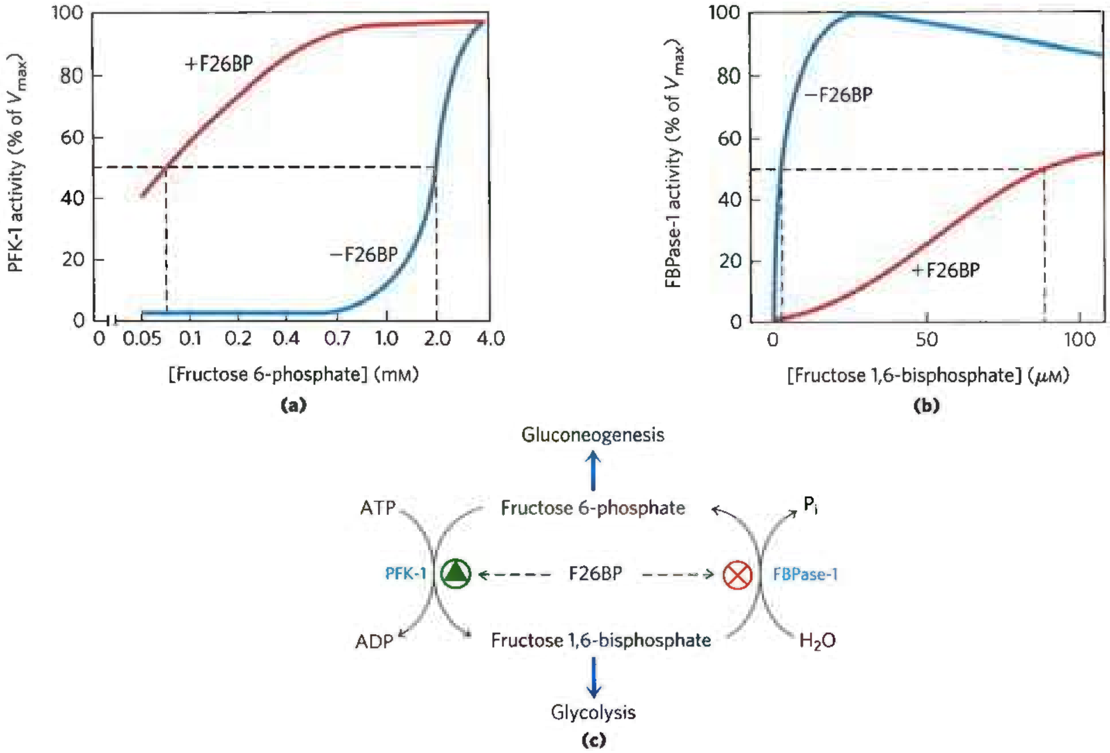
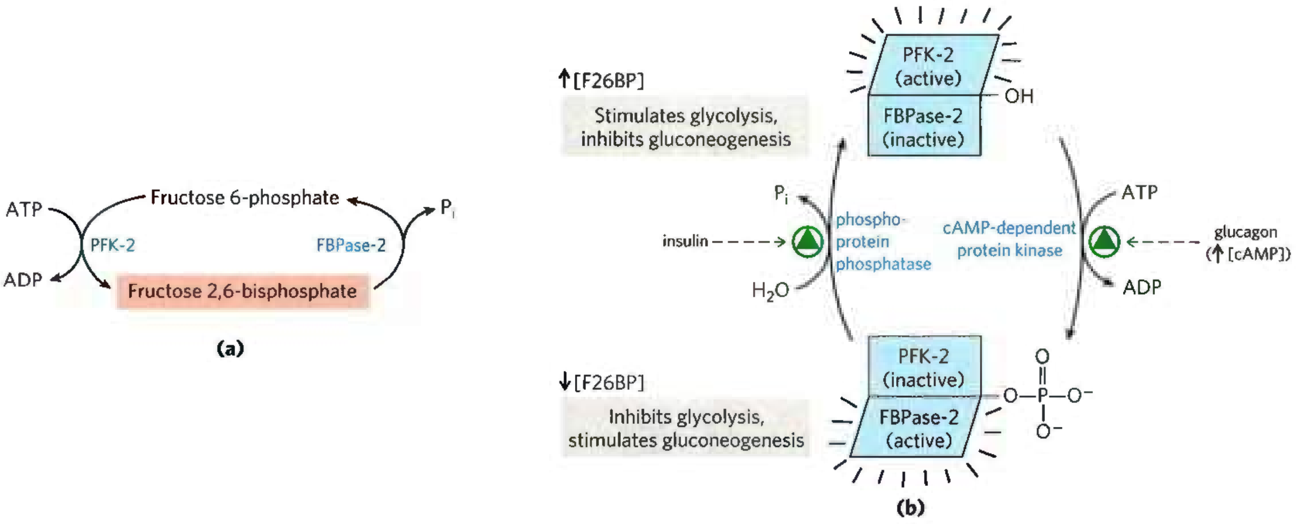


FIGURE 14-24 Role of fructose 2,6-bisphosphate in regulation of glycolysis and gluconeogenesis. Fructose 2,6-bisphosphate (F26BP) has opposite effects on the enzymatic activities of phosphofructokinase-1 (PFK-1, a glycolytic enzyme) and fructose 1,6-bisphosphatase (FBPase-1, a gluconeogenic enzyme). (a) PFK-1 activity in the absence of F26BP (blue curve) is half-maximal when the concentration of fructose 6-phosphate is 2 mM (that is, $K_{0.5}$ = 2 mM). When 0.13 μM F26BP is present (red curve), the $K_{0.5}$ for fructose 6-phosphate is only 0.08 mM. Thus F26BP activates PFK-1 by increasing its apparent affinity for fructose 6-phosphate (see Fig. 14-23b). (b) FBPase-1 activity is inhibited by as little as 1 μM F26BP and is strongly inhibited by 25 μM. In the absence of this inhibitor (blue curve), the $K_{0.5}$ for fructose 1,6-bisphosphate is 5 μM, but in the presence of 25 μM F26BP (red curve), the $K_{0.5}$ is >70 μM. Fructose 2,6-bisphosphate also makes FBPase-1 more sensitive to inhibition by another allosteric regulator, AMP. (c) Summary of regulation by F26BP.



ATP
Fructose 6-phosphate
P_i
PFK-2
FBPase-2
ADP
Fructose 2,6-bisphosphate
(a)
↑[F26BP]
Stimulates glycolysis, inhibits gluconeogenesis
PFK-2 (active)
FBPase-2 (inactive)
OH
P_i
insulin
phospho-protein phosphatase
cAMP-dependent protein kinase
ATP
glucagon (↑[cAMP])
H_2O
ADP
↓[F26BP]
Inhibits glycolysis, stimulates gluconeogenesis
PFK-2 (inactive)
FBPase-2 (active)
O
O—P—O⁻
O⁻
(b)

FIGURE 14-25 Regulation of fructose 2,6-bisphosphate level. (a) The cellular concentration of the regulator fructose 2,6-bisphosphate (F26BP) is determined by the rates of its synthesis by phosphofructokinase-2 (PFK-2) and its breakdown by fructose 2,6-bisphosphatase (FBPase-2). (b) Both enzyme activities are part of the same polypeptide chain, and they are reciprocally regulated by insulin and glucagon.

Xylulose 5-Phosphate Is a Key Regulator of Carbohydrate and Fat Metabolism

Another regulatory mechanism also acts by controlling the level of fructose 2,6-bisphosphate. In the mammalian liver, xylulose 5-phosphate, a product of the pentose phosphate pathway, mediates the increase in glycolysis that follows ingestion of a high-carbohydrate meal. The xylulose 5-phosphate concentration rises as glucose entering the liver is converted to glucose 6-phosphate and enters both the glycolytic and pentose phosphate pathways. Xylulose 5-phosphate activates phosphoprotein phosphatase 2A, which dephosphorylates the bifunctional PFK-2/FBPase-2 enzyme (Fig. 14-25). Dephosphorylation activates PFK-2 and inhibits FBPase-2, and the resulting rise in fructose 2,6-bisphosphate concentration stimulates glycolysis and inhibits gluconeogenesis. The increased glycolysis boosts the production of acetyl-CoA, while the increased flow of hexose through the pentose phosphate pathway generates NADPH. Acetyl-CoA and NADPH are the starting materials for fatty acid synthesis, which increases dramatically in response to intake of a high-carbohydrate meal. Xylulose 5-phosphate also increases the synthesis of *all* the enzymes required for fatty acid synthesis, as we shall see (Fig. 14-28).

The Glycolytic Enzyme Pyruvate Kinase Is Allosterically Inhibited by ATP

At least three isozymes of pyruvate kinase are found in vertebrates, differing in their tissue distribution and their response to modulators. High concentrations of ATP, acetyl-CoA, and long-chain fatty acids (signs of abundant energy supply) allosterically inhibit all isozymes of pyruvate kinase (**Fig. 14-26**). The liver isozyme (L form), but not the muscle isozyme (M form), is subject to further regulation by phosphorylation. When low blood glucose causes glucagon release, cAMP-dependent protein kinase phosphorylates the L isozyme of pyruvate kinase, inactivating it. This slows the use of glucose as a fuel in liver, sparing it for export to the brain and other organs. In muscle, the effect of increased [cAMP] is quite different. In response to epinephrine, cAMP activates glycogen breakdown and glycolysis and provides the fuel needed for the fight-or-flight response.

Conversion of Pyruvate to Phosphoenolpyruvate Is Stimulated When Fatty Acids Are Available

P5 In the pathway leading from pyruvate to glucose, the first control point determines the fate of pyruvate in the mitochondrion: its conversion either to acetyl-CoA (by the pyruvate dehydrogenase complex) to fuel the citric acid cycle (Chapter 16) or to oxaloacetate (by pyruvate carboxylase) to start the process of gluconeogenesis (**Fig. 14-27**). When fatty acids are readily available as fuels, their breakdown in liver mitochondria yields acetyl-CoA, a signal that further oxidation of glucose for fuel is not necessary. Acetyl-CoA is a positive allosteric modulator of pyruvate carboxylase and a negative modulator of pyruvate dehydrogenase, through stimulation of a protein kinase that inactivates the dehydrogenase. When the cell's energy needs are being met, oxidative phosphorylation slows, [NADH] rises relative to [NAD$^+$] and inhibits the citric acid cycle, and acetyl-CoA accumulates. The increased concentration of acetyl-CoA inhibits the pyruvate dehydrogenase complex, slowing the formation of acetyl-CoA from pyruvate, and stimulates gluconeogenesis by activating pyruvate carboxylase, allowing conversion of excess pyruvate to oxaloacetate (and, eventually, glucose).

Oxaloacetate formed in this way is converted to phosphoenolpyruvate (PEP) in the reaction catalyzed by PEP carboxykinase (Fig. 14-16). In mammals, the regulation of this key enzyme occurs primarily at the level of its synthesis and breakdown, in response to dietary

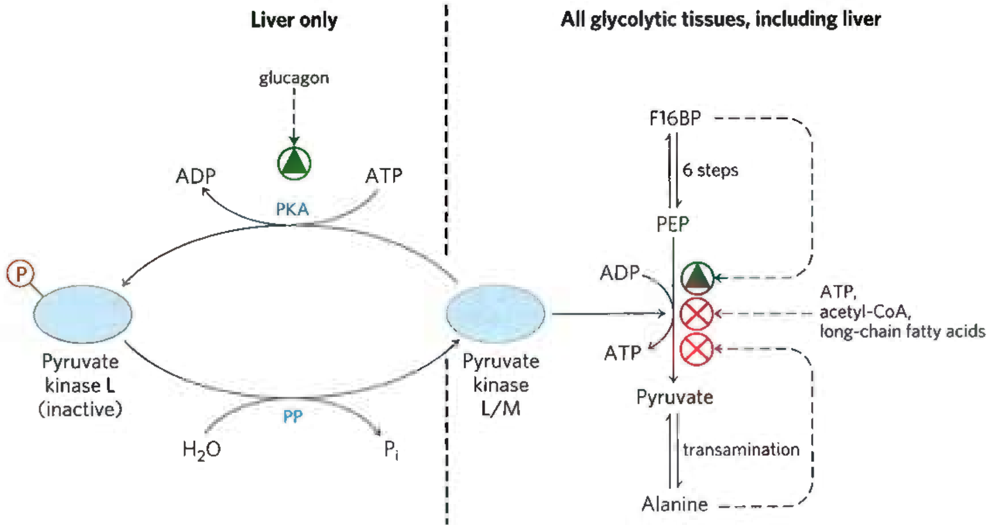


FIGURE 14-26 Regulation of pyruvate kinase. The enzyme is allosterically inhibited by ATP, acetyl-CoA, and long-chain fatty acids (all signs of an abundant energy supply), and the accumulation of fructose 1,6-bisphosphate triggers its activation. Accumulation of alanine, which can be synthesized from pyruvate in one step, allosterically inhibits pyruvate kinase, slowing the production of pyruvate by glycolysis. The liver isozyme (L form) is also regulated hormonally. Glucagon activates cAMP-dependent protein kinase (PKA; see Fig. 15-12), which phosphorylates the pyruvate kinase L isozyme, inactivating it. When the glucagon level drops, a protein phosphatase (PP) dephosphorylates pyruvate kinase, activating it. This mechanism prevents the liver from consuming glucose by glycolysis when blood glucose is low; instead, the liver exports glucose. The muscle isozyme (M form) is not affected by this phosphorylation mechanism.

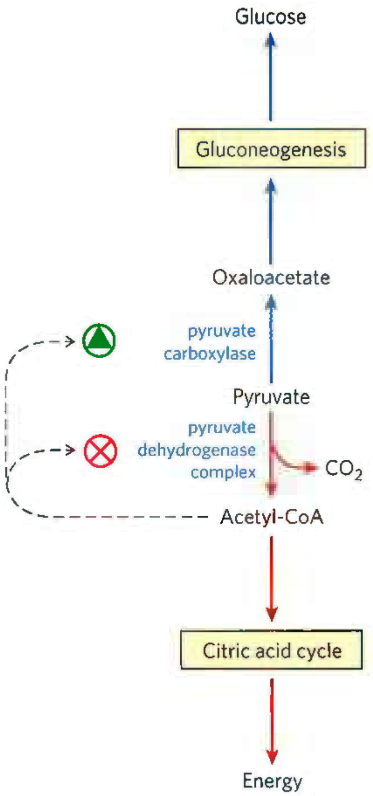


FIGURE 14-27 Two alternative fates for pyruvate. Pyruvate can be converted to glucose and glycogen via gluconeogenesis or oxidized to acetyl-CoA for energy production. The first enzyme in each path is regulated allosterically; acetyl-CoA, produced either by fatty acid oxidation or by the pyruvate dehydrogenase complex, stimulates pyruvate carboxylase and inhibits pyruvate dehydrogenase.

and hormonal signals. Fasting or high glucagon levels act through cAMP to increase the rate of transcription and to stabilize the mRNA. Insulin, or high blood [glucose], has the opposite effects. We discuss this transcriptional regulation in more detail below. Generally triggered by a signal from outside the cell, these changes take place on a time scale of minutes to days.

Transcriptional Regulation Changes the Number of Enzyme Molecules

Most of the regulatory actions discussed thus far are mediated by fast, reversible mechanisms to change the activity of existing enzyme molecules: allosteric effects, covalent alteration (phosphorylation) of the enzyme, or binding of a regulatory protein. Another set of regulatory processes involves changes in the number of molecules of an enzyme in the cell, through changes in the balance of enzyme synthesis and breakdown. Our discussion now turns briefly to regulation of transcription through signal-activated transcription factors. Transcriptional control is discussed in more detail in Chapter 28.

In Chapter 12 we encountered nuclear receptors and transcription factors in the context of insulin signaling. Insulin acts through its receptor in the plasma membrane to turn on at least two distinct signaling pathways, each involving activation of a protein kinase (MAP kinase and protein kinase B). The kinases phosphorylate transcription factors, which then act in the nucleus to stimulate the synthesis of enzymes needed for cell growth and division. More than 150 genes are transcriptionally regulated by insulin, many of which encode proteins we have described here (Table 14-5).

P5 One transcription factor important to carbohydrate metabolism is **ChREBP** (**carbohydrate response element binding protein**; Fig. 14-28), which is expressed primarily in liver, adipose tissue, and kidney. It coordinates the synthesis of enzymes needed for carbohydrate and fat synthesis. ChREBP in its phosphorylated form is inactive and is located in the cytosol. When the phosphoprotein phosphatase PP2A removes a phosphoryl group from ChREBP, the transcription factor can enter the nucleus. Here, nuclear PP2A removes

TABLE 14-5 Some of the Many Genes Regulated by Insulin

Change in gene expression	Role in glucose metabolism
Increased expression	
Hexokinase II Hexokinase IV Phosphofructokinase-1 (PFK-1) PFK-2/FBPase-2 Pyruvate kinase	Essential for glycolysis, which consumes glucose for energy
Glucose 6-phosphate dehydrogenase 6-Phosphogluconate dehydrogenase Malic enzyme	Produce NADPH, which is essential for conversion of glucose to lipids
ATP-citrate lyase Pyruvate dehydrogenase	Produce acetyl-CoA, which is essential for conversion of glucose to lipids
Acetyl-CoA carboxylase Fatty acid synthase complex Stearoyl-CoA dehydrogenase Acyl-CoA–glycerol transferases	Essential for conversion of glucose to lipids
Decreased expression	
PEP carboxykinase Glucose 6-phosphatase (catalytic subunit)	Essential for glucose production by gluconeogenesis

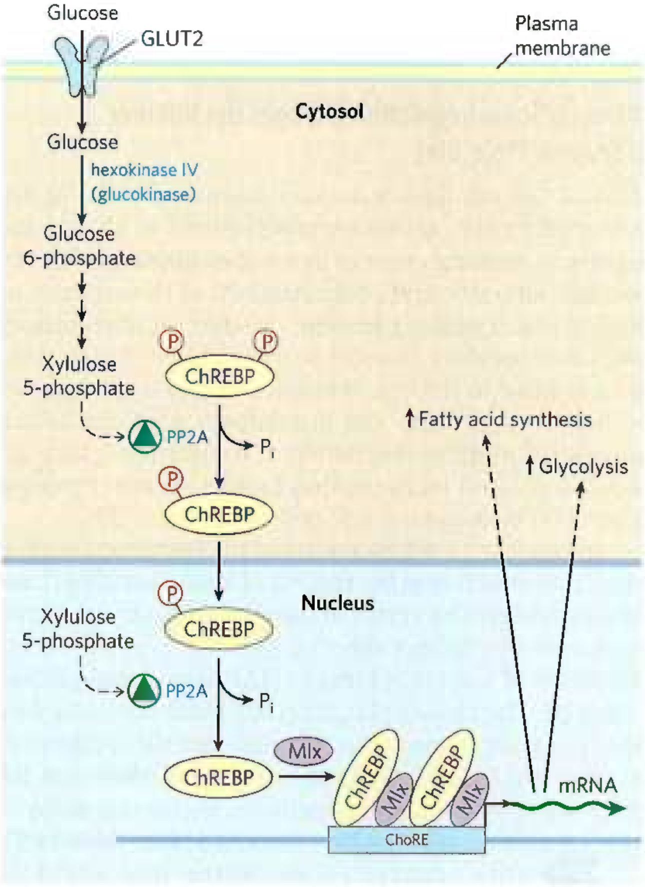


FIGURE 14-28 Mechanism of gene regulation by the transcription factor ChREBP. When ChREBP in the cytosol of a hepatocyte is phosphorylated on a Ser residue and a Thr residue, it cannot enter the nucleus. Dephosphorylation of Ⓟ—Ser by protein phosphatase PP2A allows ChREBP to enter the nucleus, where a second dephosphorylation, of Ⓟ—Thr, activates ChREBP so that it can associate with its partner protein, Mlx. ChREBP-Mlx now binds to the carbohydrate response element (ChoRE) in the promoter and stimulates transcription. PP2A is allosterically activated by xylulose 5-phosphate, an intermediate in the pentose phosphate pathway.

another phosphoryl group, and ChREBP now joins with a partner protein, Mlx, and turns on the synthesis of several enzymes: pyruvate kinase; fatty acid synthase; and acetyl-CoA carboxylase, the first enzyme in the path to fatty acid synthesis.

Controlling the activity of PP2A—and thus, ultimately, the synthesis of this group of metabolic enzymes—is xylulose 5-phosphate, an intermediate of the pentose phosphate pathway (see Fig. 14-31). When blood glucose concentration is high, glucose enters the liver and is phosphorylated by hexokinase IV. The glucose 6-phosphate thus formed can enter either the glycolytic pathway or the pentose phosphate pathway. If the latter, two initial oxidations produce xylulose 5-phosphate, which serves as a signal that the glucose-utilizing pathways are well-supplied with substrate. It accomplishes this by allosterically activating PP2A, which then dephosphorylates ChREBP, allowing the transcription factor to turn on the expression of genes for enzymes of glycolysis and fat synthesis (Fig. 14-28).

SUMMARY 14.5 *Coordinated Regulation of Glycolysis and Gluconeogenesis*

- Glycolysis and gluconeogenesis are reciprocally regulated to prevent wasteful operation of both pathways at the same time.
- Hexokinase IV (glucokinase) has kinetic properties related to its special role in the liver: releasing glucose to the blood when blood [glucose] is low; taking up and metabolizing glucose when blood [glucose] is high. Hexokinases I, II, and III are all inhibited by their product, glucose 6-phosphate.
- PFK-1 is allosterically inhibited by high [ATP]; low [AMP] inhibits FBPase-1. High [ATP] therefore slows glycolysis and speeds gluconeogenesis.
- Reciprocal allosteric control of glycolysis and gluconeogenesis is mainly achieved by the opposing effects of fructose 2,6-bisphosphate on PFK-1 and FBPase-1. Fructose 2,6-bisphosphate formation is stimulated, indirectly, by insulin, and inhibited by epinephrine.
- Xylulose 5-phosphate, an intermediate of the pentose phosphate pathway, activates phosphoprotein phosphatase PP2A. Activated PP2A tips the balance toward glucose uptake, glycogen synthesis, and lipid synthesis in the liver.
- Pyruvate kinase is allosterically inhibited by ATP, and the liver isozyme also is inhibited by cAMP-dependent phosphorylation. When [ATP] is high, glycolysis is slowed.
- When fatty acids are readily available as fuels, their breakdown in liver mitochondria yields acetyl-CoA, a signal that further oxidation of glucose for fuel is not necessary. Acetyl-CoA activates pyruvate carboxylase, thus favoring gluconeogenesis.
- Transcription factors such as ChREBP act in the nucleus to regulate the expression of specific genes coding for enzymes of the glycolytic and gluconeogenic pathways.

14.6 Pentose Phosphate Pathway of Glucose Oxidation

P6 In most animal tissues, the major catabolic fate of glucose 6-phosphate is glycolytic breakdown to pyruvate, much of which is then oxidized via the citric acid cycle, ultimately leading to the formation of ATP. Glucose 6-phosphate does have other catabolic fates, however, which lead to specialized products needed by the cell. Of particular importance in some tissues is the oxidation of glucose 6-phosphate to pentose phosphates by the **pentose phosphate pathway** (also called the **phosphogluconate pathway** or the **hexose monophosphate pathway**; Fig. 14-29). In this oxidative pathway, $NADP^+$ is the electron acceptor, yielding NADPH. Rapidly dividing cells, such as those of bone marrow, skin, and intestinal mucosa, and those of tumors, use the pentose ribose 5-phosphate to make RNA, DNA, and such coenzymes as ATP, NADH, $FADH_2$, and coenzyme A.

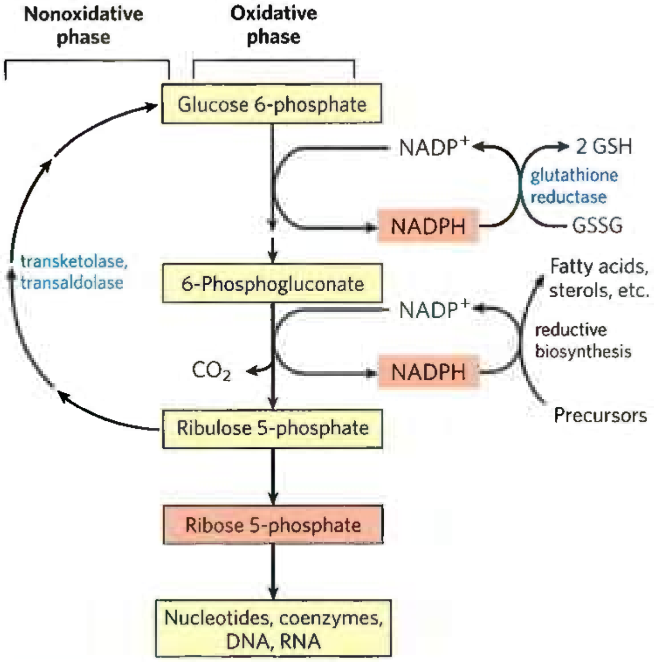

Nonoxidative phase
Oxidative phase
Glucose 6-phosphate
$NADP^+$
2 GSH
glutathione reductase
NADPH
GSSG
transketolase, transaldolase
6-Phosphogluconate
Fatty acids, sterols, etc.
$NADP^+$
reductive biosynthesis
CO_2
NADPH
Precursors
Ribulose 5-phosphate
Ribose 5-phosphate
Nucleotides, coenzymes, DNA, RNA

FIGURE 14-29 General scheme of the pentose phosphate pathway. NADPH formed in the oxidative phase is used to reduce glutathione, GSSG (see Box 14-4), and to support reductive biosynthesis. The other product of the oxidative phase is ribose 5-phosphate, which serves as a precursor for nucleotides, coenzymes, and nucleic acids. In cells that are not using ribose 5-phosphate for biosynthesis, the nonoxidative phase recycles six molecules of the pentose into five molecules of the hexose glucose 6-phosphate, allowing continued production of NADPH and converting glucose 6-phosphate (in six cycles) to CO_2.

In other tissues, the essential product of the pentose phosphate pathway is not the pentoses but the electron donor NADPH, needed for reductive biosynthesis or to counter the damaging effects of oxygen radicals. Tissues that carry out extensive fatty acid synthesis (liver, adipose, lactating mammary gland) or very active synthesis of cholesterol and steroid hormones (liver, adrenal glands, gonads) require the NADPH provided by this pathway. Erythrocytes and the cells of the lens and cornea are directly exposed to oxygen and thus to the damaging free radicals generated by oxygen. By maintaining a reducing environment (a high ratio of NADPH to $NADP^+$ and a high ratio of reduced glutathione to oxidized glutathione), such cells can prevent or undo oxidative damage to proteins, lipids, and other sensitive molecules. In erythrocytes, the NADPH produced by the pentose phosphate pathway is so important in preventing oxidative damage that a genetic defect in glucose 6-phosphate dehydrogenase, the first enzyme of the pathway, can have serious medical consequences (**Box 14-4**).

The Oxidative Phase Produces NADPH and Pentose Phosphates

P6 The first reaction of the pentose phosphate pathway (**Fig. 14-30**) is the oxidation of glucose 6-phosphate by **glucose 6-phosphate dehydrogenase (G6PD)** to form

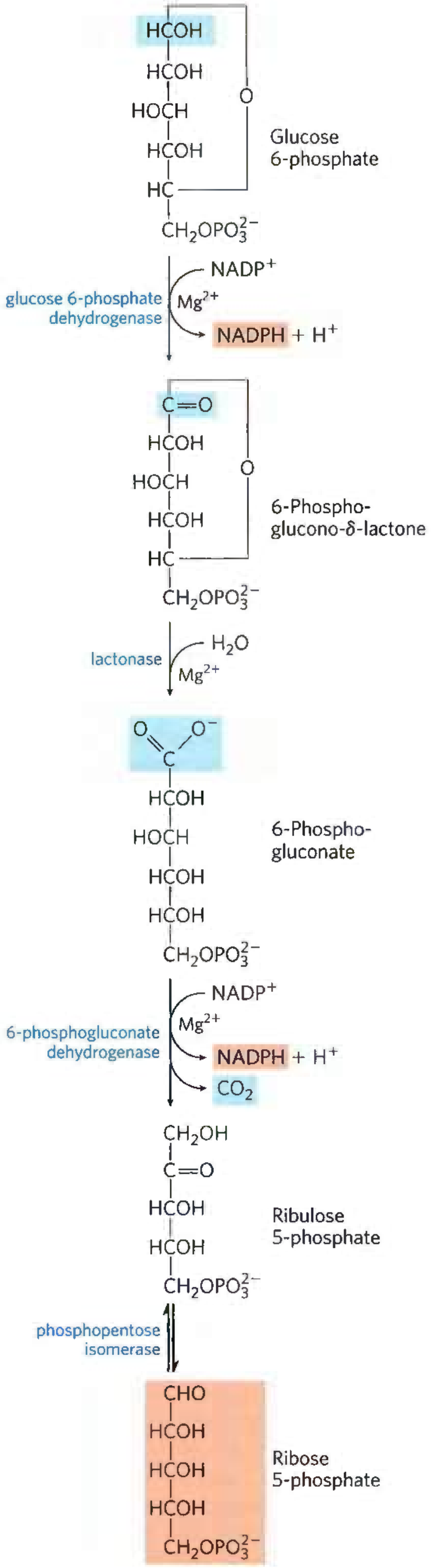

Glucose 6-phosphate
$NADP^+$
glucose 6-phosphate dehydrogenase
Mg^{2+}
$NADPH + H^+$
6-Phospho-glucono-δ-lactone
lactonase
H_2O
Mg^{2+}
6-Phospho-gluconate
$NADP^+$
6-phosphogluconate dehydrogenase
Mg^{2+}
$NADPH + H^+$
CO_2
Ribulose 5-phosphate
phosphopentose isomerase
Ribose 5-phosphate

FIGURE 14-30 Oxidative reactions of the pentose phosphate pathway. The end products are ribose 5-phosphate, CO_2, and NADPH.

BOX 14-4 MEDICINE

Why Pythagoras Wouldn't Eat Falafel: Glucose 6-Phosphate Dehydrogenase Deficiency

Fava beans, an ingredient of falafel, have been an important food source in the Mediterranean and the Middle East since antiquity. The Greek philosopher and mathematician Pythagoras prohibited his followers from dining on fava beans, perhaps because they make many people sick with a condition called favism, which can be fatal. In favism, erythrocytes begin to lyse 24 to 48 hours after ingestion of the beans, releasing free hemoglobin into the blood. Jaundice and sometimes kidney failure can result. Similar symptoms can occur with ingestion of the antimalarial drug primaquine or of sulfa antibiotics, or following exposure to certain herbicides. These symptoms have a genetic basis: glucose 6-phosphate dehydrogenase (G6PD) deficiency, which affects about 400 million people worldwide. Most G6PD-deficient individuals are asymptomatic; only the combination of G6PD deficiency and certain environmental factors produces the clinical manifestations.

Glucose 6-phosphate dehydrogenase catalyzes the first step in the pentose phosphate pathway (see Fig. 14-30), which produces NADPH. This reductant, essential in many biosynthetic pathways, also protects cells from oxidative damage by hydrogen peroxide (H_2O_2) and superoxide free radicals, highly reactive oxidants generated as metabolic byproducts and through the actions of drugs such as primaquine and natural products such as divicine—the toxic ingredient of fava beans. During normal detoxification, H_2O_2 is converted to H_2O by reduced glutathione and glutathione peroxidase, and the oxidized glutathione is converted back to the reduced form by glutathione reductase and NADPH (Fig. 1). H_2O_2 is also broken down to H_2O and O_2 by catalase, which also requires NADPH. In G6PD-deficient individuals, the NADPH production is diminished and detoxification of H_2O_2 is inhibited. Cellular damage results: lipid peroxidation leading to breakdown of erythrocyte membranes and oxidation of proteins and DNA.

The geographic distribution of G6PD deficiency is instructive. Frequencies as high as 25% occur in tropical Africa, parts of the Middle East, and Southeast Asia, areas where malaria is most prevalent. In addition to such epidemiological observations, in vitro studies show that growth of one malaria parasite, *Plasmodium falciparum*, is inhibited in G6PD-deficient erythrocytes. The parasite is very sensitive to oxidative damage and is killed by a level of oxidative stress that is tolerable to a G6PD-deficient human host. Because the advantage of resistance to malaria balances the disadvantage of lowered resistance to oxidative damage, natural selection sustains the G6PD-deficient genotype in human populations where malaria is prevalent. Only under overwhelming oxidative stress, caused by drugs, herbicides, or divicine, does G6PD deficiency cause serious medical problems.

An antimalarial drug such as primaquine is believed to act by causing oxidative stress to the parasite. It is ironic that antimalarial drugs can cause human illness through the same biochemical mechanism that provides resistance to malaria. Divicine also acts as an antimalarial drug, and ingestion of fava beans may protect against malaria. By refusing to eat falafel, many Pythagoreans with normal G6PD activity may have unwittingly increased their risk of malaria!

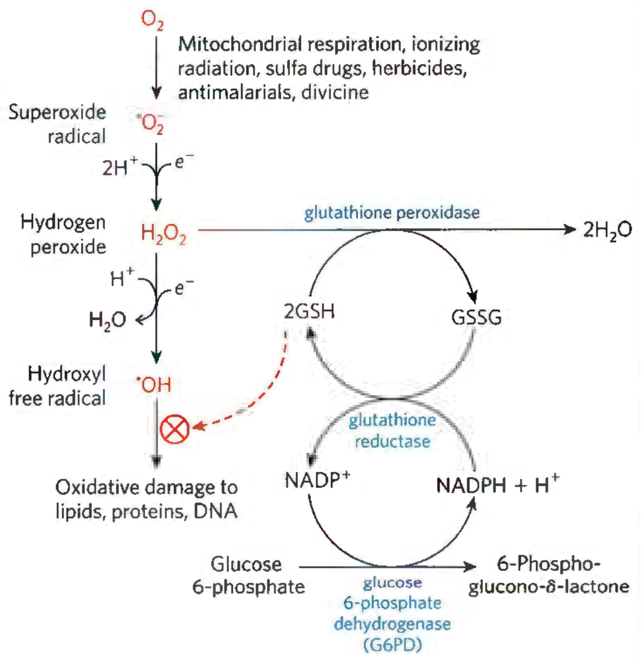


FIGURE 1 Role of NADPH and glutathione in protecting cells against highly reactive oxygen derivatives. Reduced glutathione (GSH) protects the cell by destroying hydrogen peroxide and hydroxyl free radicals. Regeneration of GSH from its oxidized form (GSSG) requires the NADPH produced in the glucose 6-phosphate dehydrogenase reaction.

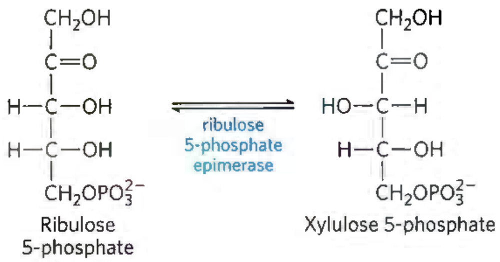
6-phosphoglucono-δ-lactone, an intramolecular ester. $NADP^+$ is the electron acceptor, and the overall equilibrium lies far in the direction of NADPH formation. The lactone is hydrolyzed to the free acid 6-phosphogluconate by a specific **lactonase**, then 6-phosphogluconate undergoes oxidation and decarboxylation by **6-phosphogluconate dehydrogenase** to form the ketopentose ribulose 5-phosphate; the reaction also generates a second molecule of NADPH. **Phosphopentose isomerase** converts ribulose 5-phosphate to its aldose isomer, ribose 5-phosphate. In some tissues, the pentose phosphate pathway ends at this point, and its overall equation is

$$\text{Glucose 6-phosphate} + 2\text{NADP}^+ + \text{H}_2\text{O} \longrightarrow \text{ribose 5-phosphate} + \text{CO}_2 + 2\text{NADPH} + 2\text{H}^+$$

The net result is the production of NADPH, a reductant for biosynthetic reactions, and ribose 5-phosphate, a precursor for nucleotide synthesis.

The Nonoxidative Phase Recycles Pentose Phosphates to Glucose 6-Phosphate

P6 In tissues that require primarily NADPH, the pentose phosphates produced in the oxidative phase of the pathway are recycled into glucose 6-phosphate. In this nonoxidative phase, ribulose 5-phosphate is first epimerized to xylulose 5-phosphate:



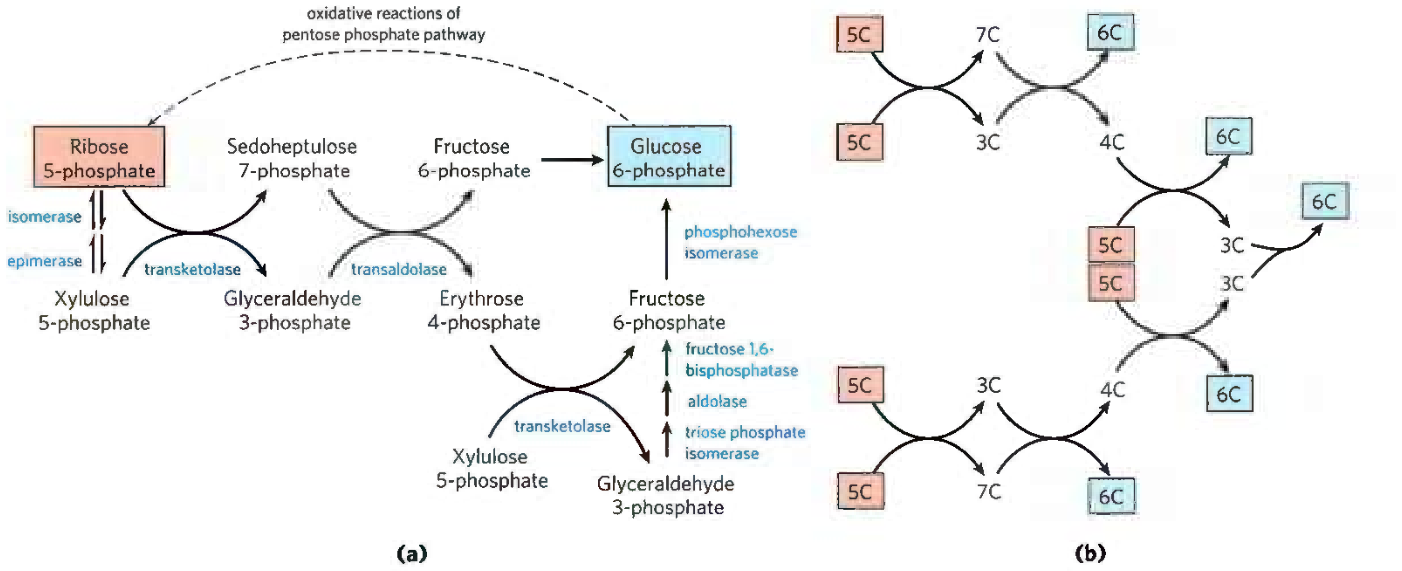
CH_2OH
C=O
H—C—OH
H—C—OH
$CH_2OPO_3^{2-}$
Ribulose 5-phosphate
ribulose 5-phosphate epimerase
CH_2OH
C=O
HO—C—H
H—C—OH
$CH_2OPO_3^{2-}$
Xylulose 5-phosphate

Then, in a series of rearrangements of the carbon skeletons (**Fig. 14-31**), six five-carbon sugar phosphates are converted to five six-carbon sugar phosphates, completing the cycle and allowing continued oxidation of glucose 6-phosphate with production of NADPH. Continued recycling leads ultimately to the conversion of glucose 6-phosphate to six CO_2. Two enzymes unique to the pentose phosphate pathway act in these interconversions of sugars: transketolase and transaldolase. **Transketolase** catalyzes the transfer of a two-carbon fragment from a ketose donor to an aldose acceptor (**Fig. 14-32a**). In its first appearance in the pentose phosphate pathway, transketolase transfers C-1 and C-2 of xylulose 5-phosphate to ribose 5-phosphate, forming the seven-carbon product sedoheptulose 7-phosphate (Fig. 14-32b). The remaining three-carbon fragment from xylulose is glyceraldehyde 3-phosphate.

Next, **transaldolase** catalyzes a reaction similar to the aldolase reaction of glycolysis: a three-carbon fragment is removed from sedoheptulose 7-phosphate and condensed with glyceraldehyde 3-phosphate, forming fructose 6-phosphate and the tetrose erythrose 4-phosphate (**Fig. 14-33**). Now transketolase acts again, forming fructose 6-phosphate and glyceraldehyde 3-phosphate from erythrose 4-phosphate and xylulose 5-phosphate (**Fig. 14-34**). Two molecules of glyceraldehyde 3-phosphate formed by two iterations of these reactions can be converted to a molecule of fructose 1,6-bisphosphate as in gluconeogenesis (Fig. 14-16), and finally FBPase-1 and phosphohexose isomerase convert fructose 1,6-bisphosphate to glucose 6-phosphate. Overall, six pentose phosphates have been converted to five hexose phosphates (Fig. 14-32b)—the cycle is now complete.

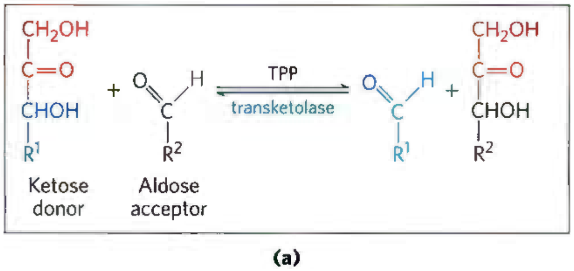
Transketolase requires the cofactor thiamine pyrophosphate (TPP), which stabilizes a two-carbon carbanion in this reaction (**Fig. 14-35a**), just as it does in the pyruvate decarboxylase reaction (Fig. 14-13). Transaldolase uses a Lys side chain to form a Schiff base with the carbonyl group of its substrate, a ketose, thereby stabilizing a carbanion (Fig. 14-35b) that is central to the reaction mechanism.

The first and third steps of the **oxidative pentose phosphate pathway** shown in Figure 14-30 are oxidations with large, negative standard free-energy changes and are essentially irreversible in the cell. The reactions of the nonoxidative part of the pentose phosphate pathway (Fig. 14-31) are readily reversible and thus also provide a means of converting hexose phosphates to pentose phosphates.



oxidative reactions of pentose phosphate pathway
Ribose 5-phosphate
isomerase
epimerase
Xylulose 5-phosphate
transketolase
Sedoheptulose 7-phosphate
Glyceraldehyde 3-phosphate
transaldolase
Fructose 6-phosphate
Erythrose 4-phosphate
Glucose 6-phosphate
phosphohexose isomerase
Fructose 6-phosphate
fructose 1,6-bisphosphatase
aldolase
triose phosphate isomerase
transketolase
Xylulose 5-phosphate
Glyceraldehyde 3-phosphate
(a)
5C
5C
7C
3C
6C
4C
6C
5C
5C
3C
3C
6C
4C
6C
5C
5C
3C
7C
6C
(b)

FIGURE 14-31 Nonoxidative reactions of the pentose phosphate pathway. (a) These reactions convert pentose phosphates to hexose phosphates, allowing the oxidative reactions to continue. Transketolase and transaldolase are specific to this pathway; the other enzymes also serve in the glycolytic or gluconeogenic pathways. (b) A schematic diagram showing the pathway from six pentoses (5C) to five hexoses (6C). Note that this involves two sets of the interconversions shown in (a). Every reaction shown here is reversible; unidirectional arrows are used only to make clear the direction of the reactions during continuous oxidation of glucose 6-phosphate. In the light-independent reactions of photosynthesis, the direction of these reactions is reversed.


Ketose donor
Aldose acceptor
TPP
transketolase
(a)

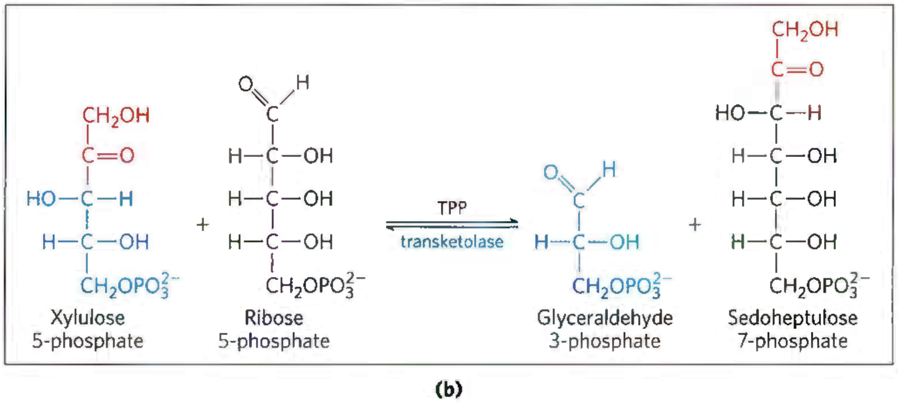

Xylulose 5-phosphate
Ribose 5-phosphate
TPP
transketolase
Glyceraldehyde 3-phosphate
Sedoheptulose 7-phosphate
(b)

FIGURE 14-32 The first reaction catalyzed by transketolase. (a) The general reaction catalyzed by transketolase is the transfer of a two-carbon group, carried temporarily on enzyme-bound TPP, from a ketose donor to an aldose acceptor. (b) Conversion of two pentose phosphates to a triose phosphate and a seven-carbon sugar phosphate, sedoheptulose 7-phosphate.

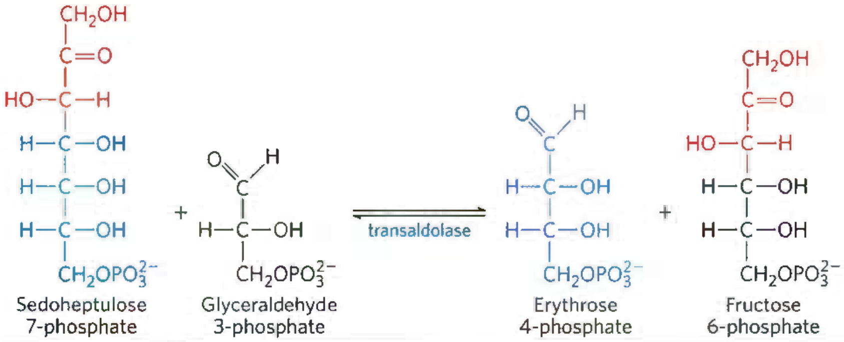

Sedoheptulose 7-phosphate
Glyceraldehyde 3-phosphate
transaldolase
Erythrose 4-phosphate
Fructose 6-phosphate

FIGURE 14-33 The reaction catalyzed by transaldolase.

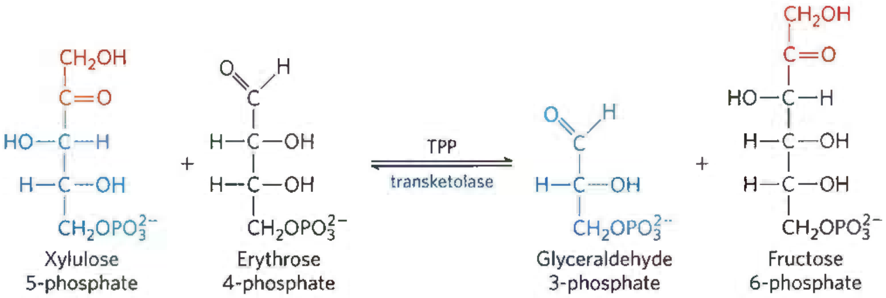

Xylulose 5-phosphate
Erythrose 4-phosphate
TPP
transketolase
Glyceraldehyde 3-phosphate
Fructose 6-phosphate

FIGURE 14-34 The second reaction catalyzed by transketolase.

As we shall see in Chapter 20, a process that converts hexose phosphates to pentose phosphates is central to the photosynthetic assimilation of CO_2 by plants. That pathway, the **reductive pentose phosphate pathway**, is essentially the reversal of the reactions shown in Figure 14-31 and employs many of the same enzymes.

All the enzymes of the pentose phosphate pathway are located in the cytosol, like those of glycolysis and most of those of gluconeogenesis. In fact, these three pathways are connected through several shared intermediates and enzymes. The glyceraldehyde 3-phosphate formed by the action of transketolase is readily converted to dihydroxyacetone phosphate by the glycolytic enzyme

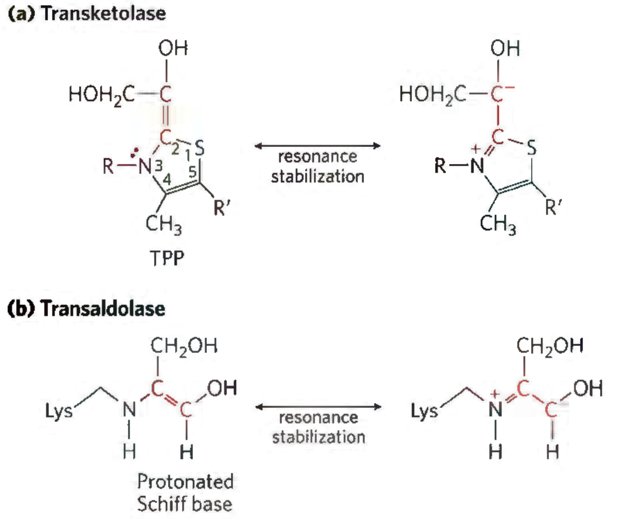


FIGURE 14-35 Carbanion intermediates stabilized by covalent interactions with transketolase and transaldolase. (a) The ring of TPP stabilizes the carbanion in the dihydroxyethyl group carried by transketolase. (b) In the transaldolase reaction, the protonated Schiff base formed between the ε-amino group of a Lys side chain and the substrate stabilizes the C-3 carbanion formed after aldol cleavage.

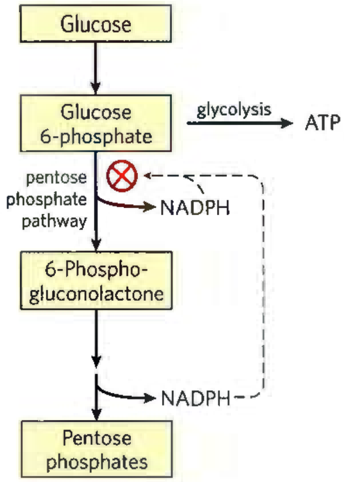


FIGURE 14-36 Role of NADPH in regulating the partitioning of glucose 6-phosphate between glycolysis and the pentose phosphate pathway. When NADPH is forming faster than it is being used for biosynthesis and glutathione reduction, [NADPH] rises and inhibits the first enzyme in the pentose phosphate pathway. As a result, more glucose 6-phosphate is available for glycolysis.

triose phosphate isomerase, and these two trioses can be joined by the aldolase as in gluconeogenesis, forming fructose 1,6-bisphosphate. Alternatively, the triose phosphates can be oxidized to pyruvate by the glycolytic reactions. The fate of the trioses is determined by the cell's relative needs for pentose phosphates, NADPH, and ATP.

Glucose 6-Phosphate Is Partitioned between Glycolysis and the Pentose Phosphate Pathway

P6 Whether glucose 6-phosphate enters glycolysis or the pentose phosphate pathway depends on the current needs of the cell and on the concentration of $NADP^+$ in the cytosol. Without this electron acceptor, the first reaction of the pentose phosphate pathway (catalyzed by glucose 6-phosphate dehydrogenase) cannot proceed. When a cell is rapidly converting NADPH to $NADP^+$ in biosynthetic reductions, $[NADP^+]$ rises, allosterically stimulating glucose 6-phosphate dehydrogenase and thereby increasing the flux of glucose 6-phosphate through the pentose phosphate pathway (**Fig. 14-36**). When the demand for NADPH slows, the level of $NADP^+$ drops, the pentose phosphate pathway slows, and glucose 6-phosphate is instead used to fuel glycolysis.

Thiamine Deficiency Causes Beriberi and Wernicke-Korsakoff Syndrome

Thiamine, precursor to the cofactor thiamine pyrophosphate (TPP), is one of the B vitamins, essential in humans. Lack of vitamin B_1 in the diet leads to a range of medical problems. The condition known as beriberi is characterized by an accumulation of body fluids (swelling), pain, paralysis, and ultimately, without treatment, death. Wernicke-Korsakoff syndrome, also caused by a severe deficiency of thiamine, typically includes problems with voluntary movements, reflected in abnormal eye movements and gait, and neurological defects. The syndrome is more common among heavy drinkers than in the general population because chronic, heavy alcohol consumption interferes with the intestinal absorption of thiamine. The syndrome can be exacerbated by a mutation in the gene for transketolase that results in an enzyme with a lowered affinity for TPP—an affinity one-tenth that of the normal enzyme. This defect makes individuals much more sensitive to a thiamine deficiency: even a moderate thiamine deficiency (tolerable in individuals with an unmutated transketolase) can result in a transketolase that is not saturated with TPP at its normal concentration. The result is a slowing down of the whole pentose phosphate pathway. In people with Wernicke-Korsakoff syndrome, this mutation results in a worsening of symptoms, which can include severe memory loss, mental confusion, and partial paralysis. ■

SUMMARY 14.6 *Pentose Phosphate Pathway of Glucose Oxidation*

- The *oxidative* pentose phosphate pathway produces NADPH and pentose phosphates. Tissues that carry out extensive fatty acid synthesis (liver, adipose, lactating mammary gland) or very active synthesis of cholesterol and steroid hormones (liver, adrenal glands, gonads) require the NADPH provided by this pathway.
- Ribose 5-phosphate is a precursor for nucleotide and nucleic acid synthesis.
- The first, oxidative phase of the pentose phosphate pathway consists of two oxidations that convert glucose

6-phosphate to ribulose 5-phosphate and reduce $NADP^+$ to NADPH.

- The second, nonoxidative phase of the pentose phosphate pathway comprises steps that convert pentose phosphates to glucose 6-phosphate, which begins the oxidative cycle again.
- Entry of glucose 6-phosphate either into glycolysis or into the pentose phosphate pathway is largely determined by the relative concentrations of $NADP^+$ and NADPH.

KEY TERMS

Terms in bold are defined in the glossary.

glycolysis	511
hexokinase	515
isozymes	515
phosphofructokinase-1 (PFK-1)	516
fructose 1,6-bisphosphate aldolase	516
aldolase	516
triose phosphate isomerase	517
glyceraldehyde 3-phosphate dehydrogenase	518
acyl phosphate	518
phosphoglycerate kinase	518
substrate-level phosphorylation	520
respiration-linked phosphorylation	520
phosphoglycerate mutase	520
enolase	520
phosphoenolpyruvate (PEP)	520
pyruvate kinase	521
glycogen phosphorylase	522
mutases	522
isomerases	522
lactose intolerance	523
galactosemia	523
hypoxia	525
fermentation	525
lactic acid fermentation	525
ethanol (alcohol) fermentation	525
lactate dehydrogenase	526
pyruvate decarboxylase	530
alcohol dehydrogenase	530
thiamine pyrophosphate (TPP)	530
gluconeogenesis	533
biotin	534
fructose 1,6-bisphosphatase (FBPase-1)	537
glucogenic	538
glyceroneogenesis	538
glucagon	542
fructose 2,6-bisphosphate	542
phosphofructokinase-2 (PFK-2)	542
fructose 2,6-bisphosphatase (FBPase-2)	542
carbohydrate response element binding protein (ChREBP)	545
pentose phosphate pathway	546
phosphogluconate pathway	546
hexose monophosphate pathway	546
glucose 6-phosphate dehydrogenase (G6PD)	547
6-phosphogluconate dehydrogenase	548

PROBLEMS

1. Is the Hexokinase Reaction at Equilibrium in Cells? For the reaction catalyzed by the enzyme hexokinase

$$\text{Glucose} + \text{ATP} \rightleftharpoons \text{glucose 6-phosphate} + \text{ADP}$$

the equilibrium constant, K_{eq}, is 7.8×10^2. In living *E. coli* cells, [ATP] = 5 mM, [ADP] = 0.5 mM, [glucose] = 2 mM, and [glucose 6-phosphate] = 1 mM. Is the reaction at equilibrium in *E. coli*?

2. Equation for the Preparatory Phase of Glycolysis Write balanced biochemical equations for all the reactions in the catabolism of glucose to two molecules of glyceraldehyde 3-phosphate (the preparatory phase of glycolysis), including the standard free-energy change for each reaction. Then write the overall or net equation for the preparatory phase of glycolysis, with the net standard free-energy change.

3. Payoff Phase of Glycolysis: Fate of Pyruvate in Active Skeletal Muscle In working skeletal muscle under anaerobic conditions, glyceraldehyde 3-phosphate is converted to pyruvate (the payoff phase of glycolysis), and the pyruvate is reduced to lactate. Write balanced biochemical equations for all the reactions in this process, with the standard free-energy change for each reaction. Then write the overall or net equation for the payoff phase of glycolysis with fermentation to lactate, including the net standard free-energy change.

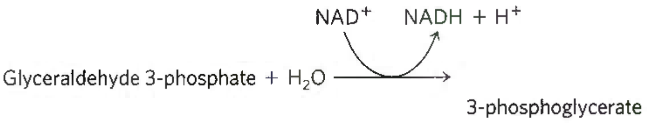
4. Energetics of the Aldolase Reaction Aldolase catalyzes the glycolytic reaction

$$\text{Fructose 1,6-bisphosphate} \longrightarrow \text{glyceraldehyde 3-phosphate} + \text{dihydroxyacetone phosphate}$$

The standard free-energy change for this reaction in the direction written is +23.8 kJ/mol. The concentrations of the three intermediates in the hepatocyte of a mammal are fructose 1,6-bisphosphate, 1.4×10^{-5} M; glyceraldehyde 3-phosphate, 3×10^{-6} M; and dihydroxyacetone phosphate, 1.6×10^{-5} M. At body temperature (37 °C), what is the actual free-energy change for the reaction?

5. Equivalence of Triose Phosphates A researcher adds ^{14}C-labeled glyceraldehyde 3-phosphate to a yeast extract. After a short time, she isolates fructose 1,6-bisphosphate labeled with ^{14}C at C-3 and C-4. What was the location of the ^{14}C label in the starting glyceraldehyde 3-phosphate? Where did the second ^{14}C label in fructose 1,6-bisphosphate come from? Explain.

6. Glycolysis Shortcut Suppose you discovered a mutant yeast whose glycolytic pathway was shorter because of the presence of a new enzyme catalyzing the reaction



$$\text{Glyceraldehyde 3-phosphate} + H_2O \xrightarrow{NAD^+ \;\; NADH + H^+} \text{3-phosphoglycerate}$$

Would shortening the glycolytic pathway in this way benefit the cell? Explain.

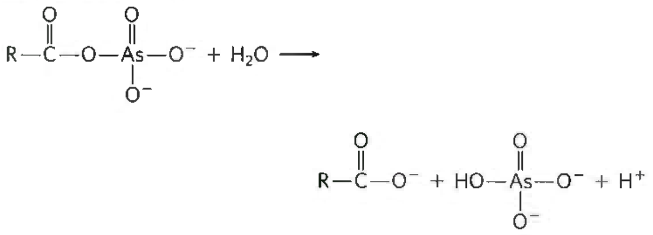
7. Role of Lactate Dehydrogenase During strenuous activity, the demand for ATP in muscle tissue vastly increases. In rabbit leg muscle or turkey flight muscle, ATP production is almost exclusively a product of lactic acid fermentation. Phosphoglycerate kinase and pyruvate kinase catalyze the two reactions that form ATP in the payoff phase of glycolysis. Suppose skeletal muscle were devoid of lactate dehydrogenase. Could it carry out strenuous physical activity; that is, could it generate ATP at a high rate by glycolysis? Explain.

8. Efficiency of ATP Production in Muscle The transformation of glucose to lactate in myocytes releases only about 7%

of the free energy released when glucose is completely oxidized to CO_2 and H_2O. Does this mean that glycolysis with lactate fermentation under anaerobic conditions in muscle is a wasteful use of glucose? Explain.

9. Free-Energy Change for Triose Phosphate Oxidation The oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate, catalyzed by glyceraldehyde 3-phosphate dehydrogenase, proceeds with an unfavorable equilibrium constant ($K'_{eq} = 0.08$; $\Delta G'^{\circ} = 6.3$ kJ/mol), yet the flow through this point in the glycolytic pathway proceeds smoothly. How does the cell overcome the unfavorable equilibrium?

10. Arsenate Poisoning Arsenate is structurally and chemically similar to inorganic phosphate (P_i), and many enzymes that require phosphate will also use arsenate. Organic compounds of arsenate are less stable than analogous phosphate compounds, however. For example, acyl *arsenates* decompose rapidly by hydrolysis, as shown.



$$R-\overset{\overset{O}{\|}}{C}-O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{As}}-O^- + H_2O \longrightarrow R-\overset{\overset{O}{\|}}{C}-O^- + HO-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{As}}-O^- + H^+$$

On the other hand, acyl *phosphates*, such as 1,3-bisphosphoglycerate, are more stable and undergo further enzyme-catalyzed transformation in cells.

(a) Predict the effect on the net reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase if phosphate were replaced by arsenate.

(b) What would be the consequence to an organism if arsenate were substituted for phosphate? Arsenate is very toxic to most organisms. Explain why.

11. Requirement for Phosphate in Ethanol Fermentation In 1906 Harden and Young, in a series of classic studies on the fermentation of glucose to ethanol and CO_2 by extracts of brewer's yeast, made the following observations: (1) Inorganic phosphate was essential to fermentation; when the supply of phosphate was exhausted, fermentation ceased before all the glucose was used. (2) During fermentation under these conditions (with no phosphate), ethanol, CO_2, and a hexose bisphosphate accumulated. (3) When arsenate was substituted for phosphate, no hexose bisphosphate accumulated, but the fermentation proceeded until all the glucose was converted to ethanol and CO_2.

(a) Why did fermentation cease when the supply of phosphate was exhausted?

(b) Why did ethanol and CO_2 accumulate? Was the conversion of pyruvate to ethanol and CO_2 essential? Why? Identify the hexose bisphosphate that accumulated. Why did it accumulate?

(c) Why did the substitution of arsenate for phosphate prevent the accumulation of the hexose bisphosphate yet allow fermentation to ethanol and CO_2 to go to completion? (See Problem 10.)

12. Role of the Vitamin Niacin Adults engaged in strenuous physical activity require an intake of about 160 g of carbohydrate daily but only about 20 mg of niacin for optimal nutrition. Given the role of niacin in glycolysis, how do you explain the observation?

13. Synthesis of Glycerol Phosphate The glycerol 3-phosphate required for the synthesis of glycerophospholipids can be synthesized from a glycolytic intermediate. Propose a reaction sequence for this conversion.

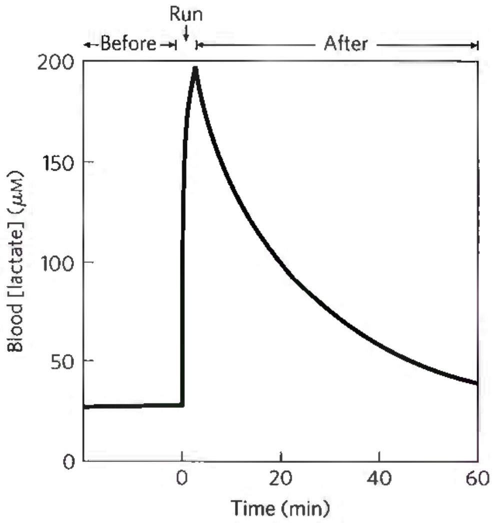
14. Severity of Clinical Symptoms Due to Enzyme Deficiency The clinical symptoms of two forms of galactosemia—galactokinase-deficiency galactosemia and transferase-deficiency galactosemia—show radically different severity. Although both types produce gastric discomfort after milk ingestion, deficiency of the transferase also leads to liver, kidney, spleen, and brain dysfunction and eventual death. What products accumulate in the blood and tissues with each type of enzyme deficiency? Estimate the relative toxicities of these products from the above information.

15. Ethanol Affects Blood Glucose Levels The consumption of alcohol (ethanol), especially after periods of strenuous activity or after not eating for several hours, results in a deficiency of glucose in the blood, a condition known as hypoglycemia. The first step in the metabolism of ethanol by the liver is oxidation to acetaldehyde, catalyzed by liver alcohol dehydrogenase:

$$CH_3CH_2OH + NAD^+ \longrightarrow CH_3CHO + NADH + H^+$$

Explain how this reaction inhibits the transformation of lactate to pyruvate. Why does this lead to hypoglycemia?

16. Blood Lactate Levels during Vigorous Exercise The graph shows the concentrations of lactate in blood plasma before, during, and after a 400 m sprint.



(a) What causes the rapid rise in lactate concentration?

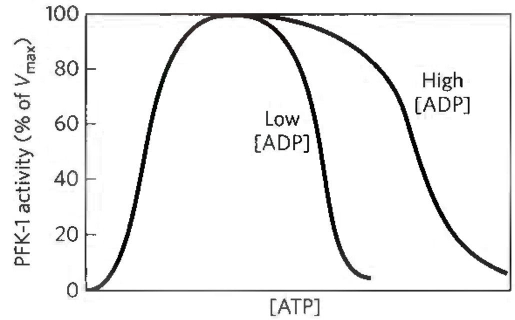
(b) What causes the decline in lactate concentration after completion of the sprint? Why does the decline occur more slowly than the increase?

(c) Why is the concentration of lactate not zero during the resting state?

17. Relationship between Fructose 1,6-Bisphosphatase and Blood Lactate Levels A congenital defect in the liver enzyme fructose 1,6-bisphosphatase results in abnormally high levels of lactate in the blood plasma. Explain.

18. Effect of O_2 Supply on Glycolytic Rates The regulated steps of glycolysis in intact cells can be identified by studying the catabolism of glucose in whole tissues or organs. For example, the glucose consumption by heart muscle can be measured by artificially circulating blood through an isolated intact heart and measuring the concentration of glucose before and after the blood passes through the heart. If the circulating blood is deoxygenated, heart muscle consumes glucose at a steady rate. When oxygen is added to the blood, the rate of glucose consumption drops dramatically, then is maintained at the new, lower rate. Explain.

19. Regulation of PFK-1 The graph shows the effect of ATP on the allosteric enzyme PFK-1. For a given concentration of fructose 6-phosphate, the PFK-1 activity increases with increasing concentrations of ATP, but there is a point beyond which increasing the concentration of ATP inhibits the enzyme.



(a) Explain how ATP can be both a substrate and an inhibitor of PFK-1. How is the enzyme regulated by ATP?
(b) How do ATP levels regulate glycolysis?
(c) The inhibition of PFK-1 by ATP diminishes when the ADP concentration is high, as shown in the graph. What explains this observation?

20. Cellular Glucose Concentration Homeostatic mechanisms maintain the concentration of glucose in human blood at about 5 mM. The concentration of free glucose inside a myocyte is much lower. Why is the concentration so low in the cell? What happens to glucose after entry into the cell? Physicians administer glucose intravenously as a food source in certain clinical situations. Given that the transformation of glucose to glucose 6-phosphate consumes ATP, why not administer intravenous glucose 6-phosphate instead?

21. Ethanol Production in Yeast When grown anaerobically on glucose, yeast (*S. cerevisiae*) converts pyruvate to acetaldehyde, then reduces acetaldehyde to ethanol using electrons from NADH. Write the equation for the second reaction, and calculate its equilibrium constant at 25 °C, given the standard reduction potentials in Table 13-7.

22. Pathway of Atoms in Fermentation An investigator carries out a "pulse-chase" experiment using ^{14}C-labeled carbon sources on a yeast extract maintained under strictly anaerobic conditions to produce ethanol. The experiment consists of incubating a small amount of ^{14}C-labeled substrate (the pulse) with the yeast extract just long enough for each intermediate in the fermentation pathway to become labeled. The addition of excess unlabeled glucose then "chases" the label through the pathway. The chase effectively prevents any further entry of labeled glucose into the pathway.
(a) If the investigator uses [1-^{14}C]glucose (glucose labeled at C-1 with ^{14}C) as a substrate, what is the location of ^{14}C in the product ethanol? Explain.
(b) Where would ^{14}C have to be located in the starting glucose to ensure that all the ^{14}C activity is liberated as $^{14}CO_2$ during fermentation to ethanol? Explain.

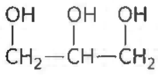
23. Heat from Fermentations Large-scale industrial fermenters generally require constant, vigorous cooling. Why?

24. Fermentation to Produce Soy Sauce Soy sauce preparation involves fermenting a salted mixture of soybeans and wheat with several microorganisms, including yeast, over a period of 8 to 12 months. The resulting sauce (after solids are removed) is rich in lactate and ethanol. How are these two compounds produced? To prevent the soy sauce from having a strong vinegary taste (vinegar is dilute acetic acid), oxygen must be kept out of the fermentation tank. Why?

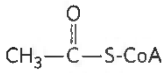
25. Glucogenic Substrates A common procedure for determining the effectiveness of compounds as precursors of glucose in mammals is to starve the animal until the liver glycogen stores are depleted and then administer the compound in question. A substrate that leads to a *net* increase in liver glycogen is termed glucogenic, because it must first be converted to glucose 6-phosphate. Show by means of known enzymatic reactions which of these substances are glucogenic:

(a) Succinate $^-OOC{-}CH_2{-}CH_2{-}COO^-$

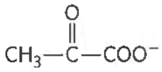
(b) Glycerol


$$\mathrm{\overset{OH}{\overset{|}{CH_2}}{-}\overset{OH}{\overset{|}{CH}}{-}\overset{OH}{\overset{|}{CH_2}}}$$

(c) Acetyl-CoA


$$\mathrm{CH_3{-}\overset{O}{\overset{\|}{C}}{-}S\text{-}CoA}$$

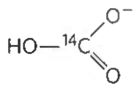
(d) Pyruvate


$$\mathrm{CH_3{-}\overset{O}{\overset{\|}{C}}{-}COO^-}$$

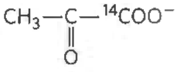
(e) Butyrate $CH_3{-}CH_2{-}CH_2{-}COO^-$

26. Pathway of Atoms in Gluconeogenesis An investigator briefly incubates a liver extract capable of carrying out all the normal metabolic reactions of the liver in separate experiments with two different ^{14}C-labeled precursors: [^{14}C]bicarbonate and [^{14}C]pyruvate.

(a) [^{14}C]Bicarbonate



(b) [1-^{14}C]Pyruvate


$$\mathrm{CH_3{-}\underset{\underset{O}{\|}}{C}{-}{}^{14}COO^-}$$

Trace the pathway of each precursor through gluconeogenesis. Indicate the location of ^{14}C in all intermediates and in the product, glucose.

27. Energy Cost of a Cycle of Glycolysis and Gluconeogenesis What is the cost (in ATP equivalents) of transforming glucose to pyruvate via glycolysis and back again to glucose via gluconeogenesis?

28. Relationship between Gluconeogenesis and Glycolysis Why is it important that gluconeogenesis is not the exact reversal of glycolysis?

29. Energetics of the Pyruvate Kinase Reaction Explain in bioenergetic terms how the conversion of pyruvate to phosphoenolpyruvate in gluconeogenesis overcomes the large, negative, standard free-energy change of the pyruvate kinase reaction in glycolysis.

30. Muscle Wasting in Starvation One consequence of starvation is a reduction in muscle mass. What happens to the muscle proteins?

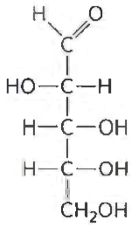
31. Effect of Phloridzin on Carbohydrate Metabolism Phloridzin, a toxic glycoside from the bark of the pear tree, blocks the normal reabsorption of glucose from the kidney tubule, thus causing blood glucose to be almost completely excreted in the urine. In an experiment, rats fed phloridzin and sodium succinate excreted about 0.5 mol of glucose (made by gluconeogenesis) for every 1 mol of sodium succinate ingested. How do rats transform the succinate to glucose? Explain the stoichiometry.

32. Excess O_2 Uptake during Gluconeogenesis The conversion of lactate to glucose in the liver requires the input of 6 mol of ATP for every mol of glucose produced. Investigators can monitor the extent of this process in a rat liver preparation by administering [^{14}C]lactate and measuring the amount of [^{14}C]glucose produced. Because the stoichiometry of O_2 consumption and ATP production is known (about 5 ATP per O_2), investigators can predict the extra O_2 consumption above the normal rate after administering a given amount of lactate. However, when they actually measure extra O_2 used in the synthesis of glucose from lactate, it is always higher than what the stoichiometric relationships predict. Suggest a possible explanation for this observation.

33. Role of the Pentose Phosphate Pathway If the oxidation of glucose 6-phosphate via the pentose phosphate pathway were being used primarily to generate NADPH for biosynthesis, the other product, ribose 5-phosphate, would accumulate. What problems might this cause?

DATA ANALYSIS PROBLEM

34. Engineering a Fermentation System Fermentation of plant matter to produce ethanol for fuel is one potential method for reducing the use of fossil fuels and thus the CO_2 emissions that lead to global warming. Many microorganisms can break down cellulose, then ferment the glucose to ethanol. However, many potential cellulose sources, including agricultural residues and switchgrass, also contain substantial amounts of arabinose, which is not as easily fermented.



D-Arabinose

Escherichia coli is capable of fermenting arabinose to ethanol, but it is not naturally tolerant of high ethanol levels, thus limiting its utility for commercial ethanol production. Another bacterium, *Zymomonas mobilis*, is naturally tolerant of high levels of ethanol but cannot ferment arabinose. Deanda, Zhang, Eddy, and Picataggio (1996) described their efforts to combine the most useful features of these two organisms by introducing the *E. coli* genes for the arabinose-metabolizing enzymes into *Z. mobilis*.

(a) Why is this a simpler strategy than the reverse: engineering *E. coli* to be more ethanol-tolerant?

Deanda and colleagues inserted five *E. coli* genes into the *Z. mobilis* genome: *araA*, coding for L-arabinose isomerase, which interconverts L-arabinose and L-ribulose; *araB*, L-ribulokinase, which uses ATP to phosphorylate L-ribulose at C-5; *araD*, L-ribulose 5-phosphate epimerase, which interconverts L-ribulose 5-phosphate and L-xylulose 5-phosphate; *talB*, transaldolase; and *tktA*, transketolase.

(b) For each of the three *ara* enzymes, briefly describe the chemical transformation it catalyzes and, where possible, name an enzyme discussed in this chapter that carries out an analogous reaction.

The five *E. coli* genes inserted in *Z. mobilis* allowed the entry of arabinose into the nonoxidative phase of the pentose phosphate pathway (Fig. 14-31), where it was converted to glucose 6-phosphate and fermented to ethanol.

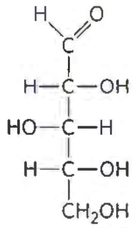
(c) The three *ara* enzymes eventually converted arabinose into which sugar?

(d) The product from part (c) feeds into the pathway shown in Figure 14-31a. Combining the five *E. coli* enzymes listed above with the enzymes of this pathway, describe the overall pathway for the fermentation of six molecules of arabinose to ethanol.

(e) What is the stoichiometry of the fermentation of six molecules of arabinose to ethanol and CO_2? How many ATP molecules would you expect this reaction to generate?

(f) *Z. mobilis* uses a pathway for ethanol fermentation that is slightly different from the one described in this chapter. As a result, the expected ATP yield is only 1 ATP per molecule of arabinose. Although this is less beneficial for the bacterium, it is better for ethanol production. Why?

Another sugar commonly found in plant matter is xylose.



D-Xylose

(g) What additional enzymes would you need to introduce into the modified *Z. mobilis* strain described above to enable it to use xylose as well as arabinose to produce ethanol? You don't need to name the enzymes (they may not even exist in the real world); just give the reactions they would need to catalyze.

Reference

Deanda, K., M. Zhang, C. Eddy, and S. Picataggio. 1996. Development of an arabinose-fermenting *Zymomonas mobilis* strain by metabolic pathway engineering. *Appl. Environ. Microbiol. 62*:4465–4470.

Chapter 15

THE METABOLISM OF GLYCOGEN IN ANIMALS

15.1 The Structure and Function of Glycogen 557
15.2 Breakdown and Synthesis of Glycogen 558
15.3 Coordinated Regulation of Glycogen Breakdown and Synthesis 565

In Chapter 14 we examined universal pathways by which hexoses are metabolized through glycolysis, fermentation, gluconeogenesis, and the pentose phosphate pathways to provide energy and components for the biosynthesis of amino acids, fats, and nucleotides. In this chapter, we focus more narrowly on the metabolism of glycogen, the polymeric storage form of glucose employed by animals. These are the principles that guide our discussion:

P1 **Glycogen provides vertebrate animals with a ready source of glucose to supply the brain and skeletal muscles with energy.** Although animals store about 100 times more energy as fat than as glycogen, they cannot metabolize fat into glucose. The highly branched polymeric structure of glycogen granules allows cells in the liver and muscle to make large numbers of glucose and glucose phosphate monomers available quickly, without raising the osmolarity of the cytosol by storing them in monomeric form.

P2 **Monomers are released from glycogen granules by a phosphorolysis reaction that creates phosphorylated glucose molecules that can enter glycolysis to supply energy to the cell.** Skeletal muscle cells especially require stores of glycogen to supply energy for bursts of activity. In the liver, the phosphate can be removed, allowing free glucose to be transported out of the cell to the blood for use in the brain and other tissues when dietary glucose is not sufficient.

P3 **Glycogen synthesis requires a protein primer and an activated glucose precursor.** Individual glucose molecules activated as sugar nucleotides are added to the nonreducing end of the growing linear chains in the outer tiers of the glycogen β-granules, and a branching enzyme adds branches periodically.

P4 **Regulation of the balance between the formation of glycogen from excess glucose and the release of glucose from glycogen polymers when it is needed in metabolism is a critical function of cellular and organismal homeostasis.** This balance, ultimately controlled by the hormones epinephrine, glucagon, and insulin, is achieved through allosteric regulation and phosphorylation of the synthetic and degradative enzymes. These enzymes, and the regulatory proteins that act on them, are integral parts of the glycogen granule.

Glycogen was discovered in the mid-1800s by Claude Bernard. The French physiologist also found that a liver "ferment" (enzyme) released a reducing sugar from liver tissue. He named this reducing sugar *matière glycogène*—sugar-forming substance. In the first half of the twentieth century, scientists in laboratories around the world followed up this early work, purifying the "ferments" that synthesize and degrade glycogen and characterizing the regulation of these enzymes by insulin and epinephrine. These studies characterized the enzymes and also uncovered multiple regulatory mechanisms that proved to be universal: second messengers responsive to extracellular signals, protein kinase cascades, and protein phosphorylation, for example. In this chapter, we begin by exploring the structure and function of glycogen particles, describe the pathways of glycogen breakdown and synthesis, and finally, dig into the complex web of regulatory controls that exquisitely deliver the necessary amount of energy from glucose that each organ system requires to function in the moment.

15.1 The Structure and Function of Glycogen

Structure–function relationships are key to understanding biomolecules and biochemical systems, and glycogen is no exception. The compact structure of glycogen granules allows cells to store glucose when it is available in excess, and to make it available on short notice when needed. Subtle tissue-specific differences in the enzymes (isozymes) that act on glycogen determine the dynamics of glycogen metabolism in each tissue.

Vertebrate Animals Require a Ready Fuel Source for Brain and Muscle

P1 For all vertebrate animals, maintaining a ready supply of glucose for the brain and muscles is a top metabolic priority. The challenge for cells is to be able to store glucose in a form that rapidly sequesters it when the glucose concentration in the blood is high (say, after a meal) but allows it to be accessed quickly for use particularly by the brain and skeletal muscle. Recall from our discussion in Chapter 7 (p. 242) that a typical hepatocyte in the fed state stores an amount of glucose, polymerized as glycogen, that in monomeric form would be equivalent to about 0.4 M. At this concentration, the osmolarity of the cell would be so high relative to the surrounding fluid that water would enter the cell and likely rupture it.

When the diet temporarily provides more carbohydrate than is needed immediately as fuel, excess glucose is polymerized into glycogen. Small amounts of glycogen are present in all animal cells, but it is stored primarily in liver and muscle, where it is a significant portion of the wet weight of the organ (5% to 10% of liver and 1% to 2% of muscle). A 70 kg human stores about 100 g of glycogen in the liver and up to 400 g in skeletal muscle. The total amount of energy stored in the body as glycogen is far less (about 1%) than the amount stored as fat (triacylglycerol), but fats cannot be converted to glucose in vertebrates and cannot be catabolized anaerobically through glycolysis, as is often required in skeletal muscle.

P1 When a sudden burst of physical activity demands a quick source of energy in muscle, the rapid breakdown of glycogen stored there provides glucose for glycolysis within seconds. Between meals or during a fast, the release of glucose from glycogen stored in the liver provides a steady supply of glucose in the blood. This is especially important for the brain, a major consumer of metabolic energy, which, unlike muscle, cannot use fatty acids as fuel; long-chain fatty acids do not cross the blood-brain barrier. The brain therefore depends on a constant supply of glucose, from the diet or from the liver.

Glycogen Granules Have Many Tiers of Branched Chains of D-Glucose

Glycogen is stored as cytosolic granules, called β-granules, that vary in size, structure, and subcellular location depending on the tissue or cell type. (In this chapter we focus on liver and muscle.) The size of β-granules also varies with the state of activity and feeding of the animal. In muscle, β-granules are 20–30 nm in diameter and have an M_r of 10^6–10^7. They consist of up to 55,000 glucose residues with about 2,000 nonreducing ends available for degradative enzymes to work on. In liver, 20 to 40 β-granules cluster together to form protein-rich α-granules as large as 300 nm in diameter and of M_r greater than 10^8. They are visible with the electron microscope in tissue samples from well-fed animals (**Fig. 15-1**), but they are essentially absent after a 24-hour fast. The β-granules of muscle release glucose more quickly than the α-granules of liver, consistent with the different needs of these tissues for glucose.

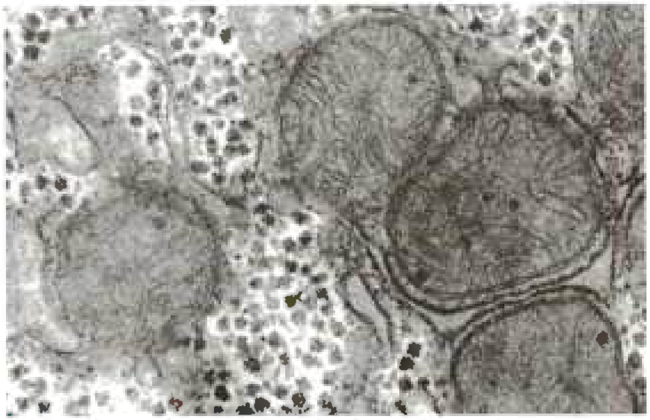


FIGURE 15-1 Glycogen granules in a hepatocyte. Glycogen β-granules appear as electron-dense particles. In liver they form larger clusters called α-granules and are often associated with tubules of the smooth endoplasmic reticulum. Four mitochondria are also evident in this micrograph. [BCC Microimaging. Reproduced with permission.]

All glycogen granules have at their core a dimer of the protein glycogenin, which serves as a primer for the synthesis of polymers of D-glucose. In the tiered β-granule model, the central glycogenin dimer is surrounded by tier upon tier of chains of about 13 glucose residues in (α1→4) linkage, with (α1→6)-linked branches. Inner B-chains contain two branch points, and outer A-chains are unbranched (**Fig. 15-2**). Granules typically have 6 or 7 tiers, with the outermost tier of unbranched A-chains making up the majority of the granule. Associated with each β-granule are patches of electron-dense, protein-rich material, called γ-particles. Among the associated proteins are the enzymes that synthesize and break down glycogen.

The general mechanisms for storing and mobilizing glycogen are the same in muscle and liver, but the enzymes involved differ in subtle yet important ways that reflect the different roles of glycogen in the two tissues. In the next two sections we will look at the enzymatic basis for glycogen synthesis and breakdown, and at the regulation of these processes.

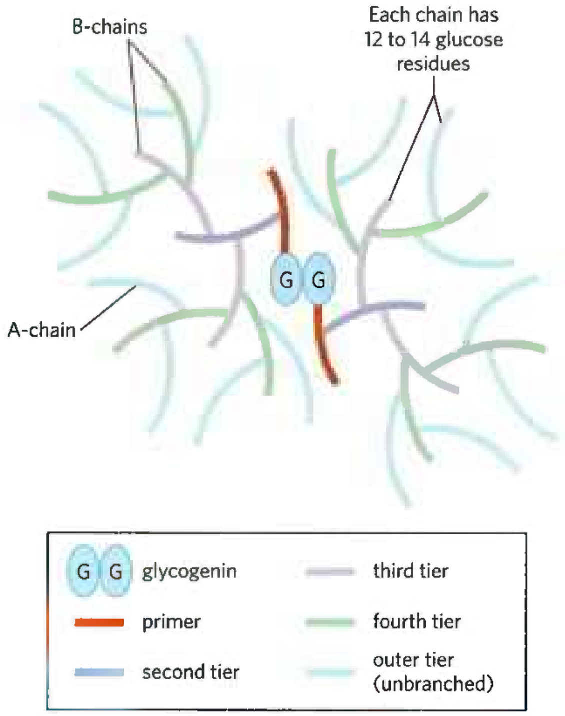

B-chains
Each chain has 12 to 14 glucose residues
G G
A-chain
G G glycogenin
primer
second tier
third tier
fourth tier
outer tier (unbranched)

FIGURE 15-2 **Structure of a glycogen β-granule.** Starting at a central glycogenin homodimer, glycogen chains (12 to 14 residues) extend in tiers. Inner chains (B-chains) have two (α1→6) branches each. A-chains in the outer tier are unbranched. There are in theory a maximum of 12 tiers in a mature glycogen β-granule (only 5 are shown here), consisting of about 55,000 glucose residues in a molecule of about 21 nm diameter and $M_r \sim 1 \times 10^7$.

SUMMARY 15.1 *The Structure and Function of Glycogen*

- All cells need ready access to glucose, either from the diet or from supplies stored in cells. Glycogen is a polymeric storage form of glucose in animals that is found primarily in muscle and liver. Glycogen breakdown in muscle delivers glucose needed for muscle contraction. Glycogen stored in the liver provides a reservoir that maintains homeostasis of blood glucose throughout the body.
- Glycogen β-granules have tiers of glucose residues in (α1→4) linkage, with (α1→6)-linked branches, providing many free nonreducing ends for synthetic and degradative enzymes to access. In liver, β-granules cluster into larger α-granules, which release glucose more slowly.

15.2 Breakdown and Synthesis of Glycogen

As we saw in Chapter 14 (Fig. 14-9), glycogen obtained in the diet is broken down by α-amylases, hydrolytic enzymes that act in the mouth and gut to convert glycogen to free glucose. (Dietary starch is hydrolyzed in a similar way.) But glycogen stored in cells (endogenous glycogen) is degraded by a different pathway. We begin with the breakdown of cellular glycogen to glucose 1-phosphate **(glycogenolysis)**, then turn to synthesis of glycogen **(glycogenesis)**.

Glycogen Breakdown Is Catalyzed by Glycogen Phosphorylase

P2 In skeletal muscle and liver, the glucose units of the outer branches of glycogen enter the glycolytic pathway through the action of three enzymes: glycogen phosphorylase, glycogen debranching enzyme, and phosphoglucomutase. Glycogen phosphorylase catalyzes the reaction in which an (α1→4) glycosidic linkage between two glucose residues at a nonreducing end of glycogen undergoes attack by inorganic phosphate (P_i), removing the terminal glucose residue as α-D-glucose 1-phosphate (**Fig. 15-3**). This *phosphorolysis* reaction is different from

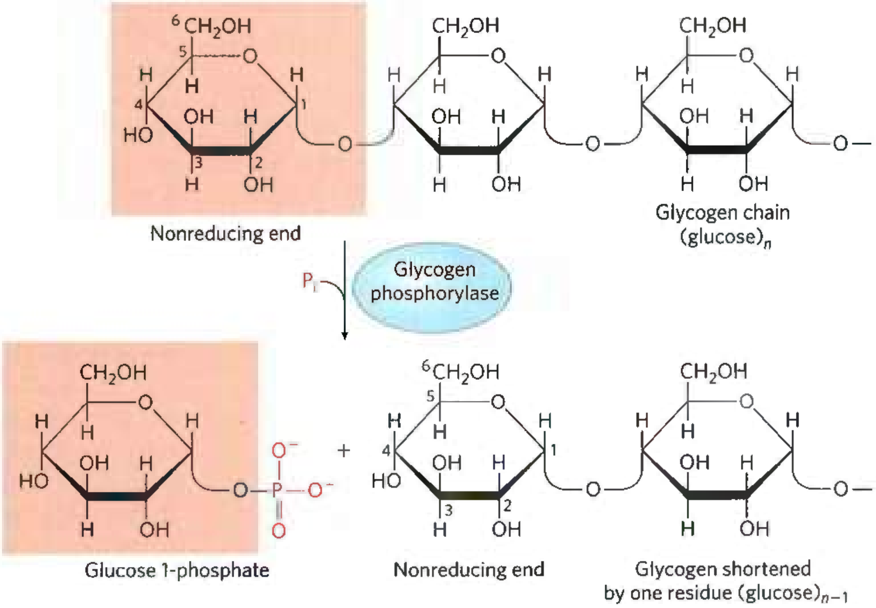

$^6CH_2OH$
5
O
H
H
H
4
OH
H
1
HO
3
2
H
OH
Nonreducing end
CH_2OH
Glycogen chain
(glucose)$_n$
P_i
Glycogen phosphorylase
CH_2OH
O$^-$
O—P—O$^-$
O
+
Glucose 1-phosphate
Nonreducing end
Glycogen shortened by one residue (glucose)$_{n-1}$

FIGURE 15-3 **Removal of a glucose residue from the nonreducing end of a glycogen chain by glycogen phosphorylase.** This process is repetitive; the enzyme removes successive glucose residues, creating a new nonreducing end, until it reaches the fourth glucose unit from a branch point (see Fig. 15-4).

the *hydrolysis* of glycosidic bonds by amylase during intestinal degradation of dietary glycogen and starch. In phosphorolysis, some of the energy of the glycosidic bond is conserved in the formation of the phosphate ester glucose 1-phosphate.

Pyridoxal phosphate is an essential cofactor in the glycogen phosphorylase reaction. It is covalently attached near the enzyme active site, where its phosphate group acts as a general acid catalyst, promoting attack by P_i on the glycosidic bond. (This is an unusual role for pyridoxal phosphate; its more typical role is as a cofactor in amino acid metabolism; see Fig. 18-6.)

Glycogen phosphorylase acts repetitively on the nonreducing ends of glycogen branches until it reaches a point four glucose residues away from an (α1→6) branch point, where its action stops. Further degradation by glycogen phosphorylase can occur only after the **debranching enzyme**, formally known as oligo (α1→6) to (α1→4) glucantransferase, catalyzes two successive reactions that transfer branches (**Fig. 15-4**) to form straight chains. Once these branches are transferred and the glucosyl residue at C-6 is hydrolyzed, glycogen phosphorylase activity can continue.

Glucose 1-Phosphate Can Enter Glycolysis or, in Liver, Replenish Blood Glucose

Glucose 1-phosphate, the end product of the glycogen phosphorylase reaction, is converted to glucose 6-phosphate by **phosphoglucomutase**, which catalyzes the reversible reaction:

$$\text{Glucose 1-phosphate} \rightleftharpoons \text{glucose 6-phosphate}$$

Initially phosphorylated at a Ser residue, the enzyme donates its phosphoryl group to C-6 of the substrate, then accepts a phosphoryl group from C-1 (**Fig. 15-5**).

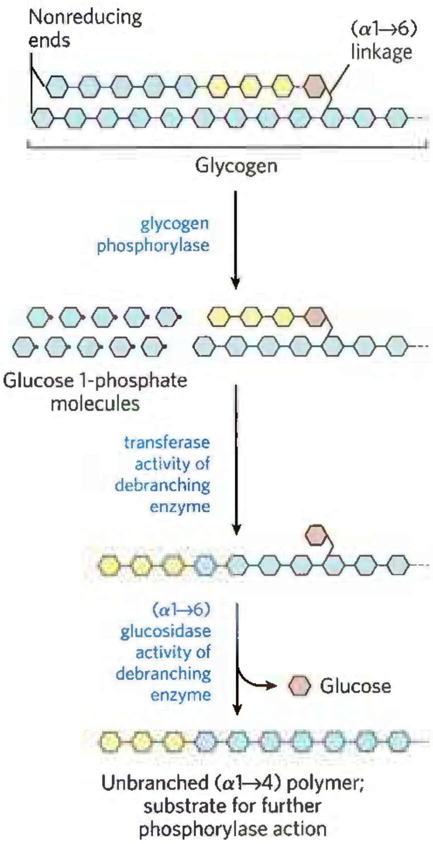

Nonreducing ends
(α1→6) linkage
Glycogen
glycogen phosphorylase
Glucose 1-phosphate molecules
transferase activity of debranching enzyme
(α1→6) glucosidase activity of debranching enzyme
Glucose
Unbranched (α1→4) polymer; substrate for further phosphorylase action

FIGURE 15-4 Glycogen breakdown near an (α1→6) branch point. Following sequential removal of terminal glucose residues by glycogen phosphorylase (see Fig. 15-3), glucose residues near a branch are removed in a two-step process that requires a bifunctional debranching enzyme. First, the transferase activity of the enzyme shifts a block of three glucose residues from the branch to a nearby nonreducing end, to which the segment is reattached in (α1→4) linkage. The single glucose residue remaining at the branch point, in (α1→6) linkage, is then released as free glucose by the (α1→6) glucosidase activity of the debranching enzyme. The glucose residues are shown in shorthand form.

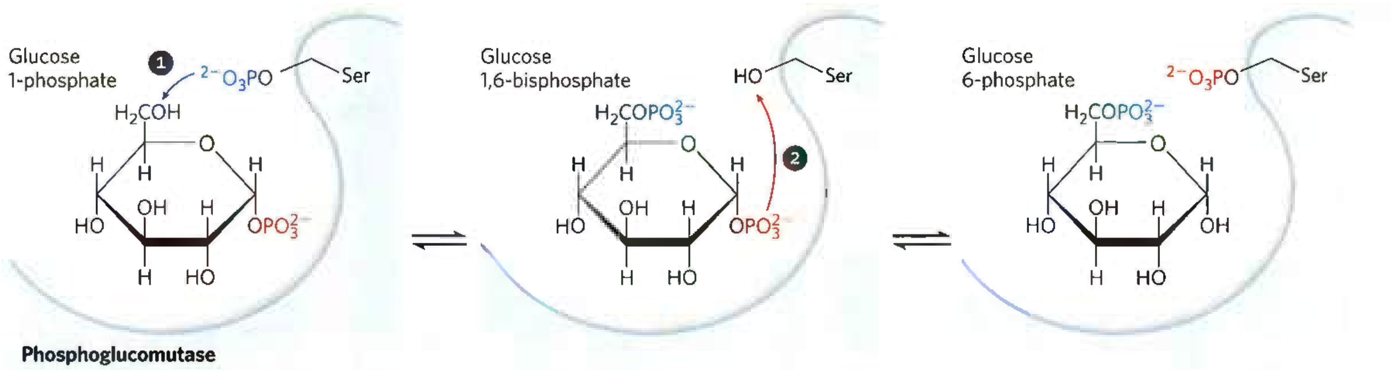

Glucose 1-phosphate
①
²⁻O₃PO
Ser
H₂COH
O
H
H
H
OH
H
HO
OPO₃²⁻
H
HO
Phosphoglucomutase
Glucose 1,6-bisphosphate
HO
Ser
H₂COPO₃²⁻
②
OPO₃²⁻
Glucose 6-phosphate
²⁻O₃PO
Ser
H₂COPO₃²⁻
OH

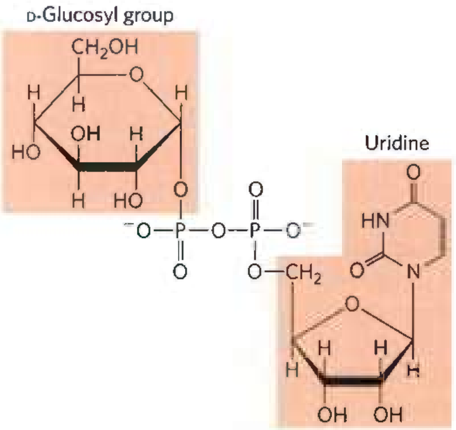
FIGURE 15-5 Reaction catalyzed by phosphoglucomutase. The reaction begins with the enzyme phosphorylated on a Ser residue. In step ①, the enzyme donates its phosphoryl group (blue) to glucose 1-phosphate, producing glucose 1,6-bisphosphate. In step ②, the phosphoryl group at C-1 of glucose 1,6-bisphosphate (red) is transferred back to the enzyme, re-forming the phosphoenzyme and producing glucose 6-phosphate.

P2 The glucose 6-phosphate formed from glycogen in skeletal muscle can enter glycolysis and serve as an energy source to support muscle contraction. In liver, glycogen breakdown serves a different purpose: to release glucose into the blood when the blood glucose level drops, as it does between meals. This requires the enzyme glucose 6-phosphatase, present in liver and kidney but not in other tissues. The enzyme is an integral protein of the endoplasmic reticulum, with its active site on the lumenal side of the ER. Glucose 6-phosphate formed in the cytosol is transported into the ER lumen by a specific transporter (T1) (**Fig. 15-6**) and hydrolyzed at the lumenal surface by glucose 6-phosphatase. The resulting P_i and glucose are carried back into the cytosol by two different transporters (T2 and T3), and the glucose enters the blood via the plasma membrane transporter, GLUT2. Notice that by having the active site of glucose 6-phosphatase in the ER lumen, the cell separates this reaction from the process of glycolysis, which takes place in the cytosol and would be aborted by the action of glucose 6-phosphatase. Genetic defects in either glucose 6-phosphatase or T1 lead to serious derangement of glycogen metabolism, resulting in type Ia glycogen storage disease (**Box 15-1**).

Because muscle and adipose tissue lack glucose 6-phosphatase, they cannot convert the glucose 6-phosphate formed by glycogen breakdown to glucose, and these tissues therefore do not contribute glucose to the blood.

The Sugar Nucleotide UDP-Glucose Donates Glucose for Glycogen Synthesis

Many of the reactions in which hexoses are transformed or polymerized involve **sugar nucleotides**, compounds in which the anomeric carbon of a sugar is activated by attachment to a nucleotide through a phosphate ester linkage. Sugar nucleotides are the substrates for polymerization of monosaccharides into disaccharides, glycogen, starch, cellulose, and more complex extracellular polysaccharides. They are also key intermediates in the production of the aminohexoses and deoxyhexoses found in some of these polysaccharides, and in the synthesis of vitamin C (L-ascorbic acid). The role of sugar nucleotides in the biosynthesis of glycogen and many other carbohydrate derivatives was discovered in 1953 by the Argentine biochemist Luis Leloir.



UDP-glucose
(a sugar nucleotide)

P3 The suitability of sugar nucleotides for biosynthetic reactions stems from several properties:

1. Their formation is metabolically irreversible, contributing to the irreversibility of the synthetic pathways in which they are intermediates. The condensation of a nucleoside triphosphate with a hexose 1-phosphate to form a sugar nucleotide has a small positive free-energy change, but the reaction releases PP_i, which is immediately hydrolyzed by inorganic

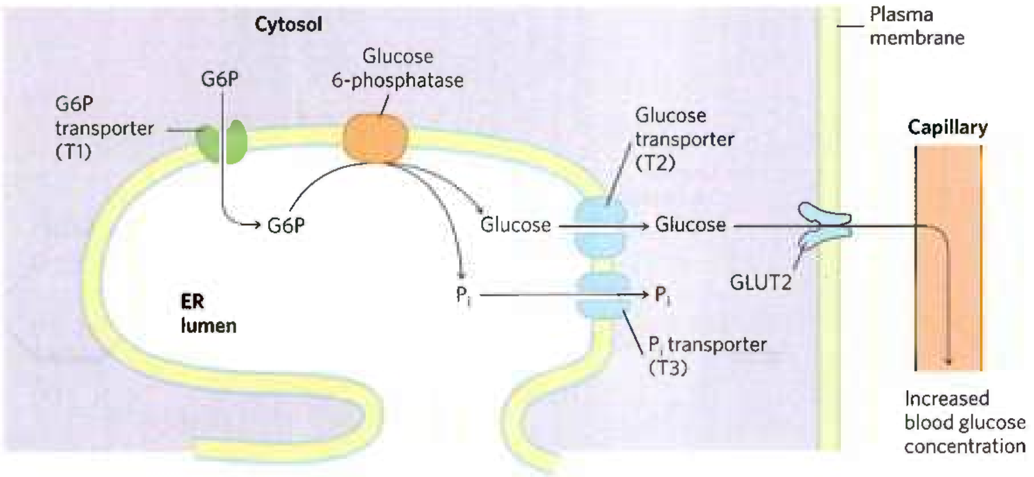


FIGURE 15-6 Hydrolysis of glucose 6-phosphate by glucose 6-phosphatase of the liver ER. The catalytic site of glucose 6-phosphatase faces the lumen of the ER. A glucose 6-phosphate (G6P) transporter (T1) carries the substrate from the cytosol to the lumen, where glucose 6-phosphatase releases P_i. The products, glucose and P_i, pass to the cytosol on specific transporters (T2 and T3). Glucose leaves the cell via the GLUT2 transporter in the plasma membrane.

BOX 15-1 MEDICINE

Carl and Gerty Cori: Pioneers in Glycogen Metabolism and Disease

Much of what is written in present-day biochemistry textbooks about the metabolism of glycogen was discovered between about 1925 and 1950 by the remarkable husband and wife team of Carl F. Cori and Gerty T. Cori. Both trained in medicine in Europe at the end of World War I (she completed premedical studies and medical school in one year!). They left Europe together in 1922 to establish research laboratories in the United States, first for nine years in Buffalo, New York, at what is now the Roswell Park Comprehensive Cancer Center, then from 1931 until the end of their lives at Washington University in St. Louis.

FIGURE 1 The Coris in Gerty Cori's laboratory, around 1947. [AP Images.]

In their early physiological studies of the origin and fate of glycogen in animal muscle, the Coris demonstrated the conversion of glycogen to lactate in tissues; movement of lactate in the blood to the liver; and, in the liver, reconversion of lactate to glycogen — a pathway that came to be known as the Cori cycle (see Fig. 23-17). Pursuing these observations at the biochemical level, they showed that glycogen was mobilized in a phosphorolysis reaction catalyzed by the enzyme they discovered, glycogen phosphorylase. They identified the product of this reaction (the "Cori ester") as glucose 1-phosphate and showed that it could be reincorporated into glycogen in the reverse reaction. Although this did not prove to be the reaction by which glycogen is synthesized in cells, it was the first in vitro demonstration of the synthesis of a macromolecule from simple monomeric subunits, and it inspired others to search for polymerizing enzymes. Arthur Kornberg, discoverer of the first DNA polymerase, said of his experience in the Coris's lab, "Glycogen phosphorylase, not base pairing, was what led me to DNA polymerase."

Gerty Cori became interested in human genetic diseases in which too much glycogen is stored in the liver. She was able to identify the biochemical defect in several of these diseases and to show that the diseases could be diagnosed by assays of the enzymes of glycogen metabolism in small samples of tissue obtained by biopsy. Table 1 summarizes what we now know about 13 genetic diseases of this sort.

Carl and Gerty Cori shared the Nobel Prize in Physiology or Medicine in 1947 with Bernardo Houssay of Argentina, who was cited for his studies of hormonal regulation of carbohydrate metabolism. The Cori laboratories in St. Louis became an international center of biochemical research in the 1940s and 1950s, and at least six scientists who trained with the Coris became Nobel laureates: Arthur Kornberg (for DNA synthesis, 1959), Severo Ochoa (for RNA synthesis,

(Continued on next page)

pyrophosphatase (**Fig. 15-7**), in a reaction that is strongly exergonic ($\Delta G'^{\circ} = -19.2$ kJ/mol). This keeps the cellular concentration of PP_i low, ensuring that the actual free-energy change in the cell is favorable. In effect, rapid removal of the product, driven by the large, negative free-energy change of PP_i hydrolysis, pulls the synthetic reaction forward. This is a common strategy in biological polymerization reactions.

2. Although the chemical transformations of sugar nucleotides do not involve the atoms of the nucleotide itself, the nucleotide moiety has many groups that can undergo noncovalent interactions with enzymes; the additional free energy of binding can contribute significantly to catalytic activity (Chapter 6; see also p. 294).
3. Like phosphate, the nucleotidyl group (UDP or ADP, for example) is an excellent leaving group, facilitating nucleophilic attack by activating the sugar carbon to which it is attached.
4. By "tagging" some hexoses with nucleotidyl groups, cells can set them aside in a pool for a particular purpose (glycogen synthesis, for example), separate from hexose phosphates destined for another purpose (such as glycolysis).

Glycogen synthesis takes place in virtually all animal tissues but is especially prominent in the liver and skeletal muscles. The starting point for synthesis of glycogen is glucose 6-phosphate. This can be derived from free glucose in a reaction catalyzed by the isozymes

BOX 15-1 MEDICINE

Carl and Gerty Cori: Pioneers in Glycogen Metabolism and Disease (*Continued*)

1959), Luis Leloir (for the role of sugar nucleotides in polysaccharide synthesis, 1970), Earl Sutherland (for the discovery of cAMP in the regulation of carbohydrate metabolism, 1971), Christian de Duve (for subcellular fractionation, 1974), and Edwin Krebs (for the discovery of phosphorylase kinase, 1991).

TABLE 1 Glycogen Storage Diseases of Humans

Type (name)	Enzyme affected	Primary organ/ cells affected	Symptoms
Type 0	Glycogen synthase	Liver	Low blood glucose, high ketone bodies, early death
Type Ia (von Gierke)	Glucose 6-phosphatase	Liver	Enlarged liver, kidney failure
Type Ib	Microsomal glucose 6-phosphate translocase	Liver	As in type Ia; also high susceptibility to bacterial infections
Type Ic	Microsomal P_i transporter	Liver	As in type Ia
Type II (Pompe)	Lysosomal glucosidase	Skeletal and cardiac muscle	Infantile form: death by age 2; juvenile form: muscle defects (myopathy); adult form: as in muscular dystrophy
Type IIIa (Cori or Forbes)	Debranching enzyme	Liver, skeletal and cardiac muscle	Enlarged liver in infants; myopathy
Type IIIb	Liver debranching enzyme (muscle enzyme normal)	Liver	Enlarged liver in infants
Type IV (Andersen)	Branching enzyme	Liver, skeletal muscle	Enlarged liver and spleen, myoglobin in urine
Type V (McArdle)	Muscle phosphorylase	Skeletal muscle	Exercise-induced cramps and pain; myoglobin in urine
Type VI (Hers)	Liver phosphorylase	Liver	Enlarged liver
Type VII (Tarui)	Muscle PFK-1	Muscle, erythrocytes	As in type V; also hemolytic anemia
Type VIb, VIII, or IX	Phosphorylase kinase	Liver, leukocytes, muscle	Enlarged liver
Type XI (Fanconi-Bickel)	Glucose transporter (GLUT2)	Liver	Failure to thrive, enlarged liver, rickets, kidney dysfunction

hexokinase I and II in muscle and hexokinase IV (glucokinase) in liver:

$$\text{Glucose} + \text{ATP} \longrightarrow \text{glucose 6-phosphate} + \text{ADP}$$

However, some ingested glucose takes a more roundabout path to glycogen. It is first taken up by erythrocytes and converted to lactate via glycolysis; the lactate is then taken up by the liver and converted to glucose 6-phosphate by gluconeogenesis.

To start glycogen synthesis, the glucose 6-phosphate is converted to glucose 1-phosphate in the phosphoglucomutase reaction:

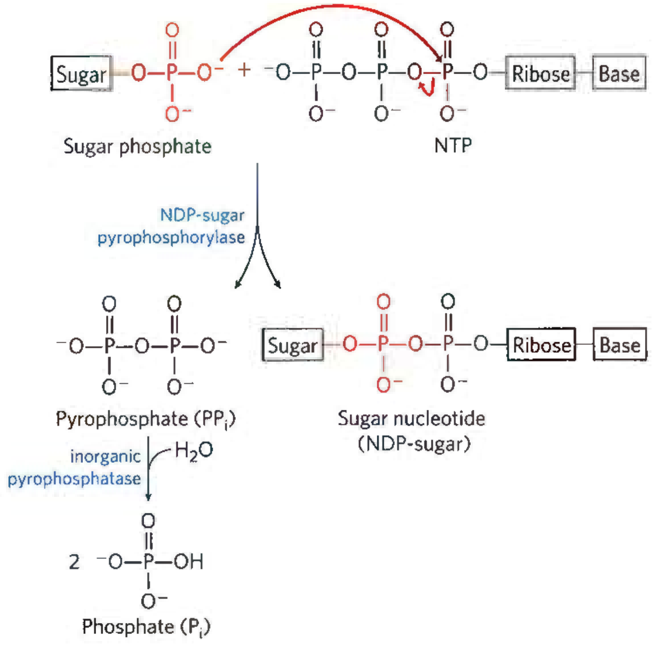
$$\text{Glucose 6-phosphate} \rightleftharpoons \text{glucose 1-phosphate}$$

The product is then converted to UDP-glucose by the action of **UDP-glucose pyrophosphorylase**, in a key step of glycogen biosynthesis:

$$\text{Glucose 1-phosphate} + \text{UTP} \longrightarrow \text{UDP-glucose} + \text{PP}_i$$

Notice that this enzyme is named for the reverse reaction; in the cell, the reaction proceeds in the direction of UDP-glucose formation, because the pyrophosphate concentration is kept low by its immediate hydrolysis by inorganic pyrophosphatase (Fig. 15-7).

P3 UDP-glucose is the immediate donor of glucose residues in the reaction catalyzed by **glycogen synthase**, which promotes the transfer of the glucose residue from UDP-glucose to a nonreducing end of a branched glycogen


Sugar
Ribose
Base
Sugar phosphate
NTP
NDP-sugar pyrophosphorylase
Pyrophosphate (PP$_i$)
Sugar nucleotide (NDP-sugar)
inorganic pyrophosphatase
H_2O
2
Phosphate (P$_i$)

Net reaction: Sugar phosphate + NTP ⟶ NDP-sugar + $2P_i$

FIGURE 15-7 Formation of a sugar nucleotide. A condensation reaction occurs between a nucleoside triphosphate (NTP) and a sugar phosphate. The negatively charged oxygen on the sugar phosphate serves as a nucleophile, attacking the α phosphate of the nucleoside triphosphate and displacing pyrophosphate. The reaction is pulled in the forward direction by the hydrolysis of PP$_i$ by inorganic pyrophosphatase.

molecule, forming an (α1→4) linkage (**Fig. 15-8**). The overall equilibrium of the path from glucose 6-phosphate to glycogen lengthened by one glucose unit greatly favors synthesis of glycogen.

Glycogen synthase cannot make the (α1→6) bonds found at the branch points of glycogen; these are formed by the **glycogen-branching enzyme**, also called amylo (1→4) to (1→6) transglycosylase, or glycosyl (4→6) transferase. The glycogen-branching enzyme catalyzes transfer of a terminal fragment of 6 or 7 glucose residues from the nonreducing end of a glycogen branch having at least 11 residues to the C-6 hydroxyl group of a glucose residue at a more interior position of the same or another glycogen chain, thus creating a new branch (**Fig. 15-9**). Further glucose residues may be added to the new branch by glycogen synthase. The biological effect of branching is to increase the number of nonreducing ends. This increases the number of sites accessible to glycogen phosphorylase and glycogen synthase, both of which act only at nonreducing ends.

Glycogenin Primes the Initial Sugar Residues in Glycogen

Glycogen synthase cannot initiate a new glycogen chain de novo. It requires a primer, usually a preformed (α1→4) polyglucose chain. P3 So, how is a *new* glycogen molecule initiated? The intriguing protein **glycogenin** (**Fig. 15-10**) is both the primer on which new chains are

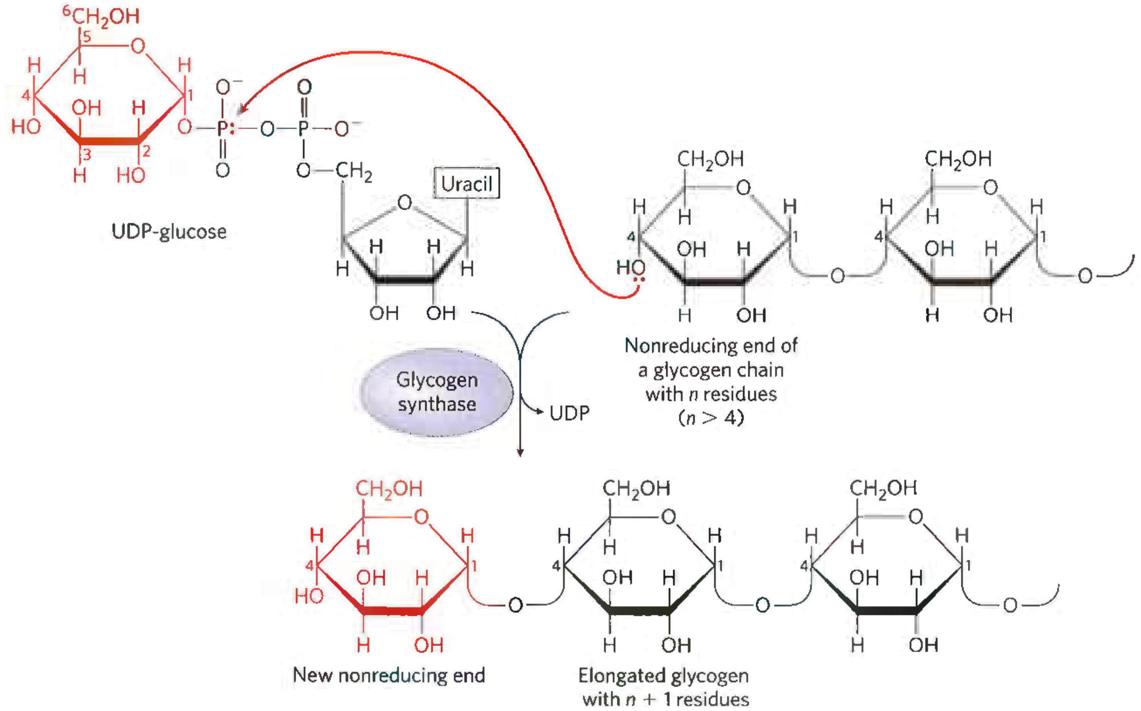

$^6CH_2OH$
UDP-glucose
Uracil
Glycogen synthase
UDP
Nonreducing end of a glycogen chain with n residues (n > 4)
New nonreducing end
Elongated glycogen with n + 1 residues

FIGURE 15-8 Glycogen synthesis. A glycogen chain is elongated by glycogen synthase. The enzyme transfers the glucose residue of UDP-glucose to the nonreducing end of a glycogen branch to make a new (α1→4) linkage.

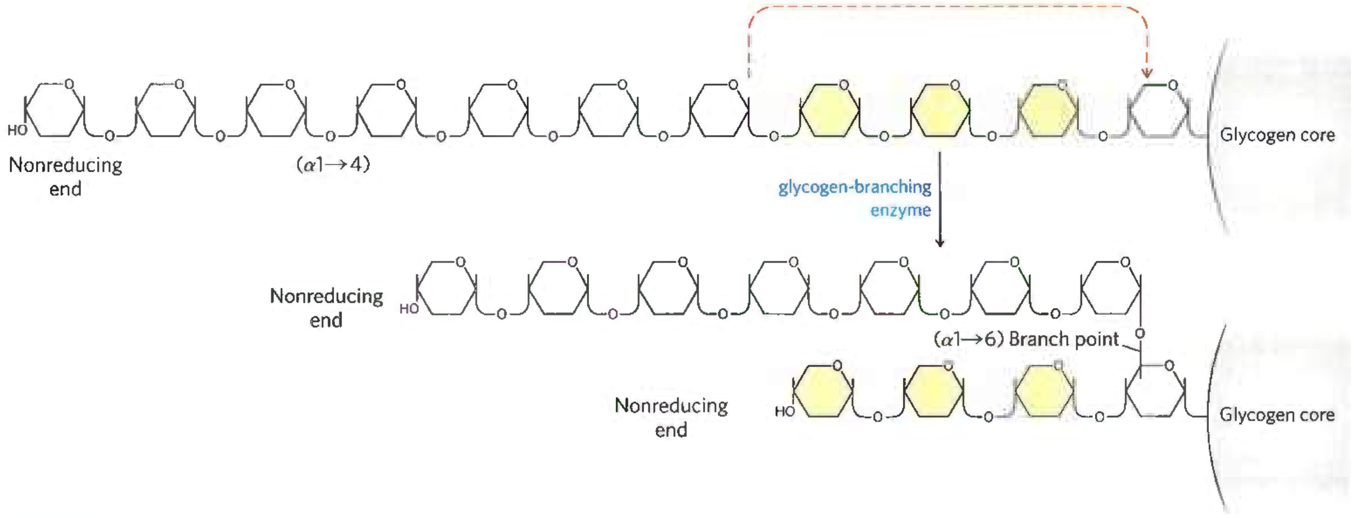

Nonreducing end
(α1→4)
Glycogen core
glycogen-branching enzyme
Nonreducing end
(α1→6) Branch point
Nonreducing end
Glycogen core

FIGURE 15-9 Branch synthesis in glycogen. The glycogen-branching enzyme forms a new branch point during glycogen synthesis.

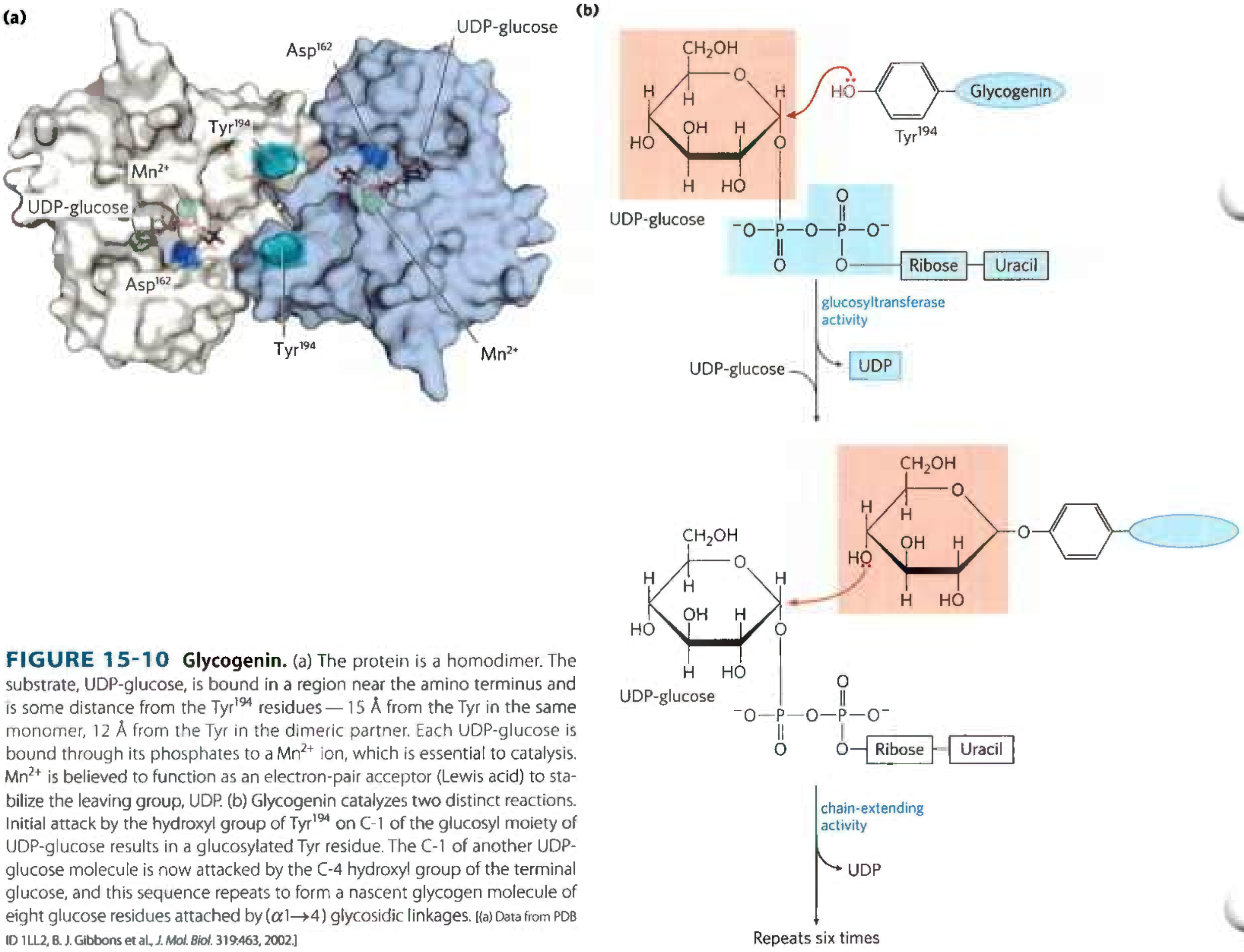

(a)
UDP-glucose
Asp162
Tyr194
Mn2+
UDP-glucose
Asp162
Tyr194
Mn2+
(b)
UDP-glucose
Glycogenin
Tyr194
Ribose
Uracil
glucosyltransferase activity
UDP-glucose
UDP
UDP-glucose
Ribose
Uracil
chain-extending activity
UDP
Repeats six times

FIGURE 15-10 Glycogenin. (a) The protein is a homodimer. The substrate, UDP-glucose, is bound in a region near the amino terminus and is some distance from the Tyr^{194} residues — 15 Å from the Tyr in the same monomer, 12 Å from the Tyr in the dimeric partner. Each UDP-glucose is bound through its phosphates to a Mn^{2+} ion, which is essential to catalysis. Mn^{2+} is believed to function as an electron-pair acceptor (Lewis acid) to stabilize the leaving group, UDP. (b) Glycogenin catalyzes two distinct reactions. Initial attack by the hydroxyl group of Tyr^{194} on C-1 of the glucosyl moiety of UDP-glucose results in a glucosylated Tyr residue. The C-1 of another UDP-glucose molecule is now attacked by the C-4 hydroxyl group of the terminal glucose, and this sequence repeats to form a nascent glycogen molecule of eight glucose residues attached by (α1→4) glycosidic linkages. [(a) Data from PDB ID 1LL2, B. J. Gibbons et al., *J. Mol. Biol.* 319:463, 2002.]

assembled and the enzyme that catalyzes their assembly. The first step in the synthesis of a new glycogen molecule is the transfer of a glucose residue from UDP-glucose to the hydroxyl group of Tyr^{194} of glycogenin. Each subunit of the glycogenin homodimer glycosylates Tyr^{194} of the other subunit. The glycosidic bond in the product has the same configuration about the C-1 of glucose as the substrate UDP-glucose, suggesting that the transfer of glucose from UDP to Tyr^{194} occurs in two steps. The first step is probably a nucleophilic attack by Asp^{162}, forming a temporary intermediate with inverted configuration. A second nucleophilic attack by Tyr^{194} then restores the starting configuration. The nascent chains are extended by the sequential addition of seven more glucose residues, each derived from UDP-glucose; the reactions are catalyzed by the chain-extending activity of glycogenin. At this point, glycogen synthase takes over, further extending the glycogen chain. Glycogenin remains buried within the β-particle, covalently attached to the two reducing ends of the glycogen molecule.

SUMMARY 15.2 *Breakdown and Synthesis of Glycogen*

- Glycogen phosphorylase catalyzes phosphorolytic cleavage at the nonreducing ends of glycogen chains, producing glucose 1-phosphate. The debranching enzyme transfers branches onto main chains and releases the residue at the ($\alpha 1 \rightarrow 6$) branch as free glucose.
- Phosphoglucomutase interconverts glucose 1-phosphate and glucose 6-phosphate. Glucose 6-phosphate can enter glycolysis or, in liver, it can be converted to free glucose by glucose 6-phosphatase in the endoplasmic reticulum, then exported to replenish blood glucose.
- The sugar nucleotide UDP-glucose donates glucose residues to the nonreducing end of glycogen in the reaction catalyzed by glycogen synthase, producing short ($\alpha 1 \rightarrow 4$)-linked segments. A separate branching enzyme produces the ($\alpha 1 \rightarrow 6$) linkages at branch points.
- New glycogen particles begin with the autocatalytic formation of a glycosidic bond between the glucose of UDP-glucose and a Tyr residue of the protein glycogenin, followed by addition of several glucose residues to form a primer that can be acted on by glycogen synthase.

15.3 Coordinated Regulation of Glycogen Breakdown and Synthesis

As we have seen, the mobilization of stored glycogen is brought about by glycogen phosphorylase, which degrades glycogen to glucose 1-phosphate (Fig. 15-3). Glycogen phosphorylase provides an especially instructive case of enzyme regulation. It was one of the first known examples of an allosterically regulated enzyme and the first enzyme shown to be controlled by reversible phosphorylation. It was also one of the first allosteric enzymes for which the detailed three-dimensional structures of the active and inactive forms were revealed by x-ray crystallographic studies. Glycogen phosphorylase also illustrates how isozymes play their tissue-specific roles.

Glycogen Phosphorylase Is Regulated by Hormone-Stimulated Phosphorylation and by Allosteric Effectors

P4 In the late 1930s, Carl and Gerty Cori (Box 15-1) discovered that the glycogen phosphorylase of skeletal muscle exists in two interconvertible forms: **glycogen phosphorylase *a***, which is catalytically active, and **glycogen phosphorylase *b***, which is much less active. (Note that glycogen phosphorylase is often referred to simply as phosphorylase — so honored because it was the first phosphorylase to be **discovered**; the shortened name has persisted in common usage and in the literature.) Subsequent studies by Earl Sutherland showed that phosphorylase *b* predominates in resting muscle, but during vigorous muscular activity, epinephrine triggers phosphorylation of phosphorylase *b*, converting it to its more active form, phosphorylase *a* (**Fig. 15-11**). In the liver,

Earl W. Sutherland, Jr., 1915–1974 [NLM/Science Source]

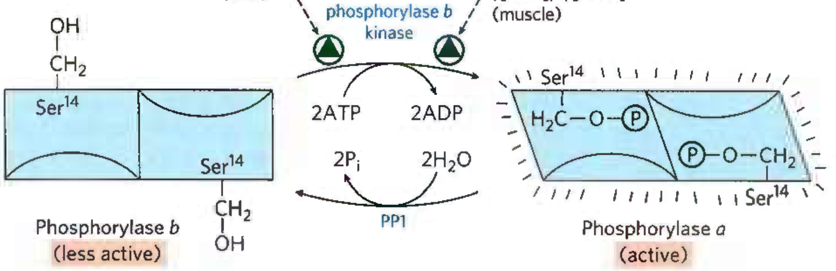


FIGURE 15-11 Regulation of muscle glycogen phosphorylase by covalent modification. In the more active form of the enzyme, phosphorylase *a*, Ser^{14} residues, one on each subunit, are phosphorylated. Phosphorylase *a* is converted to the less active form, phosphorylase *b*, by enzymatic loss of these phosphoryl groups, catalyzed by phosphoprotein phosphatase 1 (PP1). Phosphorylase *b* can be reconverted (reactivated) to phosphorylase *a* by the action of phosphorylase *b* kinase.

glucagon triggers phosphorylation of phosphorylase *b*, converting it to its active form.

Sutherland discovered the second messenger cAMP, which increases in concentration in response to stimulation by epinephrine (in muscle; see Fig. 12-4) or glucagon (in liver). Elevated [cAMP] initiates an **enzyme cascade** (**Fig. 15-12**), in which a catalyst activates a catalyst, which activates a catalyst. As we saw in Chapter 12, such cascades allow exponential amplification of the initial signal. The rise in [cAMP] activates cAMP-dependent protein kinase, also called protein kinase A (PKA). PKA then phosphorylates and activates **phosphorylase *b* kinase**, which catalyzes the phosphorylation of glycogen phosphorylase *b*, activating it and thus stimulating glycogen breakdown.

In muscle, this provides fuel for glycolysis to sustain muscle contraction for the fight-or-flight response signaled by epinephrine. In liver, glycogen breakdown counters the low blood glucose signaled by glucagon, by releasing glucose into the blood. These different roles are reflected in subtle differences in the regulatory mechanisms in muscle and liver. The glycogen phosphorylases of liver and muscle are isozymes, encoded by different genes and differing in their regulatory properties.

Figure 15-12 shows the pathway for hormonal regulation of glycogen phosphorylase activity: epinephrine or glucagon initiates the cascade that activates phosphorylase *b* kinase. Phosphorylase *b* kinase activates phosphorylase by transferring a phosphoryl group to Ser^{14} on each of the two identical subunits of phosphorylase *b*, triggering a conformation change from the T state (phosphorylase *b*) to the R state (phosphorylase *a*), shown in detail in Figure 6-40. **P4** Superimposed on the hormonal activation of phosphorylase *b* kinase are allosteric control mechanisms (Fig. 15-12). In muscle, Ca^{2+}, the signal for muscle contraction, binds

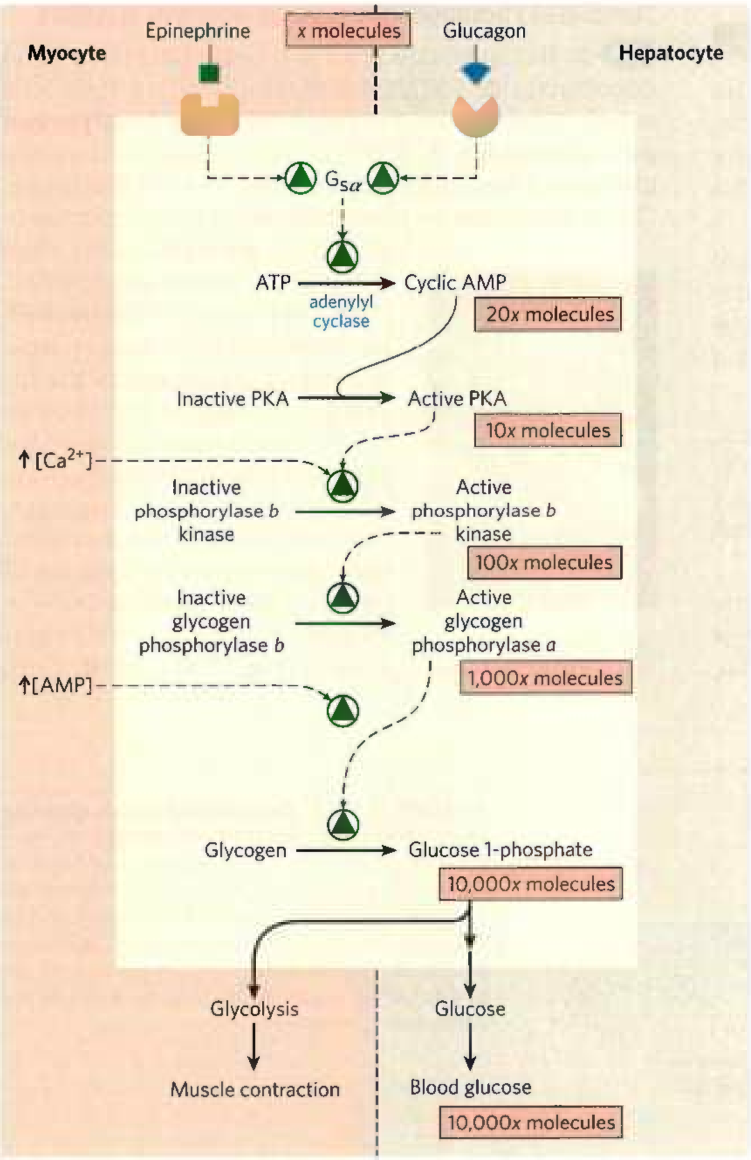

Myocyte
Epinephrine
x molecules
Glucagon
Hepatocyte
$G_{s\alpha}$
ATP
adenylyl cyclase
Cyclic AMP
20x molecules
Inactive PKA
Active PKA
10x molecules
↑[Ca^{2+}]
Inactive phosphorylase b kinase
Active phosphorylase b kinase
100x molecules
Inactive glycogen phosphorylase b
Active glycogen phosphorylase a
1,000x molecules
↑[AMP]
Glycogen
Glucose 1-phosphate
10,000x molecules
Glycolysis
Glucose
Muscle contraction
Blood glucose
10,000x molecules

FIGURE 15-12 Cascade mechanism of epinephrine and glucagon action. By binding to specific surface receptors, either epinephrine acting on a myocyte (left) or glucagon acting on a hepatocyte (right) activates a GTP-binding protein, $G_{s\alpha}$. Active $G_{s\alpha}$ triggers a rise in [cAMP], activating PKA. This sets off a cascade of phosphorylations; PKA activates phosphorylase *b* kinase, which then activates glycogen phosphorylase. Such cascades effect a large amplification of the initial signal; the figures in pink boxes are certainly low estimates of the actual increase in number of molecules at each stage of the cascade. The resulting breakdown of glycogen provides glucose, which in the myocyte can supply ATP (via glycolysis) for muscle contraction and in the hepatocyte is released into the blood to counter the low blood glucose.

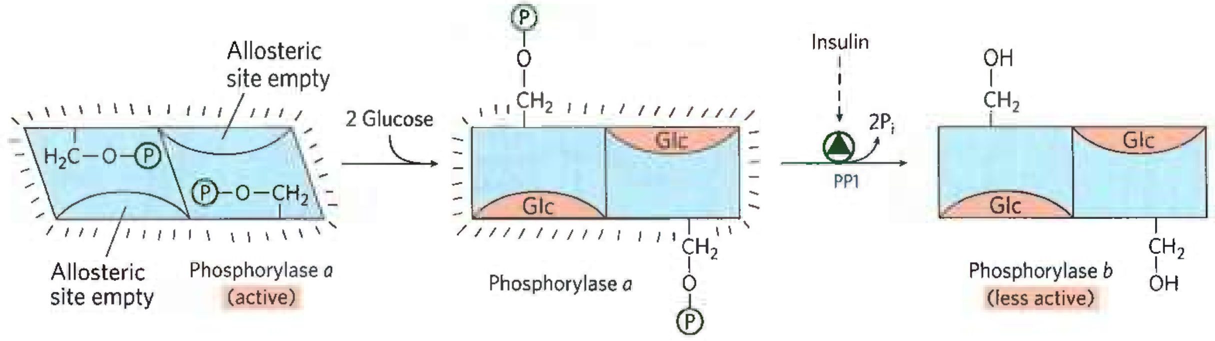

Allosteric
site empty
$H_2C-O-Ⓟ$
$Ⓟ-O-CH_2$
Allosteric
site empty
Phosphorylase *a*
(active)
2 Glucose
Ⓟ
O
CH_2
Glc
Glc
CH_2
O
Ⓟ
Phosphorylase *a*
Insulin
$2P_i$
PP1
OH
CH_2
Glc
Glc
CH_2
OH
Phosphorylase *b*
(less active)

FIGURE 15-13 Glycogen phosphorylase of liver as a glucose sensor. Glucose binding to an allosteric site of the phosphorylase *a* isozyme of liver induces a conformational change that exposes its phosphorylated Ser residues to the action of phosphoprotein phosphatase 1 (PP1). This phosphatase converts phosphorylase *a* to phosphorylase *b*, sharply reducing the activity of phosphorylase and slowing glycogen breakdown in response to high blood glucose. Insulin also acts indirectly to stimulate PP1 and slow glycogen breakdown.

to and activates phosphorylase *b* kinase. Ca^{2+} binds to phosphorylase *b* kinase through its δ subunit, which is calmodulin (see Fig. 12-17). AMP, which accumulates in vigorously contracting muscle as a result of ATP breakdown, binds to and activates phosphorylase, speeding the release of glucose 1-phosphate from glycogen. When ATP levels are adequate, ATP blocks the allosteric site to which AMP binds (see Fig. 6-40), inactivating phosphorylase.

When the muscle returns to rest, a second enzyme, **phosphoprotein phosphatase 1 (PP1)**, removes the phosphoryl groups from phosphorylase *a*, converting it to the less active form, phosphorylase *b* (see Fig. 15-11).

Like the enzyme of muscle, the glycogen phosphorylase of liver is regulated hormonally (by phosphorylation/dephosphorylation) and allosterically. The dephosphorylated form is essentially inactive. When the blood glucose level is too low, glucagon (acting through the cascade mechanism shown in Fig. 15-12) activates phosphorylase *b* kinase, which in turn converts phosphorylase *b* to its active *a* form, initiating the release of glucose into the blood. When blood glucose levels return to normal, glucose enters hepatocytes and binds to an inhibitory allosteric site on phosphorylase *a*. This binding also produces a conformational change that exposes the phosphorylated Ser residues to PP1, which catalyzes their dephosphorylation and inactivates the phosphorylase (**Fig. 15-13**). The allosteric site for glucose allows liver glycogen phosphorylase to act as its own glucose sensor and to respond appropriately to changes in blood glucose.

Glycogen Synthase Also Is Subject to Multiple Levels of Regulation

Like glycogen phosphorylase, glycogen synthase can exist in phosphorylated and unphosphorylated forms (**Fig. 15-14**). Its active form, **glycogen synthase *a***, is unphosphorylated. P4 **Glycogen synthase kinase 3 (GSK3)** adds phosphoryl groups to three Ser residues near the carboxyl terminus of glycogen synthase *a* converting it to **glycogen synthase *b***, which is inactive unless its allosteric activator, glucose 6-phosphate, is present. The action of GSK3 is hierarchical; it cannot phosphorylate glycogen synthase until another protein kinase, **casein kinase II (CKII)**, has first phosphorylated the glycogen synthase on a nearby residue, an event called **priming** (**Fig. 15-15a**). AMP-activated protein kinase (AMPK),

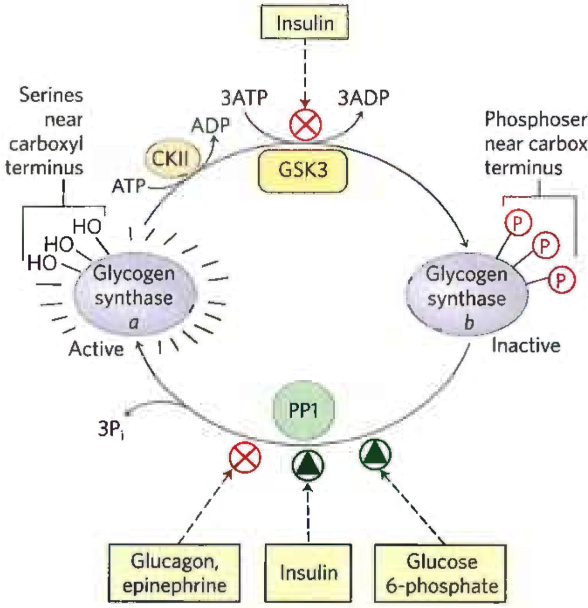

Insulin
Serines
near
carboxyl
terminus
3ATP
3ADP
ADP
CKII
ATP
GSK3
Phosphoserines
near carboxyl
terminus
HO
HO
HO
Glycogen
synthase
a
Active
Ⓟ
Ⓟ
Ⓟ
Glycogen
synthase
b
Inactive
PP1
$3P_i$
Glucagon,
epinephrine
Insulin
Glucose
6-phosphate

FIGURE 15-14 Effects of GSK3 on glycogen synthase activity. Glycogen synthase *a*, the active form, has three Ser residues near its carboxyl terminus. Their phosphorylation by glycogen synthase kinase 3 (GSK3) converts glycogen synthase to its inactive *b* form. Insulin favors the active *a* form of glycogen synthase by blocking the activity of GSK3 and activating phosphoprotein phosphatase 1 (PP1). In muscle, epinephrine activates PKA, which phosphorylates the glycogen-targeting protein GM on a site that causes dissociation of PP1 from glycogen. Glucose 6-phosphate favors dephosphorylation of glycogen synthase by binding to it and promoting a conformation that is a good substrate for PP1.

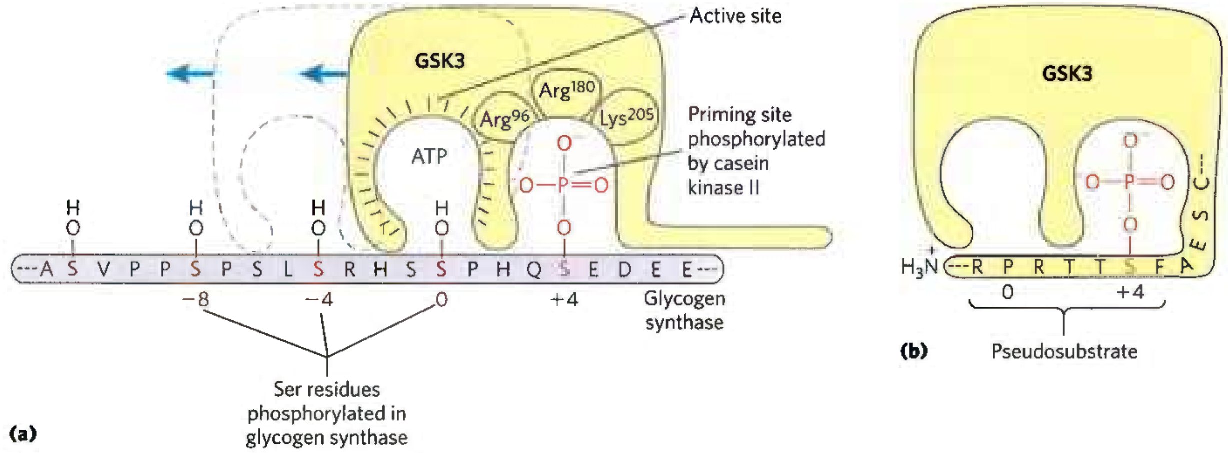

Active site
GSK3
Arg180
Arg96
Lys205
ATP
Priming site phosphorylated by casein kinase II
---A S V P P S P S L S R H S S P H Q S E D E E---
−8
−4
0
+4
Glycogen synthase
Ser residues phosphorylated in glycogen synthase
(a)
GSK3
H3N ---R P R T T S F A E S C---
0
+4
(b)
Pseudosubstrate

FIGURE 15-15 Priming of GSK3 phosphorylation of glycogen synthase. (a) Glycogen synthase kinase 3 first associates with its substrate (glycogen synthase) by interaction between three positively charged residues (Arg^{96}, Arg^{180}, Lys^{205}) and a phosphoserine residue at position +4 in the substrate. (For orientation, the Ser or Thr residue to be phosphorylated in the substrate is assigned the index 0. Residues on the amino-terminal side of this residue are numbered −1, −2, and so forth; residues on the carboxyl-terminal side are numbered +1, +2, and so forth.) This association aligns the active site of the enzyme with a Ser residue at position 0, which it phosphorylates. This creates a new priming site, and the enzyme moves down the protein to phosphorylate the Ser residue at position −4, and then the Ser at −8. (b) GSK3 has a Ser residue near its amino terminus that can be phosphorylated by PKA or PKB. This produces a "pseudosubstrate" region in GSK3 that folds into the priming site and makes the active site inaccessible to another protein substrate, inhibiting GSK3 until the priming phosphoryl group of its pseudosubstrate region is removed by PP1. Other proteins that are substrates for GSK3 also have a priming site at position +4, which must be phosphorylated by another protein kinase before GSK3 can act on them.

which associates with glycogen granules through its carbohydrate-binding domain, also phosphorylates glycogen synthase, inhibiting glycogen synthesis during periods of metabolic stress, signaled by high [AMP] and low [ATP].

Insulin favors activation of glycogen synthase by blocking the activity of GSK3 and activating PP1. In muscle, epinephrine activates PKA, which phosphorylates the glycogen-targeting protein GM (see Fig. 15-16), a regulatory subunit of PP1 in muscle, on a site that causes dissociation of PP1 from glycogen, effectively blocking its action on glycogen synthase.

In liver, conversion of glycogen synthase *b* to the active form is promoted by PP1, which is bound to the glycogen particle by its regulatory subunit in liver, GL. PP1 removes the phosphoryl groups from the three Ser residues phosphorylated by GSK3. Glucose 6-phosphate binds to an allosteric site on glycogen synthase, making the enzyme a better substrate for dephosphorylation by PP1 and causing its activation. By analogy with glycogen phosphorylase, which acts as a glucose sensor, glycogen synthase can be regarded as a glucose 6-phosphate sensor. In muscle, a different phosphatase may have the role played by PP1 in liver, activating glycogen synthase by dephosphorylating it.

As we saw in Chapter 12, one way in which insulin triggers intracellular changes is by activating a protein kinase (PKB) that, in turn, phosphorylates and inactivates GSK3 (see Fig. 12-23). Phosphorylation of a Ser residue near the amino terminus of GSK3 converts that region of the protein to a pseudosubstrate, which folds into the site at which the priming phosphorylated Ser residue normally binds (Fig. 15-15b). This prevents GSK3 from binding the priming site of a real substrate, thereby inactivating the enzyme and tipping the balance in favor of dephosphorylation of glycogen synthase by PP1. Glycogen phosphorylase can also affect the phosphorylation of glycogen synthase: active glycogen phosphorylase directly inhibits PP1, preventing it from activating glycogen synthase (Fig. 15-14).

P4 Insulin stimulates glycogen synthesis by activating PP1 and by inactivating GSK3. This single enzyme, PP1, can remove phosphoryl groups from all three of the enzymes phosphorylated in response to glucagon (liver) and epinephrine (liver and muscle): phosphorylase kinase, glycogen phosphorylase, and glycogen synthase. The catalytic subunit of PP1 (PP1c) does not exist free in the cytosol, but is tightly bound to its target proteins through a tissue-specific regulatory subunit, one of a family of **glycogen-targeting proteins** that bind glycogen and each of the three enzymes (**Fig. 15-16**). PP1 is itself subject to covalent and allosteric regulation: it is inactivated when phosphorylated by PKA and is allosterically activated by glucose 6-phosphate.

Allosteric and Hormonal Signals Coordinate Carbohydrate Metabolism Globally

Having looked at the mechanisms that regulate individual enzymes, we can now consider the overall shifts in carbohydrate metabolism that occur in the well-fed state, during fasting, and in the fight-or-flight response—signaled by insulin, glucagon, and epinephrine, respectively. We need to contrast two cases in which regulation serves different ends: (1) the role of hepatocytes in supplying glucose to the blood; and (2) the

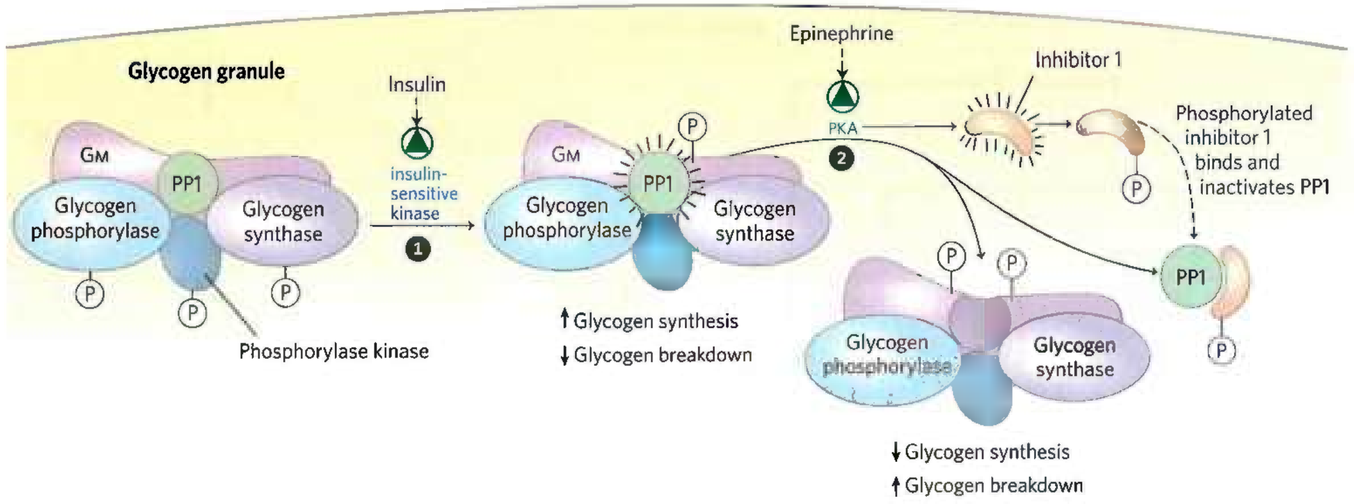


FIGURE 15-16 Glycogen-targeting protein GM. GM is a regulatory subunit of PP1 in muscle, and one of a family of glycogen-targeting proteins that serve as a scaffold, binding other proteins (including PP1) to glycogen particles. GM can be phosphorylated at two different sites in response to insulin or epinephrine. ❶ Insulin-stimulated phosphorylation of GM site 1 activates PP1, which dephosphorylates phosphorylase kinase, glycogen phosphorylase, and glycogen synthase. ❷ Epinephrine-stimulated phosphorylation of GM site 2 by PKA causes dissociation of PP1 from the glycogen particle, preventing its access to glycogen phosphorylase and glycogen synthase. PKA also phosphorylates a protein (inhibitor 1) that, when phosphorylated, inhibits PP1. By these means, insulin stimulates glycogen synthesis and inhibits glycogen breakdown, whereas epinephrine (or glucagon in the liver) has the opposite effects.

selfish use of carbohydrate fuels by extrahepatic tissues, typified by skeletal muscle (myocytes), to support their own activities.

After ingestion of a carbohydrate-rich meal, the elevation of blood glucose triggers insulin release (**Fig. 15-17**, top). In a hepatocyte, insulin has two immediate effects: it inactivates GSK3; and it activates a protein phosphatase, probably PP1. These two actions fully activate glycogen synthase. PP1 also inactivates glycogen phosphorylase *a* and phosphorylase kinase by dephosphorylating both, effectively stopping glycogen breakdown. Glucose enters the hepatocyte through the high-capacity transporter GLUT2, always present in the plasma membrane, and the elevated intracellular glucose leads to dissociation of hexokinase IV (glucokinase) from its nuclear regulatory protein (Fig. 14-21). Hexokinase IV enters the cytosol and phosphorylates glucose, stimulating glycolysis and supplying the precursor for glycogen synthesis. Under these conditions, hepatocytes use the excess glucose in the blood to synthesize glycogen, up to the limit of about 10% of the total weight of the liver.

Between meals, or during an extended fast, the blood glucose level drops, triggering the release of glucagon, which, acting through the cascade shown in Figure 15-12, activates PKA. PKA mediates all the effects of glucagon (Fig. 15-17, bottom). It phosphorylates phosphorylase kinase, activating it and leading to the activation of glycogen phosphorylase. It phosphorylates glycogen synthase, inactivating it and blocking glycogen synthesis. It phosphorylates PFK-2/FBPase-2, leading to a drop in the concentration of the regulator fructose 2,6-bisphosphate, which has the effect of inactivating the glycolytic enzyme PFK-1 and activating the gluconeogenic enzyme FBPase-1 (see Fig. 14-24). And it phosphorylates and inactivates the glycolytic enzyme pyruvate kinase. Under these conditions, the liver produces glucose 6-phosphate by glycogen breakdown and by gluconeogenesis, and it stops using glucose to fuel glycolysis or make glycogen, maximizing the amount of glucose it can release to the blood. This release of glucose is possible only in liver and kidney, because other tissues lack glucose 6-phosphatase (Fig. 15-6).

P4 The physiology of skeletal muscle differs from that of liver in three ways important to our discussion of metabolic regulation (**Fig. 15-18**): (1) muscle uses its stored glycogen only for its own needs; (2) as it goes from rest to vigorous contraction, muscle undergoes very large changes in its demand for ATP, which is provided by glycolysis; (3) muscle lacks the enzymatic machinery for gluconeogenesis. The regulation of carbohydrate metabolism in muscle reflects these differences from liver. First, myocytes lack receptors for glucagon, which are present on hepatocytes. Second, the muscle isozyme of pyruvate kinase is not phosphorylated by PKA, so glycolysis is not turned off when [cAMP] is high. In fact, cAMP *increases* the rate of glycolysis in muscle, probably by activating glycogen phosphorylase. When epinephrine is released into the blood in a fight-or-flight situation, PKA is activated by the rise in [cAMP] and phosphorylates and activates glycogen phosphorylase kinase. The resulting phosphorylation and activation of glycogen phosphorylase results in faster glycogen breakdown. Epinephrine is not released under low-stress conditions, but with each neuronal stimulation of muscle contraction, cytosolic $[\mathrm{Ca}^{2+}]$ rises briefly and activates phosphorylase kinase through its calmodulin subunit.

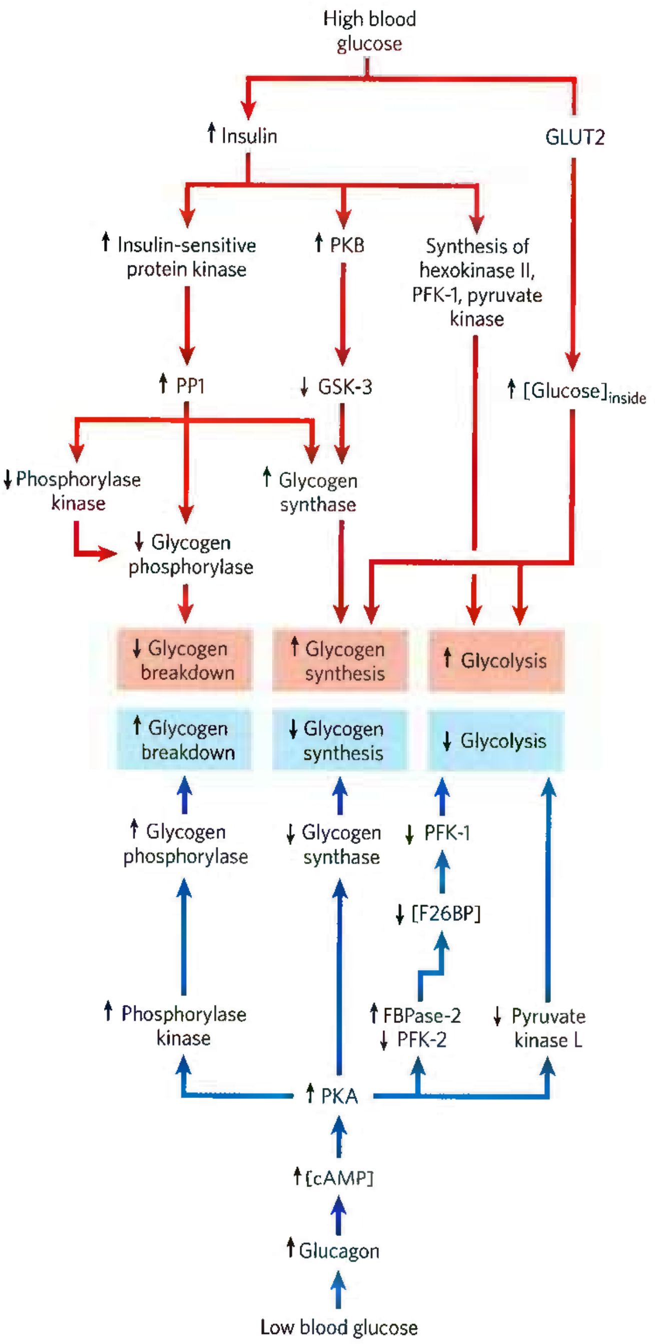

High blood glucose
↑Insulin
GLUT2
↑Insulin-sensitive protein kinase
↑PKB
Synthesis of hexokinase II, PFK-1, pyruvate kinase
↑PP1
↓GSK-3
↑[Glucose]$_{inside}$
↓Phosphorylase kinase
↑Glycogen synthase
↓Glycogen phosphorylase
↓Glycogen breakdown
↑Glycogen synthesis
↑Glycolysis
↑Glycogen breakdown
↓Glycogen synthesis
↓Glycolysis
↑Glycogen phosphorylase
↓Glycogen synthase
↓PFK-1
↓[F26BP]
↑Phosphorylase kinase
↑FBPase-2
↓PFK-2
↓Pyruvate kinase L
↑PKA
↑[cAMP]
↑Glucagon
Low blood glucose

FIGURE 15-17 Regulation of carbohydrate metabolism in the liver. Colored arrows indicate causal relationships between the changes they connect. For example, an arrow from ↓A to ↑B means that a decrease in A causes an increase in B. Red arrows connect events that result from high blood glucose; blue arrows connect events that result from low blood glucose.

Elevated insulin triggers increased glycogen synthesis in myocytes by activating PP1 and inactivating GSK3. Unlike hepatocytes, myocytes have a reserve of the glucose transporter GLUT4 sequestered in intracellular vesicles. Insulin triggers their movement to the plasma membrane (see Fig. 12-23), where they allow increased glucose uptake. In response to insulin, therefore, myocytes help to lower blood glucose by increasing their rates of glucose uptake, glycogen synthesis, and glycolysis.

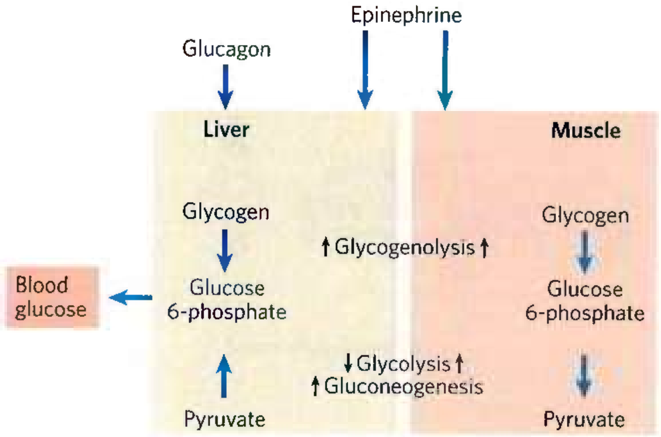

Epinephrine
Glucagon
Liver
Muscle
Glycogen
Glycogen
↑Glycogenolysis↑
Blood glucose
Glucose 6-phosphate
Glucose 6-phosphate
↓Glycolysis↑
↑Gluconeogenesis
Pyruvate
Pyruvate

FIGURE 15-18 Difference in the regulation of carbohydrate metabolism in liver and muscle. In liver, either glucagon (indicating low blood glucose) or epinephrine (signaling the need to fight or flee) has the effect of maximizing the output of glucose into the blood. In muscle, epinephrine increases glycogen breakdown and glycolysis, which together provide fuel to produce the ATP needed for muscle contraction.

Carbohydrate and Lipid Metabolism Are Integrated by Hormonal and Allosteric Mechanisms

As complex as the regulation of carbohydrate metabolism is, it is far from the whole story of fuel metabolism. **P4** The metabolism of fats and fatty acids is very closely tied to that of carbohydrates. Hormonal signals such as insulin and changes in diet or exercise are equally important in regulating fat metabolism and integrating it with that of carbohydrates. We return to this overall metabolic integration in mammals in Chapter 23, after first considering the metabolic pathways for fats and amino acids (Chapters 17 and 18). The message we wish to convey here is that metabolic pathways are overlaid with complex regulatory controls that are exquisitely sensitive to changes in metabolic circumstances. These mechanisms act to adjust the flow of metabolites through various metabolic pathways, as needed by the cell and organism, without causing major changes in the concentrations of intermediates shared with other pathways.

SUMMARY 15.3 *Coordinated Regulation of Glycogen Breakdown and Synthesis*

- Glycogen phosphorylase is activated in response to epinephrine (in muscle) or glucagon (in liver), which raise [cAMP] and thereby activate PKA. PKA phosphorylates and activates phosphorylase kinase, which converts glycogen phosphorylase *b* to its active *a* form. In muscle, Ca^{2+} and AMP act allosterically to amplify the activity of the enzymes in this cascade. In liver, glucose acts allosterically to make phosphorylase *a* more susceptible to dephosphorylation/inactivation by phosphoprotein phosphatase 1 (PP1).
- Glycogen synthase *a* is inactivated by phosphorylation, ultimately catalyzed by GSK3, but primed by phosphorylation by other kinases. Insulin, by causing inactivation of

GSK3 and activating PP1, favors glycogen synthase *a* and stimulates glycogen synthesis. Glucose 6-phosphate acts allosterically to make glycogen synthase *b* a better substrate for PP1, which dephosphorylates it to its active *a* form.

- In liver, glucagon stimulates glycogen breakdown and gluconeogenesis while blocking glycolysis, thereby sparing glucose for export to the brain and other tissues. In muscle, epinephrine stimulates glycogen breakdown and glycolysis, providing ATP to support contraction.
- Further hormonal and allosteric mechanisms regulate the use of carbohydrates and lipids as metabolic fuels.

KEY TERMS

Terms in bold are defined in the glossary.

glycogenolysis 558
glycogenesis 558
debranching enzyme 559
phosphoglucomutase 559
sugar nucleotides 560
UDP-glucose pyrophosphorylase 562
glycogen-branching enzyme 563
glycogenin 563
glycogen phosphorylase *a* 565
glycogen phosphorylase *b* 565
enzyme cascade 566
phosphorylase *b* kinase 566
phosphoprotein phosphatase 1 (PP1) 567
glycogen synthase *a* 567
glycogen synthase kinase 3 (GSK3) 567
glycogen synthase *b* 567
casein kinase II (CKII) 567
priming 567
glycogen-targeting proteins 568

PROBLEMS

1. Glycogen as Energy Storage: How Long Can a Game Bird Fly? Since ancient times, people have observed that certain game birds, such as grouse, quail, and pheasants, fatigue easily. The Greek historian Xenophon wrote: "The bustards . . . can be caught if one is quick in starting them up, for they will fly only a short distance, like partridges, and soon tire; and their flesh is delicious." The flight muscles of game birds rely almost entirely on the use of glucose 1-phosphate to drive ATP synthesis (Chapter 14). The glucose 1-phosphate derives from the breakdown of stored muscle glycogen, catalyzed by the enzyme glycogen phosphorylase. The rate of ATP production is limited by the rate at which glycogen can be broken down. During a "panic flight," the game bird's rate of glycogen breakdown is quite high, approximately 120 μmol/min of glucose 1-phosphate produced per gram of fresh tissue. Given that the flight muscles usually contain about 0.35% glycogen by weight, calculate how long a game bird can fly. (Assume the average molecular weight of a glucose residue in glycogen is 162 g/mol.)

2. Enzyme Activity and Physiological Function The $V_{\max}$ of the glycogen phosphorylase from skeletal muscle is much greater than the $V_{\max}$ of the same enzyme from liver tissue.
(a) What is the physiological function of glycogen phosphorylase in skeletal muscle? In liver tissue?
(b) Why does the $V_{\max}$ of the muscle enzyme need to be greater than that of the liver enzyme?

3. Glycogen Phosphorylase Equilibrium Glycogen phosphorylase catalyzes the removal of glucose from glycogen. The $\Delta G'^{\circ}$ for this reaction is 3.1 kJ/mol.
(a) Calculate the ratio of [P_i] to [glucose 1-phosphate] when the reaction is at equilibrium. (Hint: The removal of glucose units from glycogen does not change the glycogen concentration.)
(b) The measured ratio [P_i]/[glucose 1-phosphate] in myocytes under physiological conditions is more than 100:1. What does this indicate about the direction of metabolite flow through the glycogen phosphorylase reaction in muscle?
(c) Why are the equilibrium and physiological ratios different? What is the possible significance of this difference?

4. Regulation of Glycogen Phosphorylase In muscle tissue, the rate of conversion of glycogen to glucose 6-phosphate is determined by the ratio of phosphorylase *a* (active) to phosphorylase *b* (less active). Determine what happens to the rate of glycogen breakdown if a broken cell extract of muscle containing glycogen phosphorylase is treated with **(a)** phosphorylase kinase and ATP **(b)** PP1 **(c)** epinephrine.

5. Glycogen Breakdown in Rabbit Muscle The intracellular use of glucose and glycogen is tightly regulated at four points. To compare the regulation of glycolysis when oxygen is plentiful and when it is depleted, consider the utilization of glucose and glycogen by rabbit leg muscle in two physiological settings: a resting rabbit, with low ATP demands, and a rabbit that sights its mortal enemy, the coyote, and dashes into its burrow. For each setting, determine the relative levels (high, intermediate, or low) of AMP, ATP, citrate, and acetyl-CoA and describe how these levels affect the flow of metabolites through glycolysis by regulating specific enzymes. (Hint: In periods of stress, rabbit leg muscle produces much of its ATP by anaerobic glycolysis (lactate fermentation) and very little by oxidation of acetyl-CoA derived from fat breakdown.)

6. Glycogen Breakdown in Migrating Birds Unlike a rabbit, running all-out for a few moments to escape a predator, migratory birds require energy for extended periods of time. For example, ducks generally fly several thousand miles during their annual migration. The flight muscles of migratory birds have a high oxidative capacity and obtain the necessary ATP through the oxidation of acetyl-CoA (obtained from fats) via the citric acid cycle. Compare the regulation of muscle glycolysis during short-term intense activity, as in a fleeing rabbit, and during extended activity, as in a migrating duck. Why must the regulation in these two settings be different?

7. Enzyme Defects in Carbohydrate Metabolism Consider the four clinical case studies, A through D. For each case, determine which enzyme is defective and designate the appropriate treatment from the lists provided at the end of the problem. Justify your choices. Answer the questions contained in each case study. (You may need to refer to information in Chapter 14.)

Case A: The patient develops vomiting and diarrhea shortly after milk ingestion. The physician administers a lactose tolerance test. (The patient ingests a standard amount of lactose, and the physician measures the glucose and galactose concentrations in his blood plasma at intervals. In individuals with normal carbohydrate metabolism, the levels increase to a maximum in about 1 hour, then decline.) The patient's blood glucose and galactose concentrations do not increase during the test. Why do blood glucose and galactose increase and then decrease during the test in healthy individuals? Why do they fail to rise in the patient?

Case B: The patient develops vomiting and diarrhea after ingestion of milk. Blood tests show a low concentration of glucose but a much higher than normal concentration of reducing sugars. The urine tests positive for galactose. Why is the concentration of reducing sugar in the blood high? Why does galactose appear in the urine?

Case C: The patient complains of painful muscle cramps when performing strenuous physical exercise but has no other symptoms. A muscle biopsy indicates a muscle glycogen concentration much higher than normal. Why does glycogen accumulate?

Case D: The patient is lethargic, her liver is enlarged, and a biopsy of the liver shows large amounts of excess glycogen. She also has a lower than normal blood glucose level. What is the reason for the low blood glucose in this patient?

Defective Enzyme

(a) Muscle PFK-1
(b) Phosphomannose isomerase
(c) Galactose 1-phosphate uridylyltransferase
(d) Liver glycogen phosphorylase
(e) Triose kinase
(f) Lactase in intestinal mucosa
(g) Maltase in intestinal mucosa
(h) Muscle debranching enzyme

Treatment

1. Jogging 5 km each day
2. Fat-free diet
3. Low-lactose diet
4. Avoiding strenuous exercise
5. Large doses of niacin (the precursor of NAD)
6. Frequent feedings (smaller portions) of a normal diet

8. Effects of Insufficient Insulin in a Person with Diabetes A man with insulin-dependent diabetes is brought to the hospital emergency department in a near-comatose state. While vacationing in an isolated place, he lost his insulin medication and has not taken any insulin for two days.

(a) For each tissue listed at the end of the problem, is each pathway faster, slower, or unchanged in this patient, compared with the normal level when he is getting appropriate amounts of insulin?

(b) For each pathway, describe at least one control mechanism responsible for the change you predict.

Tissue and Pathways

1. Adipose: fatty acid synthesis
2. Muscle: glycolysis; fatty acid synthesis; glycogen synthesis
3. Liver: glycolysis; gluconeogenesis; glycogen synthesis; fatty acid synthesis; pentose phosphate pathway

9. Blood Metabolites in Insulin Insufficiency For the patient described in Problem 8, predict the levels of each listed metabolite in his blood *before* treatment in the emergency room, relative to levels maintained during adequate insulin treatment: **(a)** glucose **(b)** ketone bodies **(c)** free fatty acids.

10. Metabolic Effects of Mutant Enzymes Predict and explain the effect on glycogen metabolism of each of the listed defects caused by mutation: **(a)** Loss of the cAMP-binding site on the regulatory subunit of protein kinase A (PKA) **(b)** Loss of the protein phosphatase inhibitor (inhibitor 1 in Fig. 15-16) **(c)** Overexpression of phosphorylase *b* kinase in liver **(d)** Defective glucagon receptors in liver.

11. Hormonal Control of Metabolic Fuel Between your evening meal and breakfast, your blood glucose drops and your liver becomes a net producer rather than consumer of glucose. Describe the hormonal basis for this switch, and explain how the hormonal change triggers glucose production by the liver.

12. Altered Metabolism in Genetically Manipulated Mice Researchers can manipulate the genes of a mouse so that a single gene in a single tissue either produces an inactive protein (a "knockout" mouse) or produces a protein that is always (constitutively) active. What effects on metabolism would you predict for mice with the listed genetic changes? **(a)** Knockout of glycogen debranching enzyme in the liver **(b)** Knockout of hexokinase IV in liver **(c)** Knockout of FBPase-2 in liver **(d)** Constitutively active FBPase-2 in liver **(e)** Constitutively active AMPK in muscle **(f)** Constitutively active ChREBP in liver (see Fig. 14-28)

DATA ANALYSIS PROBLEM

13. Optimal Glycogen Structure Muscle cells need rapid access to large amounts of glucose during heavy exercise. This glucose is stored in liver and skeletal muscle in polymeric form as particles of glycogen. The typical glycogen β-particle contains about 55,000 glucose residues (see Fig. 15-2). Meléndez-Hevia, Waddell, and Shelton (1993) explored some theoretical aspects of the structure of glycogen, as described in this problem.

(a) The cellular concentration of glycogen in liver is about 0.01 μM. What cellular concentration of free glucose would be required to store an equivalent amount of glucose? Why would this concentration of free glucose present a problem for the cell?

Glucose is released from glycogen by glycogen phosphorylase, an enzyme that can remove glucose molecules, one at a time, from one end of a glycogen chain (see Fig. 15-3). Glycogen chains are branched (see Fig. 15-2), and the degree of branching—the number of branches per chain—has a powerful influence on the rate at which glycogen phosphorylase can release glucose.

(b) Why would a degree of branching that was too low (i.e., below an optimum level) reduce the rate of glucose release? (Hint: Consider the extreme case of no branches in a chain of 55,000 glucose residues.)

(c) Why would a degree of branching that was too high also reduce the rate of glucose release? (Hint: Think of the physical constraints.)

Meléndez-Hevia and colleagues did a series of calculations and found that two branches per chain (see Fig. 15-2) was optimal for the constraints described above. This is what is found in glycogen stored in muscle and liver.

To determine the optimum number of glucose residues per chain, Meléndez-Hevia and coauthors considered two key parameters that define the structure of a glycogen particle: t = the number of tiers of glucose chains in a particle (the molecule in Fig. 15-2 has five tiers); g_c = the number of glucose residues in each chain. They set out to find the values of t and g_c that would maximize three quantities: (1) the amount of glucose stored in the particle (G_T) per unit volume; (2) the number of unbranched glucose chains (C_A) per unit volume (i.e., number of A chains in the outermost tier, readily accessible to glycogen phosphorylase); and (3) the amount of glucose available to phosphorylase in these unbranched chains (G_{PT}).

(d) Show that $C_A = 2^{t-1}$. This is the number of chains available to glycogen phosphorylase before the action of the debranching enzyme.

(e) Show that C_T, the total number of chains in the particle, is given by $C_T = 2^t - 1$. For purposes of this calculation, consider the primers to be a single chain. Thus $G_T = g_c(C_T) = g_c(2^t - 1)$, the total number of glucose residues in the particle.

(f) Glycogen phosphorylase cannot remove glucose from glycogen chains that are shorter than five glucose residues. Show that $G_{PT} = (g_c - 4)(2^{t-1})$. This is the amount of glucose readily available to glycogen phosphorylase.

(g) Based on the size of a glucose residue and the location of branches, the thickness of one tier of glycogen is $0.12\ g_c$ nm + 0.35 nm. Show that the volume of a particle, V_s, is given by the equation $V_s = \frac{4}{3}\pi t^3(0.12g_c + 0.35)^3$ nm^3.

Meléndez-Hevia and coauthors then determined the optimum values of t and g_c—those that gave the maximum value of a quality function, f, that maximizes G_T, C_A, and G_{PT}, while minimizing V_s: $f = \dfrac{G_T C_A G_{PT}}{V_s}$. They found that the optimum value of g_c is independent of t.

(h) Choose a value of t between 5 and 15 and find the optimum value of g_c. How does this compare with the g_c found in liver glycogen (see Fig. 15-2)? (Hint: You may find it useful to use a spreadsheet program.)

Reference

Meléndez-Hevia, E., T.G. Waddell, and E.D. Shelton. 1993. Optimization of molecular design in the evolution of metabolism: the glycogen molecule. *Biochem. J.* 295:477–483.

Chapter 16

THE CITRIC ACID CYCLE

16.1 Production of Acetyl-CoA (Activated Acetate) *575*
16.2 Reactions of the Citric Acid Cycle *578*
16.3 The Hub of Intermediary Metabolism *590*
16.4 Regulation of the Citric Acid Cycle *593*

As we saw in Chapter 14, some cells obtain energy (ATP) by fermentation, breaking down glucose in the absence of oxygen. For most eukaryotic cells and many bacteria, which live under aerobic conditions and oxidize their organic fuels to carbon dioxide and water, glycolysis is but the first stage in the complete oxidation of glucose. Rather than being reduced to lactate, ethanol, or some other fermentation product, the pyruvate produced by glycolysis is further oxidized to H_2O and CO_2 through the process of **cellular respiration**.

Cellular respiration occurs in three major stages (**Fig. 16-1**), the first two of which we discuss in this chapter. In the first, organic fuel molecules—glucose, fatty acids, and some amino acids—are oxidized to yield two-carbon fragments in the form of the acetyl group of acetyl-coenzyme A (acetyl-CoA). In the second stage, the acetyl groups are oxidized to CO_2 in the **citric acid cycle**, also called the **tricarboxylic acid (TCA) cycle** or the **Krebs cycle** (after its discoverer, Hans Krebs). Much of the energy of these oxidations is conserved in the reduced electron carriers NADH and $FADH_2$. In the third stage of respiration, these reduced coenzymes are themselves oxidized, giving up protons (H^+) and electrons. The electrons are transferred to O_2 via a series of electron-carrying molecules known as the respiratory chain, resulting in the formation of water. In the course of electron transfer, much of the energy available from redox reactions is conserved in the form of ATP, by a process called oxidative phosphorylation. We discuss this third stage in Chapter 19.

Hans Krebs, 1900–1981
(Keystone Pictures USA/Alamy.)

We use these principles as our guide to this chapter:

P1 **Pyruvate is the metabolite that links two central catabolic pathways, glycolysis and the citric acid cycle.** It is therefore a logical point for regulation that determines the rate of catabolic activity and the partitioning of pyruvate among its possible uses.

P2 **The reactions of the citric acid cycle follow a chemical logic.** In its catabolic role the citric acid cycle oxidizes acetyl-CoA to CO_2 and H_2O. Energy from the oxidations in the cycle drives the synthesis of ATP. The chemical strategies for activating groups for oxidation and for conserving energy in the form of reducing power and high-energy compounds are used in many other biochemical pathways.

P3 **The citric acid cycle is a hub of metabolism, with catabolic pathways leading in and anabolic pathways leading out.** Acetate groups (acetyl-CoA) from the catabolism of various fuels are used in the synthesis of such metabolites as amino acids, fatty acids, and sterols. The breakdown products of many amino acids and nucleotides are intermediates of the cycle, and they can be fed in or siphoned off as needed by the cell.

P4 **The central role of the citric acid cycle in metabolism requires that it be regulated in coordination with many other pathways.** Regulation occurs by both allosteric and covalent mechanisms that overlap and interact to achieve homeostasis. Some mutations that affect the reactions of the citric acid cycle lead to tumor formation.

P5 **Enzymes have evolved to form complexes to efficiently achieve a series of chemical transformations without releasing the intermediates into the bulk solvent.** This strategy, seen in the pyruvate dehydrogenase complex and the metabolons of the citric acid cycle, is ubiquitous in other pathways of metabolism, in respiration, and in the many "—somes" that assemble and disassemble informational macromolecules.

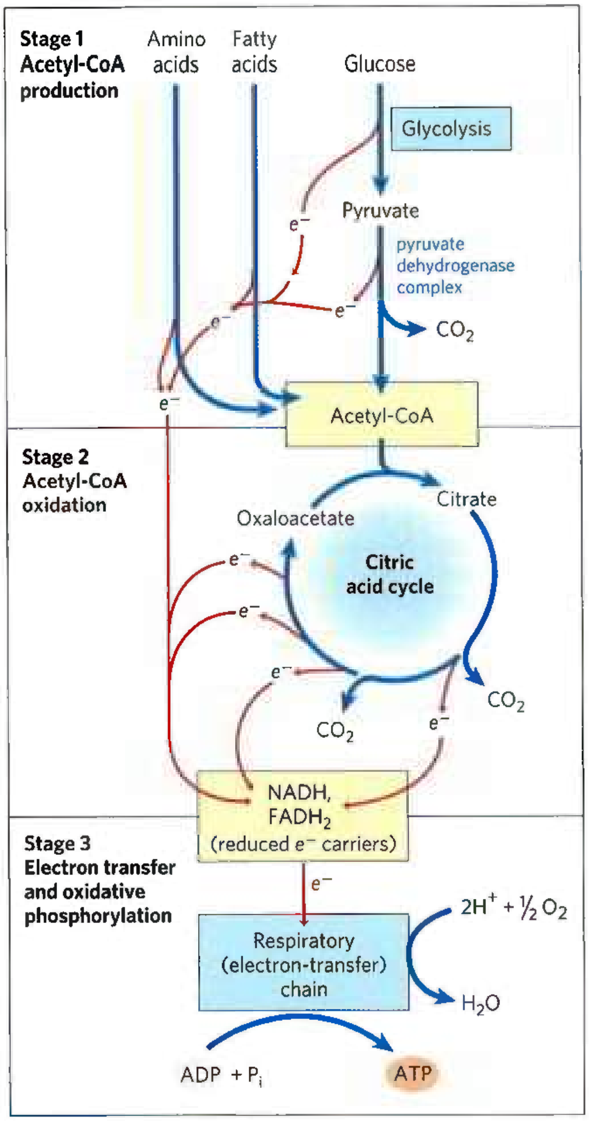


FIGURE 16-1 Catabolism of proteins, fats, and carbohydrates in the three stages of cellular respiration. Stage 1: oxidation of fatty acids, glucose, and some amino acids yields acetyl-CoA. Stage 2: oxidation of acetyl groups in the citric acid cycle includes four steps in which electrons are abstracted. Stage 3: electrons carried by NADH and $FADH_2$ are funneled into a chain of mitochondrial (or, in bacteria, plasma membrane–bound) electron carriers—the respiratory chain—ultimately reducing O_2 to H_2O. This electron flow drives the production of ATP.

16.1 Production of Acetyl-CoA (Activated Acetate)

Coenzyme A, or CoA (**Fig. 16-2**), has a reactive thiol (—SH) group that is critical to its role as an acyl carrier in many metabolic processes, including the citric acid cycle. Acyl groups are covalently linked to the thiol group, forming **thioesters**. Because of their relatively high standard free energies of hydrolysis (see Figs. 13-16, 13-17), thioesters have a high acyl group transfer potential—that is, donation of their acyl groups to a variety of acceptor molecules is a favorable reaction. The acyl group attached to coenzyme A may thus be thought of as "activated" for group transfer.

In aerobic organisms, glucose and other sugars, fatty acids, and most amino acids are ultimately oxidized to CO_2 and H_2O via the citric acid cycle and the respiratory chain. Before entering the citric acid cycle, the carbon skeletons of monosaccharides and fatty acids are degraded to the acetyl group of acetyl-CoA, the form in which the cycle accepts much of its fuel input. Many amino acid carbons also enter the cycle as acetate, although several amino acids are degraded to other cycle intermediates such as succinate and malate, which then enter the cycle.

Pyruvate Is Oxidized to Acetyl-CoA and CO_2

P1 Pyruvate generated in the cytosol by glycolysis represents a node in the metabolism of carbohydrates, proteins, and fats. Under anaerobic conditions, pyruvate may simply be reduced to lactate in the cytosol, regenerating NAD^+ for continued ATP production by glycolysis. Pyruvate may serve as a precursor for the synthesis of amino acids (Chapter 22). In eukaryotes, pyruvate may diffuse into mitochondria, first through large openings in the outer mitochondrial membrane and then into the matrix via an H^+-coupled pyruvate-specific symporter in the inner mitochondrial membrane, the **mitochondrial pyruvate carrier (MPC)**. Pyruvate that enters mitochondria may be oxidized by the citric acid cycle to generate energy or, after conversion to acetyl-CoA, may be used as the starting material for synthesis of fatty acids and sterols.

Pyruvate in the mitochondrial matrix is oxidized to acetyl-CoA and CO_2 by the **pyruvate dehydrogenase (PDH) complex**, a highly ordered cluster of enzymes and cofactors. **P5** In the PDH complex, a series of chemical intermediates remain bound to the enzyme subunits as a substrate (pyruvate) is transformed into the final product (acetyl-CoA). Five cofactors, four of which are derived from vitamins, participate in the reaction mechanism. **P4** The regulation of this enzyme complex illustrates how a combination of covalent modification and allosteric mechanism results in precisely regulated flux through a metabolic step.

The overall reaction catalyzed by the pyruvate dehydrogenase complex is an **oxidative decarboxylation**, an irreversible oxidation process in which the carboxyl group is removed from pyruvate as a molecule of CO_2 and the two remaining carbons become the acetyl group of acetyl-CoA (**Fig. 16-3**). The NADH formed in this reaction gives up a hydride ion ($:H^-$) to the respiratory chain (Fig. 16-1), which carries the two electrons to oxygen or, in anaerobic microorganisms, to an alternative electron acceptor such as nitrate or sulfate. The transfer of electrons from NADH to oxygen ultimately generates 2.5 molecules of ATP per pair of electrons. The irreversibility of the PDH complex reaction has been demonstrated by isotopic labeling experiments: the complex cannot reattach radioactively labeled CO_2 to acetyl-CoA to yield carboxyl-labeled pyruvate.

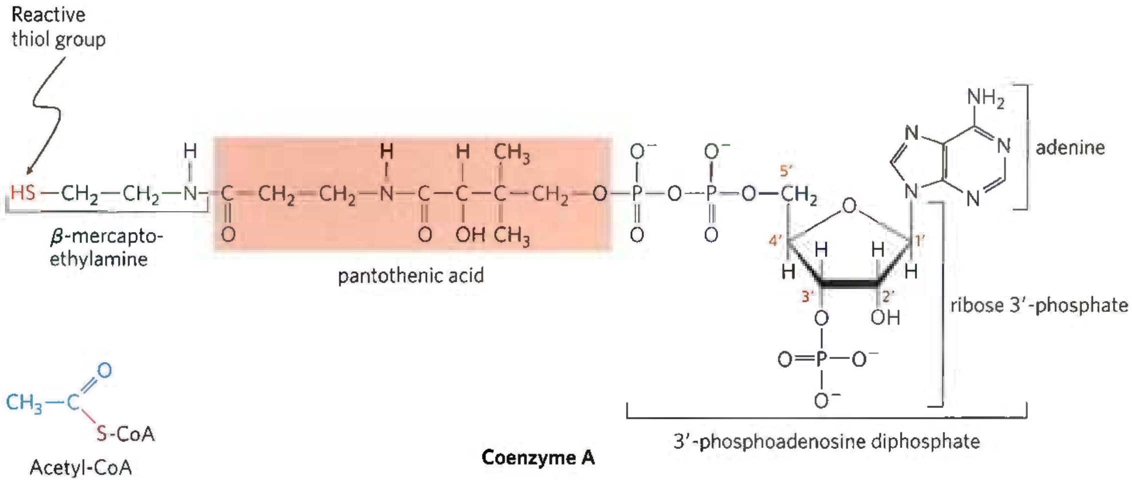

Reactive thiol group
HS
β-mercapto-ethylamine
pantothenic acid
adenine
ribose 3′-phosphate
3′-phosphoadenosine diphosphate
Coenzyme A
Acetyl-CoA
S-CoA

FIGURE 16-2 Coenzyme A (CoA-SH). A hydroxyl group of pantothenic acid is joined to a modified ADP moiety by a phosphate ester bond, and its carboxyl group is attached to β-mercaptoethylamine in amide linkage. The hydroxyl group at the 3′ position of the ADP moiety has a phosphoryl group not present in free ADP. The —SH group of the mercaptoethylamine moiety forms a thioester with acetate in acetyl-coenzyme A (acetyl-CoA) (lower left).

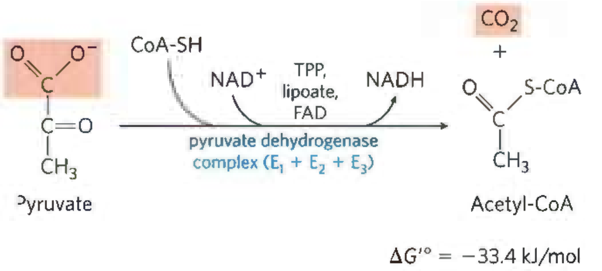

CoA-SH
NAD+
TPP, lipoate, FAD
NADH
CO2
pyruvate dehydrogenase complex (E1 + E2 + E3)
Pyruvate
Acetyl-CoA
ΔG′° = −33.4 kJ/mol

FIGURE 16-3 Overall reaction catalyzed by the pyruvate dehydrogenase complex. The five coenzymes participating in this reaction, and the three enzymes that make up the enzyme complex, are discussed in the text.

The PDH Complex Employs Three Enzymes and Five Coenzymes to Oxidize Pyruvate

The combined dehydrogenation and decarboxylation of pyruvate to the acetyl group of acetyl-CoA (Fig. 16-3) requires the sequential action of three different enzymes and five different coenzymes or prosthetic groups—thiamine pyrophosphate (TPP), lipoate, coenzyme A (CoA, sometimes denoted CoA-SH, to emphasize the role of the —SH group), flavin adenine dinucleotide (FAD), and nicotinamide adenine dinucleotide (NAD). We encountered TPP as the coenzyme of pyruvate decarboxylase (see Fig. 14-13). **Lipoate** (**Fig. 16-4**), has two thiol groups that can undergo reversible oxidation to a disulfide bond (—S—S—), similar to that between two Cys residues in a protein. Because of its capacity to undergo oxidation-reduction reactions, lipoate can serve both as an electron (hydrogen) carrier and as an acyl carrier, as we shall see. CoA serves as the carrier of the activated acyl group. We described the roles of FAD and NAD as electron carriers in Chapter 13.

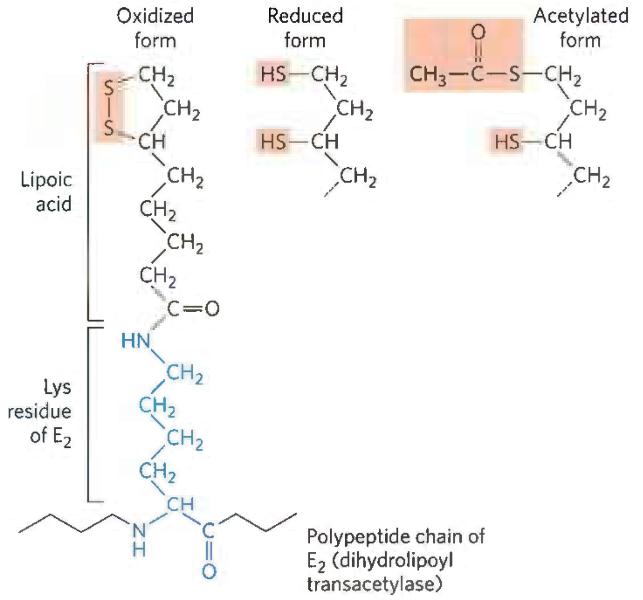

Oxidized form
Reduced form
Acetylated form
Lipoic acid
Lys residue of E2
Polypeptide chain of E2 (dihydrolipoyl transacetylase)

FIGURE 16-4 Lipoic acid (lipoate) in amide linkage with a Lys residue. The lipoyllysyl moiety is the prosthetic group of dihydrolipoyl transacetylase (E_2 of the PDH complex). The lipoyl group occurs in oxidized (disulfide) and reduced (dithiol) forms and acts as a carrier of both hydrogen and an acetyl (or other acyl) group.

The PDH complex contains multiple copies of three enzymes—**pyruvate dehydrogenase** (E_1), **dihydrolipoyl transacetylase** (E_2), and **dihydrolipoyl dehydrogenase** (E_3)—that catalyze the oxidation of pyruvate. The number of copies of each enzyme and therefore the size of the complex varies among species. A central core is formed of many copies (24 to 60) of E_2 surrounded by multiple and variable numbers of copies of E_1 and E_3 (**Fig. 16-5**). Two regulatory proteins are also part of the

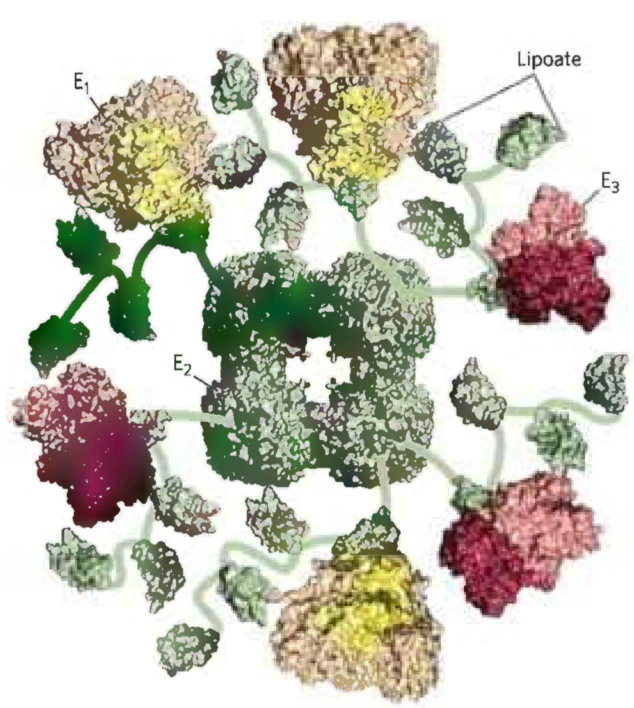


FIGURE 16-5 Structure of the pyruvate dehydrogenase complex. The complex is so big and so flexible that solving its structure required a combination of methods, including x-ray crystallography and NMR spectroscopy; once the individual pieces were solved, cryo-EM of the whole structure was used to assemble the pieces from several organisms to get this view. The core (E_2) is from the gram-negative bacterium *Azotobacter vinelandii*. E_1 and E_3 are from the thermophilic gram-positive bacterium *Geobacillus stearothermophilus*. E_1, pyruvate dehydrogenase (yellow); E_2, dihydrolipoyl transacetylase (green); and E_3, dihydrolipoyl dehydrogenase (red). The central core of the *Azotobacter* PDH complex consists of 24 copies of E_2, but to simplify the structure, only six are shown here. Multiple copies of E_1 and E_3 surround the central core, and flexible arms (shown schematically) reach out from E_2 to E_1 and E_3, carrying the lipoyl moiety (pink) from the active site of one enzyme to that of the next. The amino acid sequences and three-dimensional structures of individual domains show that both catalytic mechanism and structure have been conserved in evolution. [Information from D. Goodsell, doi:10.2210/rcsb_pdb/mom_2012_9. Data from PDB ID 1LAC F. Dardel et al., *J. Mol. Biol.* 229:1037, 1993; PDB ID 1EAA A. Mattevi et al., *Biochemistry* 32:3887, 1993; PDB ID 1W85 R. A. Frank et al., *Science* 306:872, 2004; PDB ID 1EBD S. S. Mande et al., *Structure* 4:277, 1996.]

complex: a protein kinase and a phosphoprotein phosphatase, discussed below.

The active site of E_1 has noncovalently bound TPP. E_2 has the prosthetic group lipoate, attached through an amide bond to the ε-amino group of a Lys residue (Fig. 16-4). E_2 has three functionally distinct domains: one or more (depending on the species) amino-terminal *lipoyl domains*, containing the lipoyl-Lys residue(s); the central E_1- and E_3-*binding domain*; and the inner-core *acyltransferase domain*, which contains the acyltransferase active site. The domains of E_2 are separated by linkers, sequences of 20 to 30 amino acid residues, rich in Ala and Pro and interspersed with charged residues; these linkers tend to assume their extended forms, holding the three domains apart. The attachment of lipoate to the end of a Lys side chain in E_2 produces a long, flexible arm that can move from the active site of E_1 to the active sites of E_2 and E_3, a distance of perhaps 5 nm or more, while holding the intermediate captive throughout the reaction sequence. E_3 has tightly bound FAD.

P5 This basic E_1—E_2—E_3 structure of the PDH complex is seen in two other enzyme complexes that catalyze similar reactions: α-ketoglutarate dehydrogenase, which oxidizes α-ketoglutarate in the citric acid cycle (described below), and the branched-chain α-keto acid dehydrogenase, which oxidizes α-keto acids derived from the breakdown of the branched-chain amino acids valine, isoleucine, and leucine (see Fig. 18-28). Within a given species, E_3 is identical in all three complexes. The remarkable similarity in protein structure, cofactor requirements, and reaction mechanisms of these three complexes doubtless reflects a common evolutionary origin; they are paralogs.

The PDH Complex Channels Its Intermediates through Five Reactions

Figure 16-6 shows schematically how P5 the pyruvate dehydrogenase complex carries out the five consecutive reactions in the decarboxylation and dehydrogenation of pyruvate without allowing the intermediates to leave the surface of the complex. Step ① is essentially identical to the reaction catalyzed by pyruvate decarboxylase (see Fig. 14-13c); C-1 of pyruvate is released as CO_2, and C-2, which in pyruvate has the oxidation state of an aldehyde, is attached to TPP as a hydroxyethyl group. This first step is the slowest and therefore limits the rate of the overall reaction. In step ② the hydroxyethyl group is oxidized to the level of a carboxylic acid (acetate). The two electrons removed in this reaction reduce the —S—S— of a lipoyl group on E_2 to two thiol (—SH) groups. The acetyl moiety produced in this oxidation-reduction reaction is first esterified to one of the lipoyl —SH groups, then transesterified to CoA to form acetyl-CoA (step ③). Thus the energy of oxidation drives the formation of a high-energy thioester of acetate. The remaining reactions catalyzed by the PDH complex (by E_3, in steps ④ and ⑤) are electron transfers necessary to regenerate the oxidized (disulfide) form of the lipoyl group of E_2, to prepare the enzyme complex for another round of oxidation. The electrons removed from the hydroxyethyl group derived from pyruvate pass through FAD to NAD^+, forming NADH, which can enter the respiratory chain.

P5 The five-reaction sequence shown in Figure 16-6 is an example of **substrate channeling**. The swinging lipoyllysyl arms of E_2 accept from E_1 the two electrons and the acetyl group that it derived from pyruvate, and pass them to E_3. The intermediates of the multistep sequence never leave the complex, and the local concentration of the substrate of E_2 is kept very high. This substrate channeling also prevents theft of the

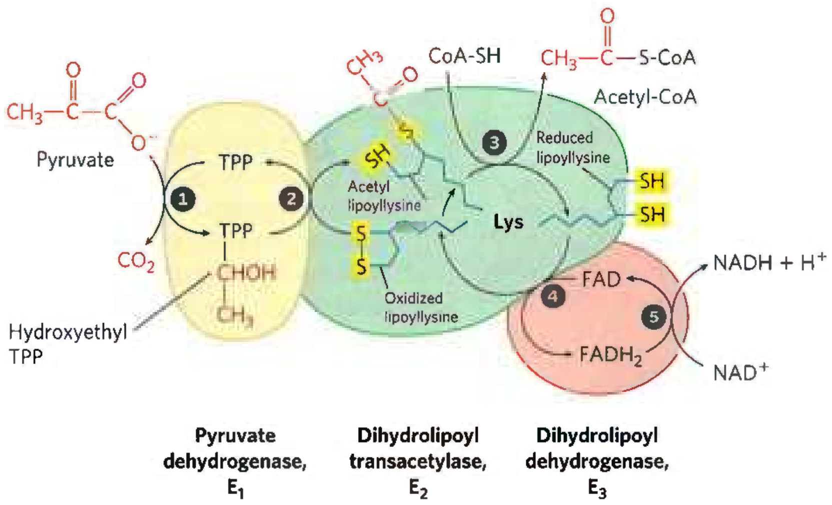


FIGURE 16-6 Oxidative decarboxylation of pyruvate to acetyl-CoA by the PDH complex. The fate of pyruvate is traced in red. In step ① pyruvate reacts with the bound thiamine pyrophosphate (TPP) of pyruvate dehydrogenase (E_1) and is decarboxylated to the hydroxyethyl derivative. Pyruvate dehydrogenase also carries out step ②, the transfer of two electrons and the acetyl group from TPP to the oxidized form of the lipoyllysyl group of the core enzyme, dihydrolipoyl transacetylase (E_2), to form the acetyl thioester of the reduced lipoyl group. Step ③ is a transesterification in which the —SH group of CoA replaces the —SH group of E_2 to yield acetyl-CoA and the fully reduced (dithiol) form of the lipoyl group. In step ④ dihydrolipoyl dehydrogenase (E_3) promotes transfer of two hydrogen atoms from the reduced lipoyl groups of E_2 to the FAD prosthetic group of E_3, restoring the oxidized form of the lipoyllysyl group of E_2. In step ⑤ the reduced $FADH_2$ of E_3 transfers a hydride ion to NAD^+, forming NADH. The enzyme complex is now ready for another catalytic cycle. (Subunit colors correspond to those in Fig. 16-5.)

activated acetyl group by other enzymes that use this group as substrate. We will encounter other enzymes that use a similar tethering mechanism to channel substrate between active sites, with lipoate, biotin, or a CoA-like moiety serving as cofactor.

Four different vitamins required in human nutrition are vital components of the pyruvate dehydrogenase complex: thiamine (TPP), pantothenate (CoA), riboflavin (FAD), and niacin (NAD). As one might predict, mutations in the genes for the subunits of the PDH complex, or a dietary vitamin deficiency, can have severe consequences. Thiamine-deficient animals are unable to oxidize pyruvate normally. This is of particular importance to the brain, which usually obtains all its energy from the aerobic oxidation of glucose in a pathway that necessarily includes the oxidation of pyruvate. Beriberi, a disease that results from thiamine deficiency, is characterized by loss of neural function. This disease occurs primarily in populations that rely on a diet consisting mainly of white (polished) rice, which lacks the hulls that contain most of the thiamine found in rice. People who habitually consume large amounts of alcohol can also develop thiamine deficiency, because much of their dietary intake consists of the vitamin-free "empty calories" of distilled spirits. An elevated level of pyruvate in the blood is often an indicator of defects in pyruvate oxidation due to one of these causes. ■

SUMMARY 16.1 *Production of Acetyl-CoA (Activated Acetate)*

- Pyruvate, the end product of glycolysis, enters the mitochondrial matrix, where the pyruvate dehydrogenase (PDH) complex oxidizes it to CO_2, acetyl-CoA—the starting material for the citric acid cycle—and NADH.
- The supramolecular PDH complex includes multiple copies of three enzymes. Pyruvate dehydrogenase, E_1 (with bound TPP), decarboxylates pyruvate, producing hydroxyethyl-TPP, which is then oxidized to an acetyl group. The electrons from this oxidation reduce the disulfide of lipoate bound to E_2, the dihydrolipoyl transacetylase, and the acetyl group is transferred to one of the —SH groups of the reduced lipoate through a thioester bond. E_2 catalyzes the transfer of the acetyl group to coenzyme A, forming acetyl-CoA. Dihydrolipoyl dehydrogenase, E_3, catalyzes the regeneration of the disulfide (oxidized) form of lipoate, passing electrons first to FAD, then to NAD^+.
- In the PDH complex we see examples of two strategies that we will see repeated in other metabolic enzyme systems. Its organization is very similar to that of the enzyme complexes that catalyze the oxidation of α-ketoglutarate and the branched-chain α-keto acids. And the long lipoyllysyl arm of E_2 is used to channel the substrate from the active site of E_1 to E_2 to E_3, tethering the intermediates to the enzyme complex and increasing the efficiency of the overall reaction.

16.2 Reactions of the Citric Acid Cycle

We are now ready to trace the process by which acetyl-CoA undergoes oxidation. This chemical transformation is carried out by the citric acid cycle, the

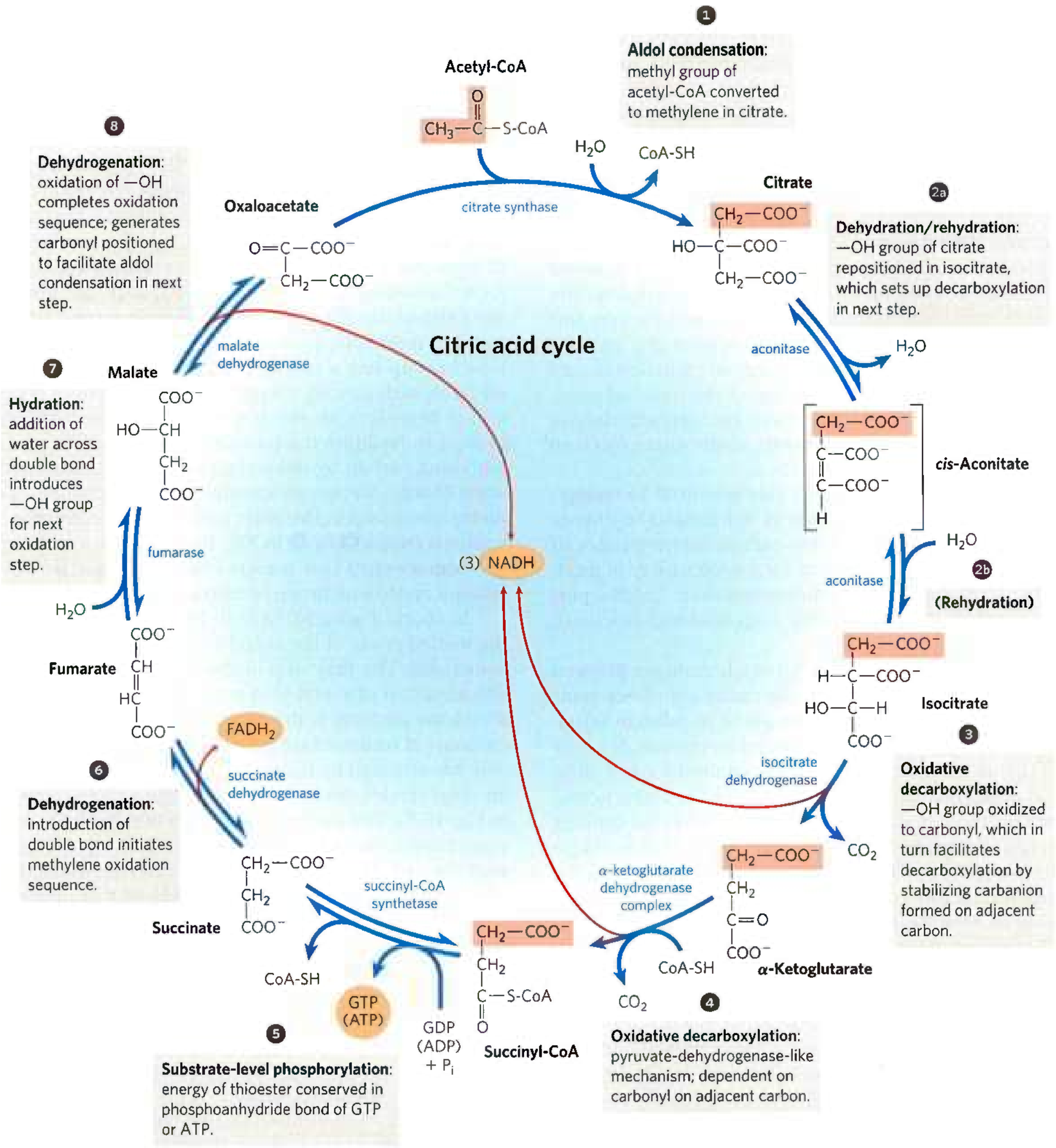

Citric acid cycle
Acetyl-CoA
$CH_3-C(=O)-S\text{-}CoA$
H₂O
CoA-SH
citrate synthase
① Aldol condensation: methyl group of acetyl-CoA converted to methylene in citrate.
Citrate
CH_2-COO^-
$HO-C-COO^-$
CH_2-COO^-
2a Dehydration/rehydration: —OH group of citrate repositioned in isocitrate, which sets up decarboxylation in next step.
aconitase
H₂O
cis-Aconitate
CH_2-COO^-
$C-COO^-$
$C-COO^-$
H
H₂O
aconitase
2b (Rehydration)
Isocitrate
CH_2-COO^-
$H-C-COO^-$
$HO-C-H$
COO^-
③ Oxidative decarboxylation: —OH group oxidized to carbonyl, which in turn facilitates decarboxylation by stabilizing carbanion formed on adjacent carbon.
isocitrate dehydrogenase
CO₂
α-Ketoglutarate
CH_2-COO^-
CH_2
$C=O$
COO^-
α-ketoglutarate dehydrogenase complex
CoA-SH
CO₂
④ Oxidative decarboxylation: pyruvate-dehydrogenase-like mechanism; dependent on carbonyl on adjacent carbon.
Succinyl-CoA
CH_2-COO^-
CH_2
$C(=O)-S\text{-}CoA$
succinyl-CoA synthetase
GDP (ADP) + Pᵢ
GTP (ATP)
CoA-SH
⑤ Substrate-level phosphorylation: energy of thioester conserved in phosphoanhydride bond of GTP or ATP.
Succinate
CH_2-COO^-
CH_2
COO^-
succinate dehydrogenase
FADH₂
⑥ Dehydrogenation: introduction of double bond initiates methylene oxidation sequence.
Fumarate
COO^-
CH
HC
COO^-
H₂O
fumarase
⑦ Hydration: addition of water across double bond introduces —OH group for next oxidation step.
Malate
COO^-
$HO-CH$
CH_2
COO^-
malate dehydrogenase
⑧ Dehydrogenation: oxidation of —OH completes oxidation sequence; generates carbonyl positioned to facilitate aldol condensation in next step.
Oxaloacetate
$O=C-COO^-$
CH_2-COO^-
(3) NADH

FIGURE 16-7 Reactions of the citric acid cycle. The carbon atoms shaded in pink are those derived from the acetate of acetyl-CoA in the first turn of the cycle; these are *not* the carbons released as CO_2 in the first turn. Note that in succinate and fumarate, the two-carbon group derived from acetate can no longer be specifically denoted; because succinate and fumarate are symmetric molecules, C-1 and C-2 are indistinguishable from C-4 and C-3. The red arrows show where energy is conserved by electron transfer to FAD or NAD^+, forming $FADH_2$ or NADH + H^+. Steps ①, ③, and ④ are essentially irreversible in the cell; all other steps are reversible. The nucleoside triphosphate product of step ⑤ may be either ATP or GTP, depending on which succinyl-CoA synthetase isozyme is the catalyst.

first *cyclic* pathway we have encountered (**Fig. 16-7**). To begin a turn of the cycle, acetyl-CoA donates its acetyl group to the four-carbon compound oxaloacetate to form the six-carbon citrate. Citrate is then transformed into isocitrate, also a six-carbon molecule, which is dehydrogenated with loss of CO_2 to yield the five-carbon compound α-ketoglutarate (also called 2-oxoglutarate). α-Ketoglutarate undergoes loss of a second molecule of CO_2 and ultimately yields the four-carbon compound succinate. Succinate is enzymatically converted in three steps into the four-carbon oxaloacetate—which is then ready to react with another molecule of acetyl-CoA. In each turn of the cycle, one acetyl group (two carbons) enters as acetyl-CoA and two molecules of CO_2 leave; one molecule of oxaloacetate is used to form citrate and one molecule of oxaloacetate is regenerated. No net removal of oxaloacetate occurs; P2 one molecule of oxaloacetate can theoretically bring about oxidation of an infinite number of acetyl groups, effectively acting catalytically; the steady-state concentration of oxaloacetate is very low (micromolar). Four of the eight steps in this process are oxidations, in which the energy of oxidation is very efficiently conserved in the form of the reduced coenzymes NADH and $FADH_2$. These two carriers donate their electrons to the respiratory chain where electron flow drives ATP synthesis.

Although the citric acid cycle is central to energy-yielding metabolism, its role is not limited to energy conservation. Four- and five-carbon intermediates of the cycle serve as precursors for a wide variety of products. To replace intermediates removed for this purpose, cells employ anaplerotic (replenishing) reactions, which are described below.

Eugene Kennedy and Albert Lehninger showed in 1948 that, in eukaryotes, the entire set of reactions of the citric acid cycle takes place in mitochondria. Isolated mitochondria were found to contain not only all the enzymes and coenzymes required for the citric acid cycle, but also all the enzymes and proteins necessary for the last stage of respiration—electron transfer and ATP synthesis by oxidative phosphorylation. As we shall see in later chapters, mitochondria also contain the enzymes for the oxidation of fatty acids and some amino acids to acetyl-CoA, and the oxidative degradation of other amino acids to α-ketoglutarate, succinyl-CoA, or oxaloacetate. Thus, in nonphotosynthetic eukaryotes, the mitochondrion is the site of most energy-yielding oxidative reactions and of the coupled synthesis of ATP. In photosynthetic eukaryotes, mitochondria are the major site of ATP production in the dark, but in daylight, chloroplasts produce most of the organism's ATP. In most bacteria, the enzymes of the citric acid cycle are in the cytosol, and the plasma membrane plays a role analogous to that of the inner mitochondrial membrane in ATP synthesis (Chapter 19).

The Sequence of Reactions in the Citric Acid Cycle Makes Chemical Sense

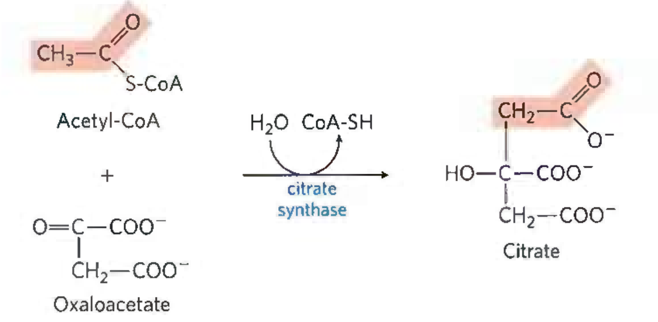
Acetyl-CoA produced in the breakdown of carbohydrates, fats, and proteins must be completely oxidized to CO_2 if the maximum potential energy is to be extracted from these fuels. P2 However, it is not biochemically feasible to directly oxidize acetate (or acetyl-CoA) to CO_2. Decarboxylation of this two-carbon acid would yield CO_2 and methane (CH_4). Methane is chemically rather stable, and except for certain methanotrophic bacteria that grow in methane-rich niches, organisms do not have the cofactors and enzymes needed to oxidize methane. Methylene groups ($—CH_2—$), however, are readily metabolized by enzyme systems present in most organisms. In typical oxidation sequences, two adjacent methylene groups ($—CH_2—CH_2—$) are involved, at least one of which is adjacent to a carbonyl group. As we noted in Chapter 13 (p. 473), carbonyl groups are particularly important in the chemical transformations of metabolic pathways. The carbon of the carbonyl group has a partial positive charge due to the electron-withdrawing property of the carbonyl oxygen and is therefore an electrophilic center. A carbonyl group can facilitate the formation of a carbanion on an adjoining carbon by delocalizing the carbanion's negative charge. We see an example of the oxidation of a methylene group in the citric acid cycle, as succinate is oxidized (steps ❻ to ❽ in Fig. 16-7) to form a carbonyl (in oxaloacetate) that is more chemically reactive than either a methylene group or methane.

In short, if acetyl-CoA is to be oxidized efficiently, the methyl group of the acetyl-CoA must be attached to something. The first step of the citric acid cycle—the condensation of acetyl-CoA with oxaloacetate—neatly solves the problem of the unreactive methyl group. The carbonyl of oxaloacetate acts as an electrophilic center, which is attacked by the methyl carbon of acetyl-CoA in an aldol condensation (p. 473) to form citrate (step ❶ in Fig. 16-7). The methyl group of acetate has been converted into a methylene in citric acid. This tricarboxylic acid then readily undergoes a series of oxidations that eliminate two carbons as CO_2. P2 Note that all steps featuring the breakage or formation of carbon–carbon bonds (steps ❶, ❸, and ❹) rely on properly positioned carbonyl groups. As in all metabolic pathways, there is a chemical logic to the sequence of steps in the citric acid cycle: each step either involves an energy-conserving oxidation or is a necessary prelude to the oxidation, placing functional groups in position to facilitate oxidation or oxidative decarboxylation. As you learn the steps of the cycle, keep in mind the chemical rationale for each; it will make the process easier to understand and remember.

The Citric Acid Cycle Has Eight Steps

In examining the eight successive reaction steps of the citric acid cycle, we place special emphasis on the chemical transformations taking place as citrate formed from acetyl-CoA and oxaloacetate is oxidized to yield CO_2 and the energy of this oxidation is conserved in the reduced coenzymes NADH and $FADH_2$.

❶ Formation of Citrate The first reaction of the cycle is the condensation of acetyl-CoA with **oxaloacetate** to form **citrate**, catalyzed by **citrate synthase**.



$\Delta G'^\circ = -32.2$ kJ/mol

In this reaction, the methyl carbon of the acetyl group is joined to the carbonyl group (C-2) of oxaloacetate. Citroyl-CoA is a transient intermediate formed on the active site of the enzyme (see Fig. 16-9). It undergoes hydrolysis to free CoA and citrate, which are released from the active site. The hydrolysis of this high-energy thioester intermediate makes the forward reaction highly exergonic. The large, negative standard free-energy change of the forward citrate synthase reaction is essential to the operation of the cycle because the concentration of oxaloacetate is normally very low (micromolar). The CoA liberated in this reaction is recycled to participate in the oxidative decarboxylation of another molecule of pyruvate by the PDH complex.

Citrate synthase from mitochondria is a homodimer (**Fig. 16-8**). Each subunit is a single polypeptide with two domains, one large and rigid, the other smaller and more flexible, with the active site between them. Oxaloacetate, the first substrate to bind to the enzyme, induces a large conformational change in the flexible domain, creating a binding site for the second substrate, acetyl-CoA. When citroyl-CoA has formed in the enzyme active site, another conformational change brings about thioester hydrolysis, releasing CoA-SH. This induced fit of the enzyme first to its substrate and then to its reaction intermediate decreases the likelihood of premature and unproductive cleavage of the thioester bond of acetyl-CoA. Kinetic studies of the enzyme are consistent with this ordered bisubstrate mechanism (see Fig. 6-15). The reaction catalyzed by citrate synthase is essentially an aldol condensation (p. 473), involving a thioester (acetyl-CoA) and a ketone (oxaloacetate) (**Fig. 16-9**).

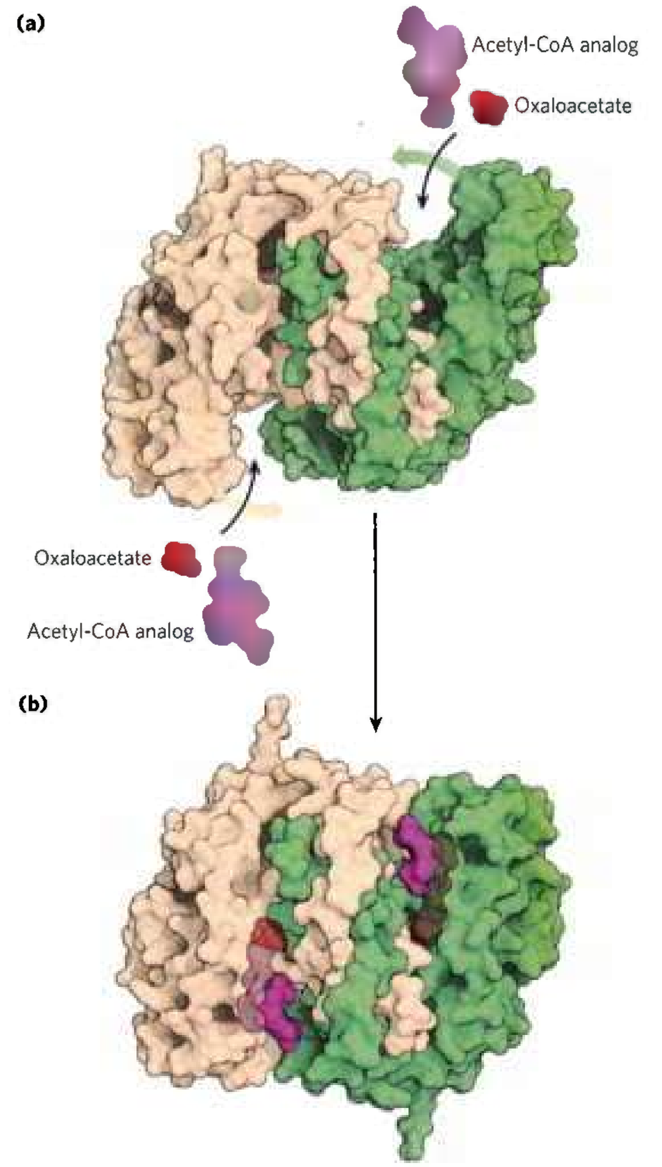
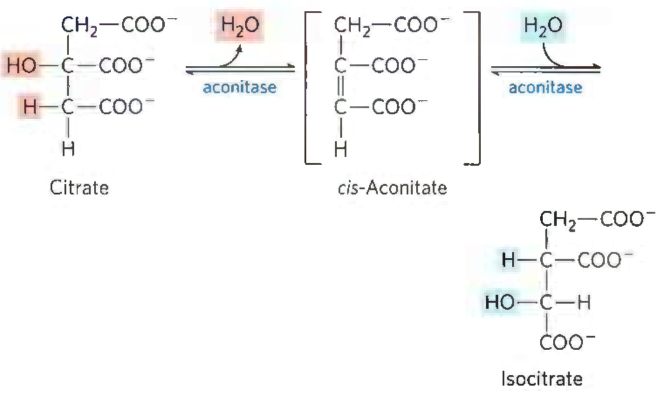


FIGURE 16-8 Structure of citrate synthase. The flexible domain of each subunit undergoes a large conformational change on binding oxaloacetate, creating a binding site for acetyl-CoA. (a) Open form of the enzyme alone; (b) closed form with bound oxaloacetate and a stable analog of acetyl-CoA (carboxymethyl-CoA). In these representations one subunit is colored tan and one green. [Data from (a) PDB ID 5CSC, D.-I. Liao et al., *Biochemistry* 30:6031, 1991; (b) PDB ID 5CTS, M. Karpusas et al., *Biochemistry* 29:2213, 1990.]

❷ Formation of Isocitrate via *cis*-Aconitate The enzyme **aconitase** (more formally, **aconitate hydratase**) catalyzes the reversible transformation of citrate to **isocitrate**, through the intermediary formation of the tricarboxylic acid ***cis*-aconitate**, which normally does not dissociate from the active site. Aconitase can promote the reversible addition of H_2O to the double bond of enzyme-bound *cis*-aconitate in two different ways, one leading to citrate and the other to isocitrate:



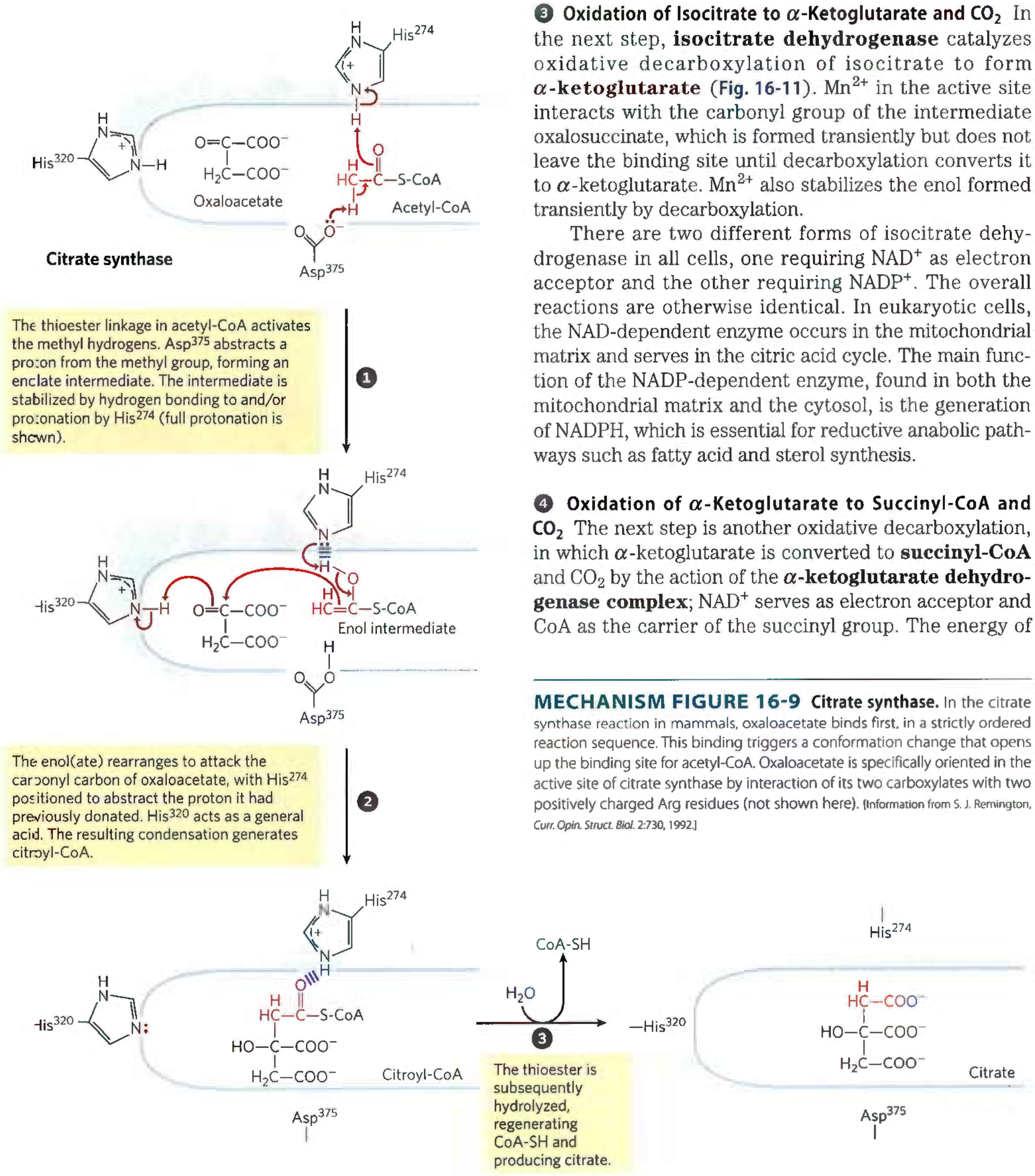
$\Delta G'^\circ = 13.3$ kJ/mol

Although the equilibrium mixture at pH 7.4 and 25 °C contains less than 10% isocitrate, in the cell the reaction is pulled to the right because isocitrate is immediately consumed in the next step of the cycle, lowering its steady-state concentration. Aconitase contains an **iron-sulfur center** (**Fig. 16-10**), which acts both in the binding of the substrate at the active site and in the catalytic addition or removal of H_2O. In iron-depleted cells, aconitase loses its iron-sulfur center and acquires a new role in the regulation of iron homeostasis. Aconitase is one of many enzymes known to "moonlight" in a second role (**Box 16-1**).

③ Oxidation of Isocitrate to α-Ketoglutarate and CO_2 In the next step, **isocitrate dehydrogenase** catalyzes oxidative decarboxylation of isocitrate to form **α-ketoglutarate** (**Fig. 16-11**). Mn^{2+} in the active site interacts with the carbonyl group of the intermediate oxalosuccinate, which is formed transiently but does not leave the binding site until decarboxylation converts it to α-ketoglutarate. Mn^{2+} also stabilizes the enol formed transiently by decarboxylation.

There are two different forms of isocitrate dehydrogenase in all cells, one requiring NAD^+ as electron acceptor and the other requiring $NADP^+$. The overall reactions are otherwise identical. In eukaryotic cells, the NAD-dependent enzyme occurs in the mitochondrial matrix and serves in the citric acid cycle. The main function of the NADP-dependent enzyme, found in both the mitochondrial matrix and the cytosol, is the generation of NADPH, which is essential for reductive anabolic pathways such as fatty acid and sterol synthesis.

④ Oxidation of α-Ketoglutarate to Succinyl-CoA and CO_2 The next step is another oxidative decarboxylation, in which α-ketoglutarate is converted to **succinyl-CoA** and CO_2 by the action of the **α-ketoglutarate dehydrogenase complex**; NAD^+ serves as electron acceptor and CoA as the carrier of the succinyl group. The energy of



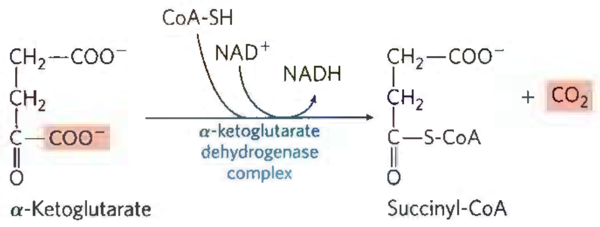
The thioester linkage in acetyl-CoA activates the methyl hydrogens. Asp^{375} abstracts a proton from the methyl group, forming an enolate intermediate. The intermediate is stabilized by hydrogen bonding to and/or protonation by His^{274} (full protonation is shown).

The enol(ate) rearranges to attack the carbonyl carbon of oxaloacetate, with His^{274} positioned to abstract the proton it had previously donated. His^{320} acts as a general acid. The resulting condensation generates citroyl-CoA.

The thioester is subsequently hydrolyzed, regenerating CoA-SH and producing citrate.

MECHANISM FIGURE 16-9 Citrate synthase. In the citrate synthase reaction in mammals, oxaloacetate binds first, in a strictly ordered reaction sequence. This binding triggers a conformation change that opens up the binding site for acetyl-CoA. Oxaloacetate is specifically oriented in the active site of citrate synthase by interaction of its two carboxylates with two positively charged Arg residues (not shown here). [Information from S. J. Remington, *Curr. Opin. Struct. Biol.* 2:730, 1992.]

oxidation of α-ketoglutarate is conserved in the formation of the thioester bond of succinyl-CoA:


CoA-SH
NAD⁺
NADH
α-ketoglutarate dehydrogenase complex
+ CO₂
α-Ketoglutarate
Succinyl-CoA

$\Delta G'^\circ = -33.5$ kJ/mol

P2 This reaction is virtually identical to the pyruvate dehydrogenase reaction discussed above and to the reaction sequence responsible for the breakdown of branched-chain amino acids (**Fig. 16-12**). The α-ketoglutarate dehydrogenase complex closely resembles the PDH complex and the complex that degrades branched-chain α-keto acids

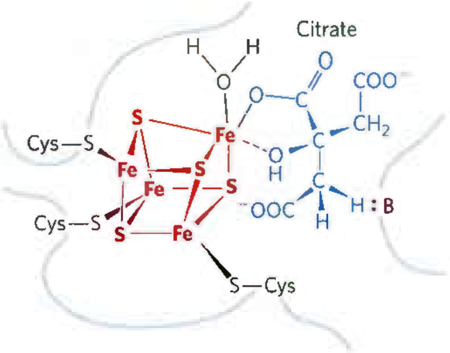
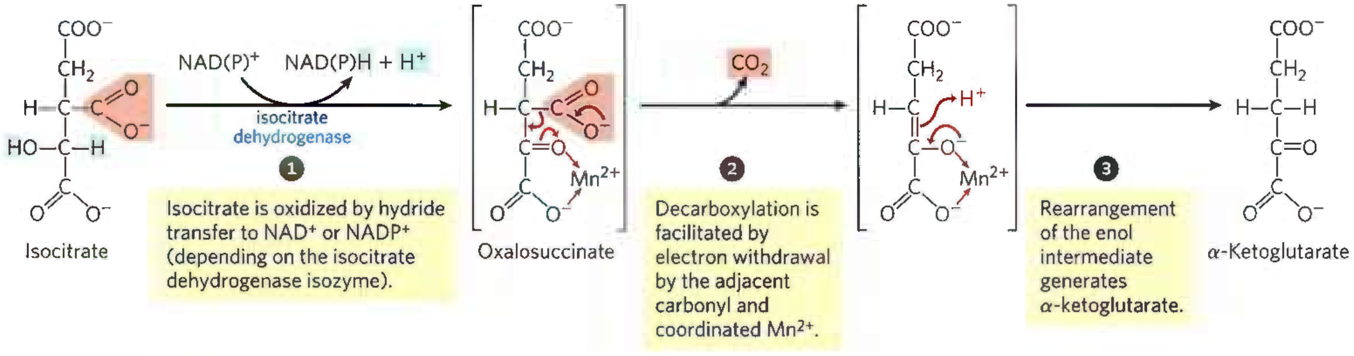

Citrate
H
H
O
O
C
COO⁻
CH₂
C
O
H
C
H
H:B
⁻OOC
Fe
S
Fe
S
Fe
S
S
Fe
Cys—S
Cys—S
S—Cys

FIGURE 16-10 Iron-sulfur center in aconitase. The iron-sulfur center is in red, the citrate molecule in blue. Three Cys residues of the enzyme bind three iron atoms; the fourth iron is bound to one of the carboxyl groups of citrate and also interacts noncovalently with a hydroxyl group of citrate (dashed bond). A basic residue (:B) in the enzyme helps to position the citrate in the active site. The iron-sulfur center acts in both substrate binding and catalysis. The general properties of iron-sulfur proteins are discussed in Chapter 19.


NAD(P)⁺
NAD(P)H + H⁺
isocitrate dehydrogenase
CO₂
H⁺
Mn²⁺
Mn²⁺
1
2
3
Isocitrate
Oxalosuccinate
α-Ketoglutarate

① Isocitrate is oxidized by hydride transfer to NAD⁺ or NADP⁺ (depending on the isocitrate dehydrogenase isozyme).

② Decarboxylation is facilitated by electron withdrawal by the adjacent carbonyl and coordinated Mn^{2+}.

③ Rearrangement of the enol intermediate generates α-ketoglutarate.

MECHANISM FIGURE 16-11 Isocitrate dehydrogenase. In this reaction, the substrate, isocitrate, loses one carbon by oxidative decarboxylation.

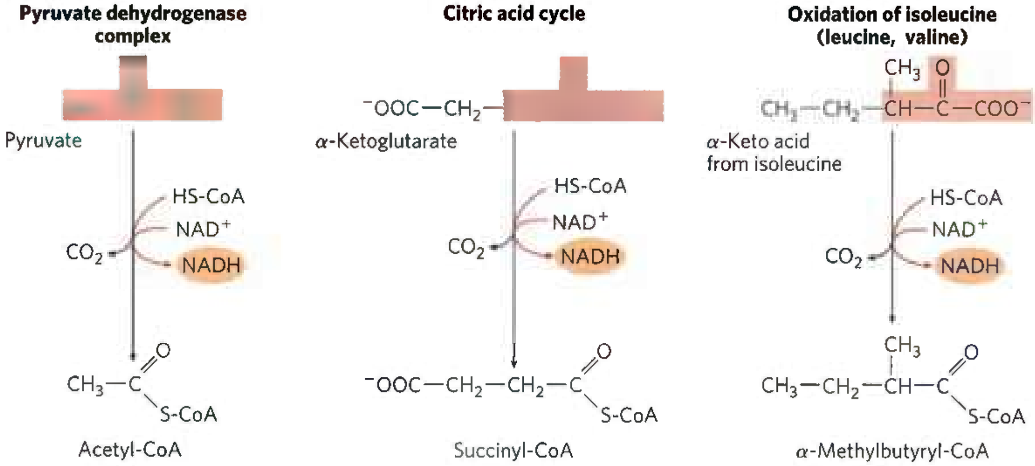

Pyruvate dehydrogenase complex
Citric acid cycle
Oxidation of isoleucine (leucine, valine)
Pyruvate
⁻OOC—CH₂—
α-Ketoglutarate
α-Keto acid from isoleucine
CH₃ O
CH₃—CH₂—CH—C—COO⁻
HS-CoA
NAD⁺
NADH
CO₂
HS-CoA
NAD⁺
NADH
CO₂
HS-CoA
NAD⁺
NADH
CO₂
CH₃—C(=O)S-CoA
Acetyl-CoA
⁻OOC—CH₂—CH₂—C(=O)S-CoA
Succinyl-CoA
CH₃—CH₂—CH(CH₃)—C(=O)S-CoA
α-Methylbutyryl-CoA

FIGURE 16-12 A conserved mechanism for oxidative decarboxylation. The pathways shown employ the same five cofactors (thiamine pyrophosphate, coenzyme A, lipoate, FAD, and NAD⁺), closely similar multienzyme complexes, and the same enzymatic mechanism to carry out oxidative decarboxylations of pyruvate (by the pyruvate dehydrogenase complex), α-ketoglutarate (in the citric acid cycle), and the carbon skeletons of the three branched-chain amino acids, isoleucine (shown here), leucine, and valine.

BOX 16-1

Moonlighting Enzymes: Proteins with More Than One Job

Before protein and DNA sequencing became routine, two groups of investigators studying two unrelated questions sometimes discovered that "their" proteins had similar properties. Further comparison sometimes showed that they were studying the *same* protein by following *different* functions. We now know that many proteins **moonlight** in a second role, a phenomenon sometimes called **gene sharing**. Today, researchers using annotated protein and DNA sequence databases can more easily find proteins of the same sequence that have been identified as having different functions, and a protein sequence annotated as having a given function doesn't necessarily have *only* that function. When a protein with a known function is made inactive by a mutation, and the resulting mutant organisms show a phenotype with no obvious relation to that function, protein moonlighting can sometimes explain the puzzling result.

One citric acid cycle enzyme is a known moonlighter. Eukaryotic cells have two isozymes of aconitase. The mitochondrial isozyme converts citrate to isocitrate in the citric acid cycle. The cytosolic isozyme has two distinct functions. It catalyzes the conversion of citrate to isocitrate, providing the substrate for a cytosolic isocitrate dehydrogenase that generates NADPH as reducing power for fatty acid synthesis and other anabolic processes in the cytosol. But it also has a role in cellular iron homeostasis.

All cells must obtain iron for the proteins that require it as a cofactor. In humans, severe iron deficiency results in anemia, an insufficient supply of erythrocytes and a reduced oxygen-carrying capacity that can be life-threatening. Too much iron is also harmful: it accumulates in and damages the liver in hemochromatosis and other diseases. Iron obtained in the diet is carried in the blood by the protein **transferrin** and enters cells via endocytosis mediated by the **transferrin receptor**. Once inside cells, iron is used in the synthesis of hemes, cytochromes, Fe-S proteins, and other Fe-dependent proteins; excess iron is stored bound to the protein **ferritin**. The levels of transferrin, transferrin receptor, and ferritin are therefore crucial to cellular iron homeostasis. The synthesis of these three proteins is regulated in response to iron availability — and aconitase, in its moonlighting job, plays a key regulatory role.

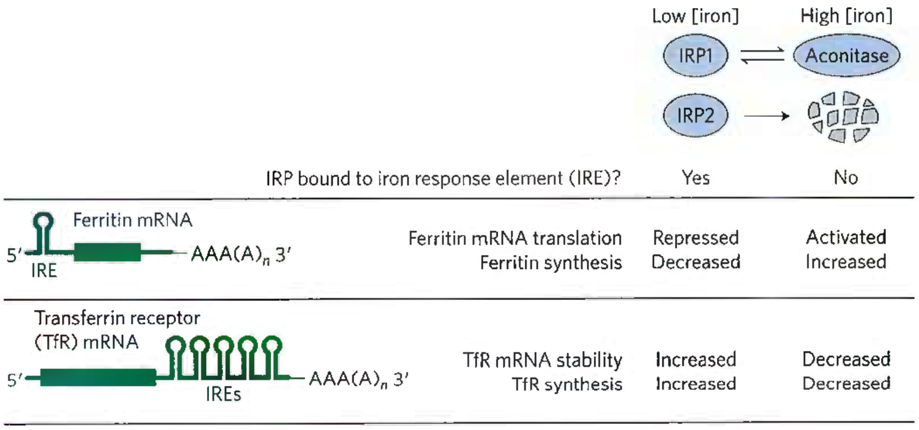
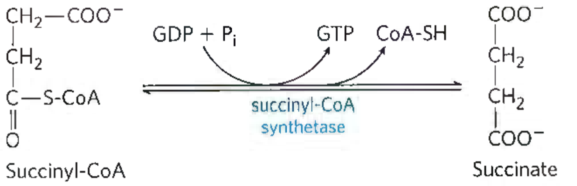


FIGURE 1 Effect of IRP1 and IRP2 on the mRNAs for ferritin and the transferrin receptor. [Information from R. S. Eisenstein, *Annu. Rev. Nutr.* 20:627, 2000, Fig. 1.]

in both structure and function. All three have homologous E_1 and E_2 components, identical E_3 components, enzyme-bound TPP and lipoate, coenzyme A, FAD, and NAD. These related enzymes can employ the same E_3 subunit because the substrate for E_3 — a reduced lipoate — is the same for both complexes. They are certainly derived from a common evolutionary ancestor by gene duplication and subsequent **divergent evolution**, as described in Figure 1-32.

⑤ Conversion of Succinyl-CoA to Succinate Succinyl-CoA, like acetyl-CoA, has a thioester bond with a strongly negative standard free energy of hydrolysis ($\Delta G'^\circ \approx -36$ kJ/mol). In the next step of the citric acid cycle, energy released in the breakage of this bond is used to drive the synthesis of a phosphoanhydride bond in either GTP or ATP, with a net $\Delta G'^\circ$ of only −2.9 kJ/mol. **Succinate** is formed in the process:



$\Delta G'^\circ = -2.9$ kJ/mol

The enzyme that catalyzes this reversible reaction is called **succinyl-CoA synthetase** or **succinic thiokinase**;

Aconitase has an essential Fe-S cluster at its active site (see Fig. 16-10). When a cell is depleted of iron, this Fe-S cluster is disassembled and the enzyme loses its aconitase activity. But the apoenzyme (apoaconitase, lacking its Fe-S cluster) so formed has now acquired its second activity: regulating iron metabolism. Cytosolic apoaconitase is identical to **iron regulatory protein 1 (IRP1)** and closely related to **IRP2**. Both IRP1 and IRP2 bind to regions in the mRNAs encoding ferritin and the transferrin receptor, with effects on iron mobilization and iron uptake. These mRNA sequences are part of hairpin structures (see Fig. 8-23) called **iron response elements (IREs)**, located at the 5′ and 3′ ends of the mRNAs (Fig. 1). When bound to the 5′-untranslated IRE sequence in the ferritin mRNA, IRPs block ferritin synthesis; when bound to the 3′-untranslated IRE sequence in the transferrin receptor mRNA, they stabilize the mRNA, preventing its degradation and thus allowing the synthesis of more copies of the receptor protein per mRNA molecule. So, in iron-deficient cells, iron uptake becomes more efficient and iron storage (bound to ferritin) is reduced. When cellular iron concentrations return to normal levels, IRP1 regains its Fe-S cluster, converting to aconitase, and IRP2 undergoes proteolytic degradation, ending the low-iron response.

The enzymatically active aconitase and the moonlighting, regulatory apoaconitase have different structures. As the active aconitase, the protein has two lobes that close around the Fe-S cluster; as IRP1, the two lobes open, exposing the mRNA-binding site (Fig. 2).

It makes evolutionary sense that the enzymes of central metabolism have had a long time to evolve additional functions. The glycolytic enzyme pyruvate kinase acts in the nucleus to regulate the transcription of genes that respond to thyroid hormone. Glyceraldehyde 3-phosphate dehydrogenase moonlights both as uracil DNA glycosylase, effecting the repair of damaged DNA, and as a regulator of histone H2B transcription. Several glycolytic enzymes, including phosphoglycerate kinase, triose phosphate isomerase, and lactate dehydrogenase, moonlight as crystallins in the lens of the vertebrate eye.

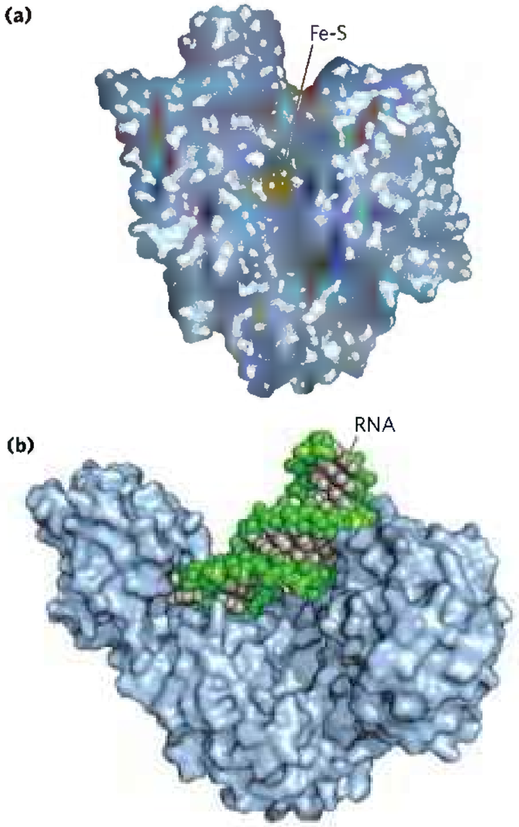


FIGURE 2 Two forms of cytosolic aconitase/IRP1 with two distinct functions. (a) In aconitase, the two major lobes are closed and the Fe-S cluster is buried; the protein has been made transparent here to show the Fe-S cluster. (b) In IRP1, the lobes open, exposing a binding site for the mRNA hairpin. [Data from (a) PDB ID 2B3Y, J. Dupuy et al., *Structure* 14:129, 2006; (b) PDB ID 2IPY, W. E. Walden et al., *Science* 314:1903, 2006.]

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both names indicate the participation of a nucleoside triphosphate in the reaction.

This energy-conserving reaction involves an intermediate step in which the enzyme molecule itself becomes phosphorylated at a His residue in the active site (**Fig. 16-13a**). This phosphoryl group, which has a high group transfer potential, is transferred to ADP (or GDP) to form ATP (or GTP). Animal cells have two isozymes of succinyl-CoA synthetase, one specific for ADP and the other for GDP. The enzyme has two subunits, α (M_r 32,000), which has the Ⓟ–His residue (His^{246}) and the binding site for CoA, and β (M_r 42,000), which confers specificity for either ADP or GDP. The active site is at the interface between subunits. The crystal structure of succinyl-CoA synthetase reveals two "power helices" (one from each subunit), oriented so that their electric dipoles situate partial positive charges close to the negatively charged Ⓟ–His (Fig. 16-13b), stabilizing the phosphoenzyme intermediate. (Recall the similar role of helix dipoles in stabilizing K^+ ions in the K^+ channel; see Fig. 11-45.)

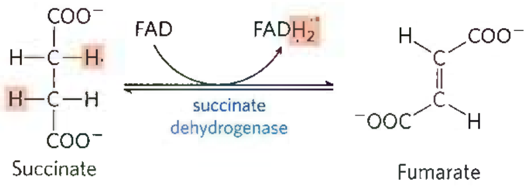
The formation of ATP (or GTP) at the expense of the energy released by the oxidative decarboxylation of α-ketoglutarate is a substrate-level phosphorylation, like the synthesis of ATP in the glycolytic reactions catalyzed by phosphoglycerate kinase and pyruvate kinase (see Fig. 14-2). The GTP formed by succinyl-CoA synthetase can donate its terminal phosphoryl group to ADP to form

ATP, in a reversible reaction catalyzed by **nucleoside diphosphate kinase** (p. 487):

$$\text{GTP} + \text{ADP} \rightleftharpoons \text{GDP} + \text{ATP} \qquad \Delta G'^{\circ} = 0 \text{ kJ/mol}$$

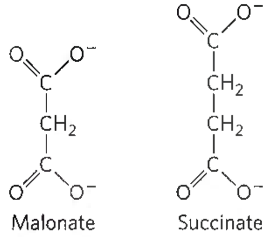
Thus the net result of the activity of either isozyme of succinyl-CoA synthetase is the conservation of energy as ATP. There is no change in free energy for the nucleoside diphosphate kinase reaction; ATP and GTP are energetically equivalent.

⑥ Oxidation of Succinate to Fumarate The succinate formed from succinyl-CoA is oxidized to **fumarate** by the flavoprotein **succinate dehydrogenase**:


COO⁻
H—C—H
H—C—H
COO⁻
Succinate
FAD
FADH₂
succinate dehydrogenase
H
COO⁻
⁻OOC
H
Fumarate

$\Delta G'^{\circ} = 0$ kJ/mol

In eukaryotes, succinate dehydrogenase is an integral protein of the mitochondrial inner membrane; in bacteria, of the plasma membrane. The enzyme contains three different iron-sulfur clusters and one molecule of covalently bound FAD (see Fig. 19-9). Electrons pass from succinate through the FAD and iron-sulfur centers before entering the chain of electron carriers in the mitochondrial inner membrane (the plasma membrane in bacteria). Electron flow from succinate through these carriers to the final electron acceptor, O_2, is coupled to the synthesis of about 1.5 ATP molecules per pair of electrons (respiration-linked phosphorylation). Malonate, an analog of succinate not normally present in cells, is a strong competitive inhibitor of succinate dehydrogenase, and its addition to mitochondria in the laboratory blocks the activity of the citric acid cycle.


Malonate
Succinate

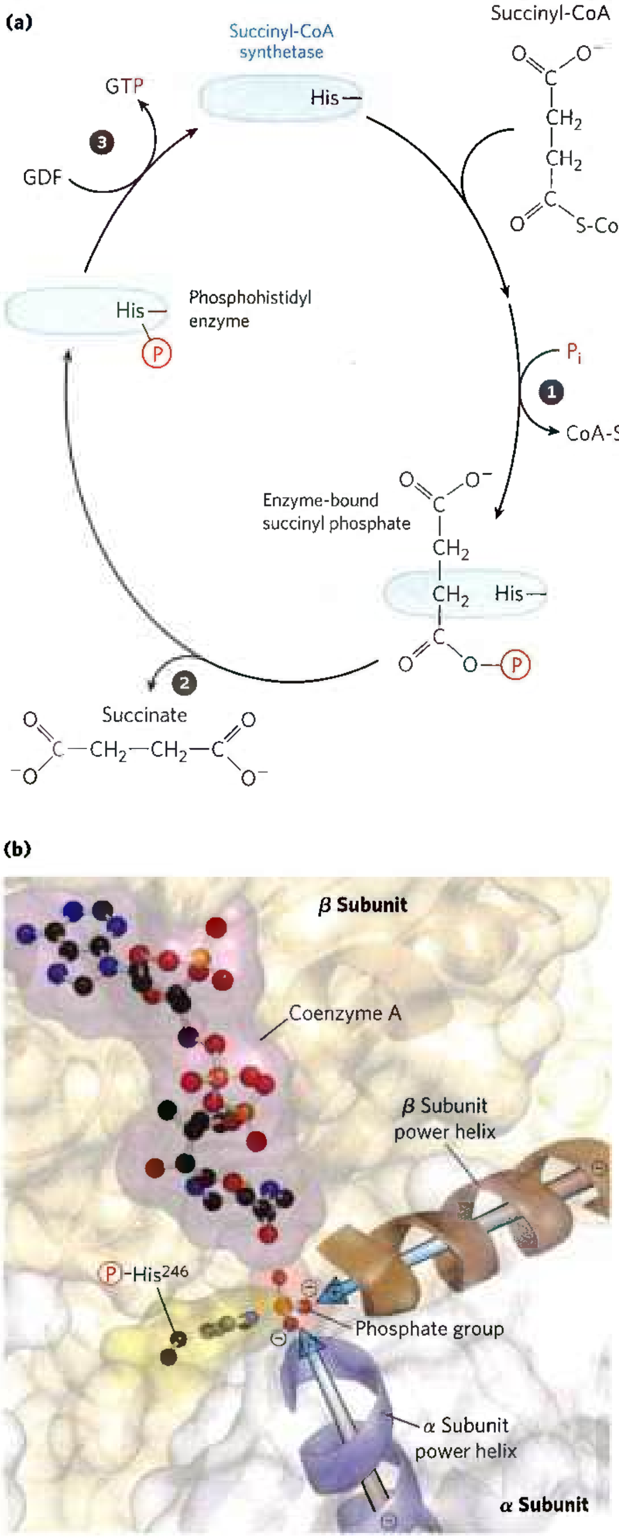
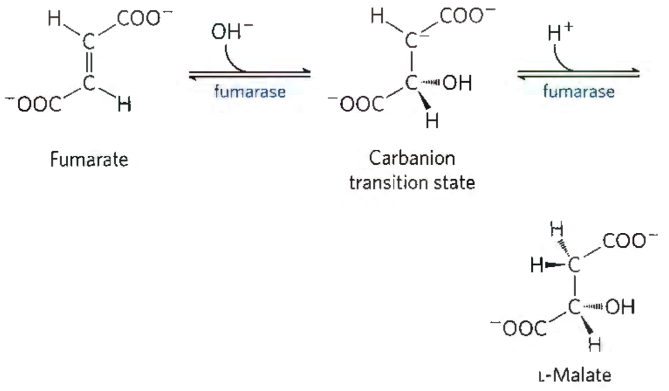

(a)
Succinyl-CoA synthetase
His—
GTP
③
GDP
Succinyl-CoA
S-CoA
Phosphohistidyl enzyme
His—
P
Pᵢ
①
CoA-SH
Enzyme-bound succinyl phosphate
His—
②
Succinate
(b)
β Subunit
Coenzyme A
β Subunit power helix
P-His²⁴⁶
Phosphate group
α Subunit power helix
α Subunit

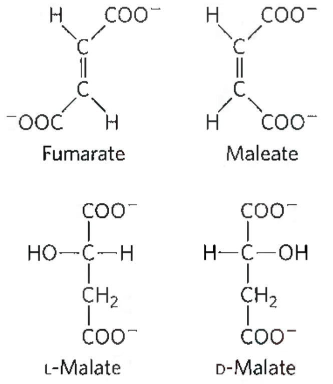
FIGURE 16-13 The succinyl-CoA synthetase reaction. (a) In step ① a phosphoryl group replaces the CoA of succinyl-CoA bound to the enzyme, forming a high-energy acyl phosphate. In step ② the succinyl phosphate donates its phosphoryl group to a His residue of the enzyme, forming a high-energy phosphohistidyl enzyme. In step ③ the phosphoryl group is transferred from the His residue to the terminal phosphate of GDP (or ADP), forming GTP (or ATP). (b) Active site of succinyl-CoA synthetase of *Escherichia coli*. The active site includes part of both the α (blue) and the β (brown) subunits. The power helices (blue, brown) place the partial positive charges of the helix dipole near the phosphate group of Ⓟ-His^{246} in the α chain, stabilizing the phosphohistidyl enzyme. The bacterial and mammalian enzymes have similar amino acid sequences and three-dimensional structures. [Data from PDB ID 1SCU, W. T. Wolodko et al., *J. Biol. Chem.* 269:10,883, 1994.]

⑦ Hydration of Fumarate to Malate The reversible hydration of fumarate to **L-malate** is catalyzed by **fumarase** (formally, **fumarate hydratase**). The transition state in this reaction is a carbanion:

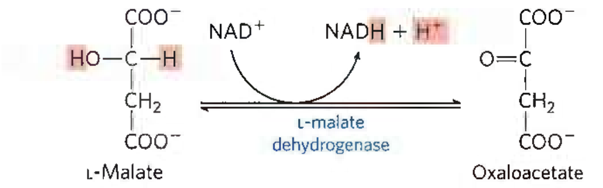


$\Delta G'^{\circ} = -3.8$ kJ/mol

This enzyme is highly stereospecific; it catalyzes hydration of the trans double bond of fumarate but not the cis double bond of maleate. In the reverse direction (from L-malate to fumarate), fumarase is equally stereospecific: D-malate is not a substrate.



⑧ Oxidation of Malate to Oxaloacetate In the last reaction of the citric acid cycle, **L-malate dehydrogenase** catalyzes the oxidation of L-malate to oxaloacetate, coupled to the reduction of NAD$^+$ to NADH:



$\Delta G'^{\circ} = 29.7$ kJ/mol

The equilibrium of this reaction lies far to the left under standard thermodynamic conditions, but in intact cells oxaloacetate is continually removed by the highly exergonic citrate synthase reaction (step ② of Fig. 16-7). This keeps the concentration of oxaloacetate in the cell extremely low (<10^{-6} M), pulling the malate dehydrogenase reaction toward the formation of oxaloacetate.

Although the individual reactions of the citric acid cycle were initially worked out in vitro, using minced muscle tissue, the pathway and its regulation have also been studied extensively in vivo. By using precursors isotopically labeled with ^{14}C, researchers have traced the fate of individual carbon atoms through the citric acid cycle. Some of the earliest experiments with ^{14}C produced an unexpected result, however, which aroused considerable controversy about the pathway and mechanism of the citric acid cycle. In fact, these experiments at first seemed to show that citrate was not the first tricarboxylic acid to be formed. **Box 16-2** gives some details of this episode in the history of citric acid cycle research. Today biochemists use ^{13}C-labeled precursors and whole-tissue NMR spectroscopy to monitor metabolic flux through the cycle in living tissue. Because the NMR signal is unique to the compound containing the ^{13}C, precursor carbons can be traced into each cycle intermediate and into compounds derived from the intermediates. This technique makes it possible to study regulation of the citric acid cycle and its interconnections with other metabolic pathways.

The Energy of Oxidations in the Cycle Is Efficiently Conserved

We have now covered one complete turn of the citric acid cycle (**Fig. 16-14**). A two-carbon acetyl group entered the cycle by combining with oxaloacetate. Two carbon atoms

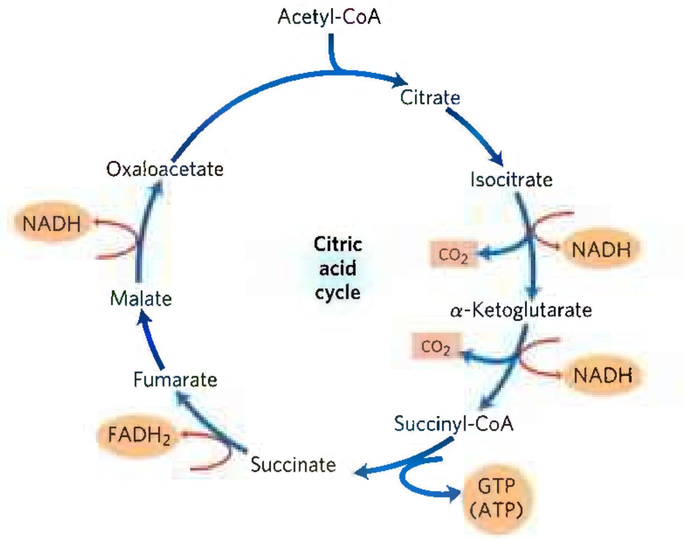


FIGURE 16-14 Products of one turn of the citric acid cycle. At each turn of the cycle, three NADH, one FADH$_2$, one GTP (or ATP), and two CO$_2$ are released in oxidative decarboxylation reactions. Here and in several of the following figures, all cycle reactions are shown as proceeding in one direction only, but keep in mind that most of the reactions are reversible.

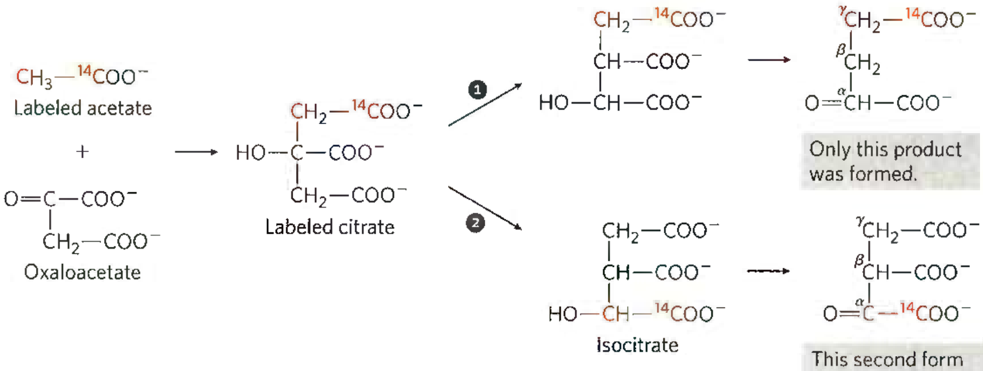
BOX 16-2

Citrate: A Symmetric Molecule That Reacts Asymmetrically

When compounds enriched in the heavy-carbon isotope ^{13}C and the radioactive carbon isotopes ^{11}C and ^{14}C became available in the mid-1940s, they were soon put to use in tracing the pathway of carbon atoms through the citric acid cycle. One such experiment initiated the controversy over the role of citrate. Acetate labeled in the carboxyl group (designated [1-^{14}C] acetate) was incubated aerobically with an animal tissue preparation. Acetate is enzymatically converted to acetyl-CoA in animal tissues, and the pathway of the labeled carboxyl carbon of the acetyl group in the cycle reactions could thus be traced. α-Ketoglutarate was isolated from the tissue after incubation, then degraded by known chemical reactions to establish the position(s) of the isotopic carbon.

Condensation of unlabeled oxaloacetate with carboxyl-labeled acetate would be expected to produce citrate labeled in one of the two primary carboxyl groups. Citrate is a symmetric molecule, its two terminal carboxyl groups being chemically indistinguishable. Therefore, half the labeled citrate molecules were expected to yield α-ketoglutarate labeled in the α-carboxyl group and the other half to yield α-ketoglutarate labeled in the γ-carboxyl group; that is, the α-ketoglutarate isolated was expected to be a mixture of the two types of labeled molecules (Fig. 1, pathways ① and ②). Contrary to this expectation, the labeled α-ketoglutarate isolated from the tissue suspension contained ^{14}C only in the γ-carboxyl group (Fig. 1, pathway ①). The investigators concluded that citrate (or any other symmetric molecule) could not be an intermediate in the pathway from acetate to α-ketoglutarate. Rather, an asymmetric tricarboxylic acid, presumably *cis*-aconitate or isocitrate, must be the first product formed from condensation of acetate and oxaloacetate.

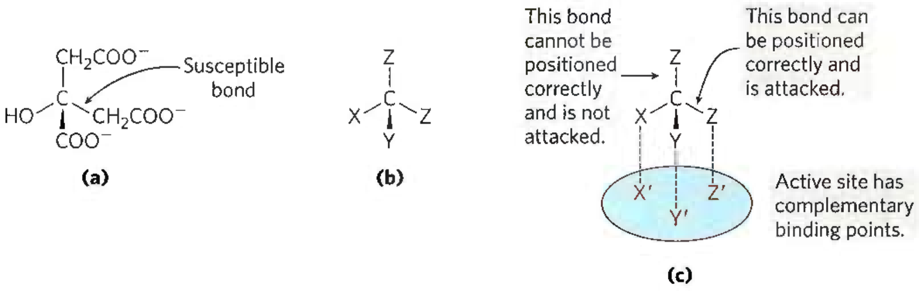
In 1948, however, Alexander Ogston pointed out that although citrate has no chiral center (see Fig. 1-19), it has the *potential* to react asymmetrically if an enzyme with which it interacts has an active site that is asymmetric. He suggested that the active site of aconitase may have three points to which the citrate must be bound and that the citrate must undergo a specific three-point attachment to these binding points. As seen in Figure 2, the binding of citrate to three such points could happen in only one way, and this would account for the formation of only one type of labeled α-ketoglutarate. Organic molecules such as citrate that have no chiral center but are potentially capable of reacting asymmetrically with an asymmetric active site are now called **prochiral molecules.**



Only this product was formed.

This second form of labeled α-ketoglutarate was also expected, but was not formed.

FIGURE 1 Incorporation of the isotopic carbon (^{14}C) of the labeled acetyl group into α-ketoglutarate by the citric acid cycle. The carbon atoms of the entering acetyl group are shown in red.



This bond cannot be positioned correctly and is not attacked.

This bond can be positioned correctly and is attacked.

Active site has complementary binding points.

FIGURE 2 The prochiral nature of citrate. (a) Structure of citrate; (b) schematic representation of citrate: X = —OH; Y = —COO$^-$; Z = —CH$_2$COO$^-$. (c) Correct complementary fit of citrate to the binding site of aconitase. There is only one way in which the three specified groups of citrate can fit on the three points of the binding site. Thus only one of the two —CH$_2$COO$^-$ groups is bound by aconitase.

TABLE 16-1 **Stoichiometry of Coenzyme Reduction and ATP Formation in the Aerobic Oxidation of Glucose via Glycolysis, the Pyruvate Dehydrogenase Complex Reaction, the Citric Acid Cycle, and Oxidative Phosphorylation**

Reaction	Number of ATP or reduced coenzyme directly formed	Number of ATP ultimately formed[a]
Glucose ⟶ glucose 6-phosphate	−1 ATP	−1
Fructose 6-phosphate ⟶ fructose 1,6-bisphosphate	−1 ATP	−1
2 Glyceraldehyde 3-phosphate ⟶ 2 1,3-bisphosphoglycerate	2 NADH	3 or 5[b]
2 1,3-Bisphosphoglycerate ⟶ 2 3-phosphoglycerate	2 ATP	2
2 Phosphoenolpyruvate ⟶ 2 pyruvate	2 ATP	2
2 Pyruvate ⟶ 2 acetyl-CoA	2 NADH	5
2 Isocitrate ⟶ 2 α-ketoglutarate	2 NADH	5
2 α-Ketoglutarate ⟶ 2 succinyl-CoA	2 NADH	5
2 Succinyl-CoA ⟶ 2 succinate	2 ATP (or 2 GTP)	2
2 Succinate ⟶ 2 fumarate	2 $FADH_2$	3
2 Malate ⟶ 2 oxaloacetate	2 NADH	5
Total		30–32

[a] This is calculated as 2.5 ATP per NADH and 1.5 ATP per $FADH_2$. A negative value indicates consumption.

[b] This number is either 3 or 5, depending on the mechanism used to shuttle NADH equivalents from the cytosol to the mitochondrial matrix; see Figures 19-31 and 19-32.

emerged from the cycle as CO_2 from the oxidation of isocitrate and α-ketoglutarate. P2 The energy released by these oxidations was conserved in the reduction of three NAD^+ and one FAD and the production of one ATP or GTP. At the end of the cycle a molecule of oxaloacetate was regenerated. Note that the two carbon atoms appearing as CO_2 are not the same two carbons that entered in the form of the acetyl group; additional turns around the cycle are required to release these carbons as CO_2 (Fig. 16-7).

P2 Although the citric acid cycle directly generates only one ATP per turn (in the conversion of succinyl-CoA to succinate), the four oxidation steps in the cycle provide a large flow of electrons into the respiratory chain via NADH and $FADH_2$ and thus lead to formation of almost 10 times more ATP during oxidative phosphorylation.

We saw in Chapter 14 that the production of two molecules of pyruvate from one molecule of glucose in glycolysis yields 2 ATP and 2 NADH. In oxidative phosphorylation (Chapter 19), passage of two electrons from NADH to O_2 drives the formation of about 2.5 ATP, and passage of two electrons from $FADH_2$ to O_2 yields about 1.5 ATP. This stoichiometry allows us to calculate the overall yield of ATP from the complete oxidation of glucose. When both pyruvate molecules are oxidized to 6 CO_2 via the pyruvate dehydrogenase complex and the citric acid cycle, and the electrons are transferred to O_2 via oxidative phosphorylation, as many as 32 ATP are obtained per glucose (**Table 16-1**). In round numbers, this represents the conservation of 32×30.5 kJ/mol = 976 kJ/mol, or 34% of the theoretical maximum of about 2,840 kJ/mol available from the complete oxidation of glucose. These calculations employ the standard free-energy changes; when corrected for the actual free energy required to form ATP within cells (see Worked Example 13-2, p. 480), the calculated efficiency of the process is closer to 65%. When cells under anaerobic conditions depend on glycolysis for ATP, one glucose yields just 2 ATP. Aerobic metabolism is far more effective in capturing the energy in glucose as a fuel.

SUMMARY 16.2 *Reactions of the Citric Acid Cycle*

- The citric acid cycle (Krebs cycle, TCA cycle) is a nearly universal central catabolic pathway in which compounds derived from the breakdown of carbohydrates, fats, and proteins are oxidized to CO_2, with most of the energy of oxidation temporarily held in the electron carriers $FADH_2$ and NADH. During aerobic metabolism, these electrons are transferred to O_2 and the energy of electron flow is conserved as ATP.
- Acetyl-CoA enters the citric acid cycle as citrate synthase catalyzes its condensation with oxaloacetate to form citrate. In seven sequential reactions, including two decarboxylations, the citric acid cycle converts citrate to oxaloacetate and releases two CO_2. The pathway is cyclic in that the intermediates of the cycle are not used up; for each oxaloacetate consumed in the path, one is produced.
- For each acetyl-CoA oxidized by the citric acid cycle, the energy gain consists of three molecules of NADH, one $FADH_2$, and one nucleoside triphosphate (either ATP or GTP).

16.3 The Hub of Intermediary Metabolism

The eight-step cyclic process for oxidation of simple two-carbon acetyl groups to CO_2 may seem unnecessarily complex and not in keeping with the biological principle of maximum economy. Remember, though, that the role of the citric acid cycle is not confined to the oxidation of acetate from carbohydrates, fatty acids, or amino acids. P3 The cycle also accepts 3-, 4-, and 5-carbon skeletons, especially from the **breakdown of amino acids**, at other points in the pathway. For example, when deaminated, the amino acids aspartate and glutamate become the cycle intermediates oxaloacetate and α-ketoglutarate, respectively.

The Citric Acid Cycle Serves in Both Catabolic and Anabolic Processes

In aerobic organisms, the citric acid cycle is an **amphibolic pathway**, one that serves in both catabolic and anabolic processes. In the cycle's anabolic role, oxaloacetate and α-ketoglutarate can be withdrawn from the cycle to serve as precursors of aspartate and glutamate by simple transamination (Chapter 22). Through aspartate and glutamate, the carbons of oxaloacetate and α-ketoglutarate are then used to build other amino acids, as well as purine and pyrimidine nucleotides. Succinyl-CoA is a central intermediate in the synthesis of the porphyrin ring of heme groups, which serve as oxygen carriers (in hemoglobin and myoglobin) and electron carriers (in cytochromes) (see Figs. 22-25, 22-26). Finally, oxaloacetate can be converted to glucose via gluconeogenesis (see Fig. 14-16).

P3 One biosynthetic process is not possible for animals: the conversion of acetate or acetyl-CoA to glucose. Given that the carbon atoms of acetate molecules entering the citric acid cycle appear eight steps later in oxaloacetate, it might seem that the cycle could generate oxaloacetate from acetate, then the oxaloacetate could be used to synthesize glucose by gluconeogenesis. However, there is no *net* conversion of acetate to oxaloacetate; for every two carbons that enter the cycle as acetate (acetyl-CoA), two leave as CO_2. In bacteria, plants, fungi, and protists, another reaction sequence, the **glyoxylate cycle**, serves as a mechanism for converting acetate to carbohydrate. The glyoxylate cycle, which shares some reactions with the citric acid cycle, converts *two* molecules of acetate to one of oxaloacetate in a variant of the citric acid cycle in which the two decarboxylation steps are bypassed (see Fig. 20-45). Thus, plants and many simpler organisms can synthesize glucose from fatty acids, but humans and other animals cannot.

Anaplerotic Reactions Replenish Citric Acid Cycle Intermediates

Under most circumstances, there is a dynamic steady state between reactions that siphon intermediates away from the citric acid cycle and those that supply additional carbon skeletons. P3 When the withdrawal of cycle intermediates for use in biosynthesis lowers the concentrations of citric acid cycle intermediates enough to slow the cycle, the intermediates are replenished by **anaplerotic reactions** (Greek, "to refill") (**Fig. 16-15**). **Table 16-2** shows the most common anaplerotic reactions, all of which, in various tissues and organisms, convert either pyruvate or phosphoenolpyruvate to oxaloacetate or malate. The most important anaplerotic reaction in mammalian liver, kidney, and brown adipose tissue is the reversible carboxylation of pyruvate by HCO_3^- to form oxaloacetate, catalyzed by **pyruvate carboxylase**. The enzymatic addition of a carboxyl group to pyruvate requires energy, which is supplied by ATP.

Pyruvate carboxylase is a regulatory enzyme and is virtually inactive in the absence of acetyl-CoA, its positive allosteric modulator. Whenever acetyl-CoA, the fuel for the citric acid cycle, is present in excess, it stimulates the pyruvate carboxylase reaction to produce more oxaloacetate, enabling the cycle to use more acetyl-CoA in the citrate synthase reaction.

Biotin in Pyruvate Carboxylase Carries One-Carbon (CO_2) Groups

The pyruvate carboxylase reaction requires the vitamin **biotin** (**Fig. 16-16**), which is the prosthetic group of the enzyme. Biotin, which plays a key role in many carboxylation reactions, is a specialized carrier of one-carbon groups in their most oxidized form: CO_2. (The transfer of one-carbon groups in more reduced forms is mediated by other cofactors, notably tetrahydrofolate and *S*-adenosylmethionine, as described in Chapter 18.) Carboxyl groups are activated in a reaction that consumes ATP and joins CO_2 to enzyme-bound biotin. This "activated" CO_2 is then passed to an acceptor (pyruvate in this case) in a carboxylation reaction.

Pyruvate carboxylase has four identical subunits, each containing a molecule of biotin covalently attached through an amide linkage to the ε-amino group of a specific Lys residue in the enzyme active site. Carboxylation of pyruvate proceeds in two steps (Fig. 16-16): first, a carboxyl group derived from HCO_3^- is attached to biotin, then the carboxyl group is transferred to pyruvate to form oxaloacetate. P5 These two steps occur at separate active sites; the long flexible arm of biotin transfers activated carboxyl groups from the first active site (on one monomer of the tetramer) to the

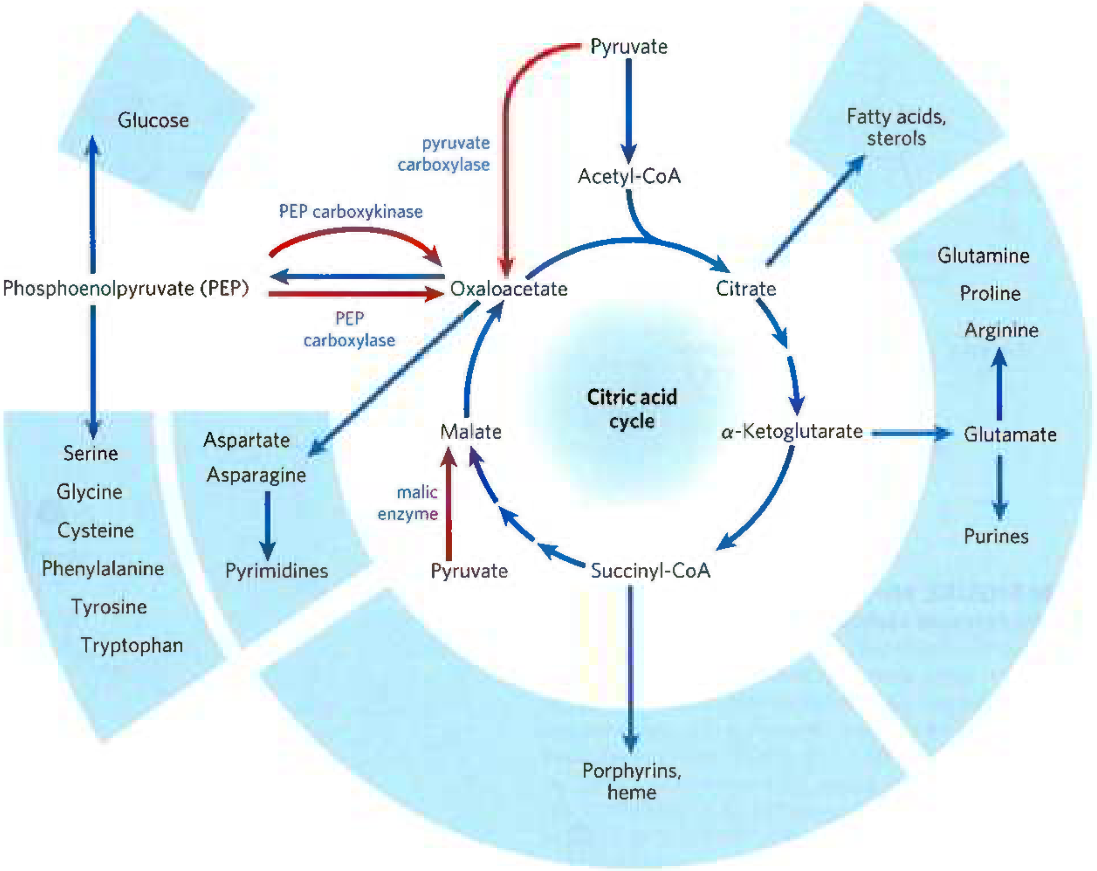

Pyruvate
pyruvate carboxylase
Acetyl-CoA
Glucose
Fatty acids, sterols
PEP carboxykinase
Phosphoenolpyruvate (PEP)
Oxaloacetate
Citrate
Glutamine
Proline
Arginine
PEP carboxylase
Citric acid cycle
Malate
α-Ketoglutarate
Glutamate
Serine
Glycine
Cysteine
Phenylalanine
Tyrosine
Tryptophan
Aspartate
Asparagine
Pyrimidines
malic enzyme
Pyruvate
Succinyl-CoA
Purines
Porphyrins, heme

FIGURE 16-15 Role of the citric acid cycle in anabolism. Intermediates of the citric acid cycle are drawn off as precursors in many biosynthetic pathways. Shown in red are four anaplerotic reactions that replenish depleted cycle intermediates (see Table 16-2).

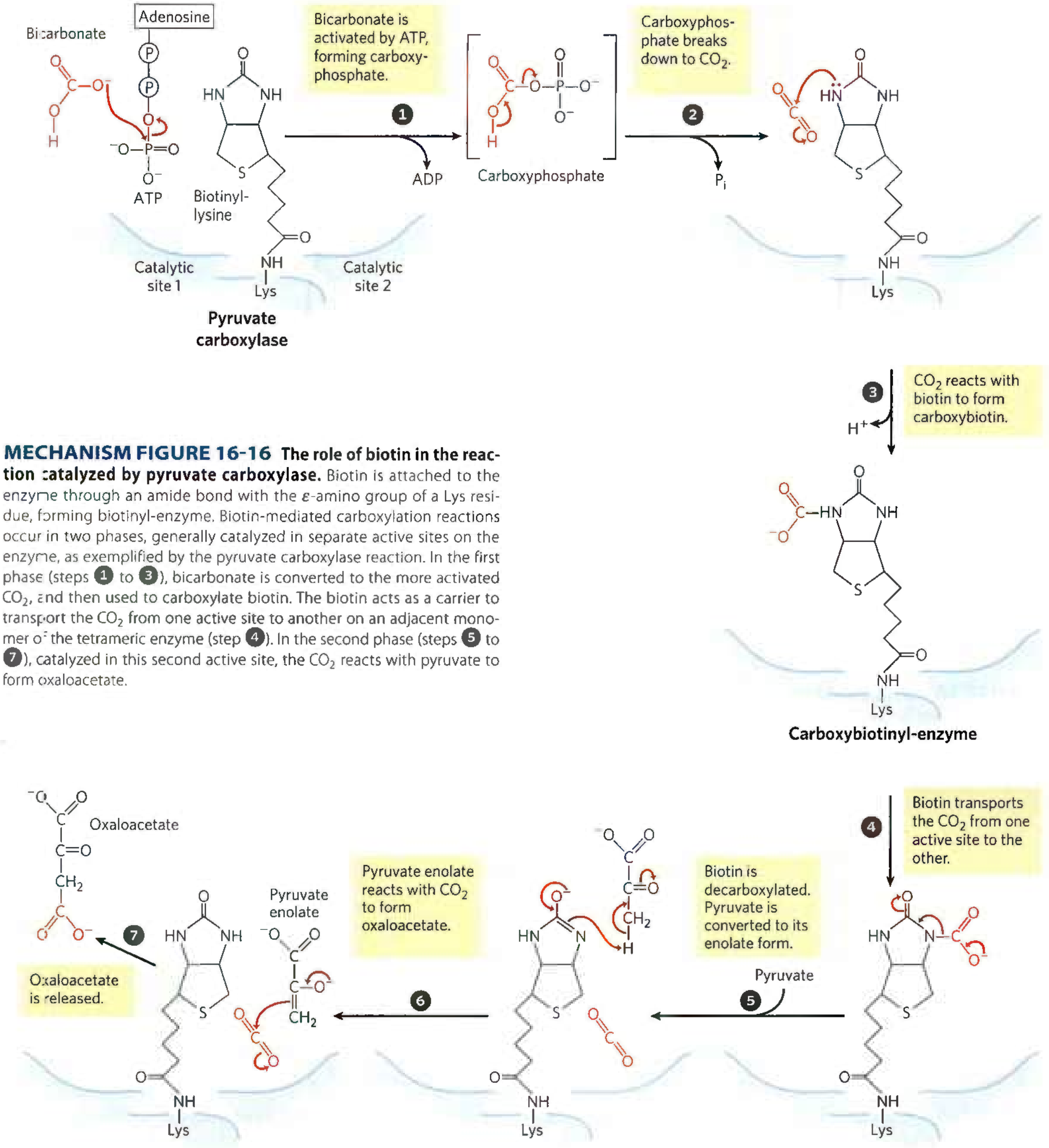
TABLE 16-2 Anaplerotic Reactions

Reaction	Tissue(s)/organism(s)
Pyruvate + HCO_3^- + ATP $\xrightleftharpoons{\text{pyruvate carboxylase}}$ oxaloacetate + ADP + P_i	Liver, kidney
Phosphoenolpyruvate + CO_2 + GDP $\xrightleftharpoons{\text{PEP carboxykinase}}$ oxaloacetate + GTP	Heart, skeletal muscle
Phosphoenolpyruvate + HCO_3^- $\xrightleftharpoons{\text{PEP carboxylase}}$ oxaloacetate + P_i	Higher plants, yeast, bacteria
Pyruvate + HCO_3^- + NAD(P)H $\xrightleftharpoons{\text{malic enzyme}}$ malate + $NAD(P)^+$	Widely distributed in eukaryotes and bacteria

second (on the adjacent monomer), functioning much like the long lipoyllysyl arm of E_2 in the PDH complex (Fig. 16-6) and the long arm of the CoA-like moiety in the acyl carrier protein involved in fatty acid synthesis (see Fig. 21-5); these are compared in **Figure 16-17**. Lipoate, biotin, and pantothenate all enter cells on the same transporter; all become covalently attached to proteins by similar reactions; and all provide a flexible tether that allows bound reaction intermediates to move from one active site to another in an enzyme complex, without dissociating from it. That is, all participate in substrate channeling.

SUMMARY 16.3 *The Hub of Intermediary Metabolism*

- The citric acid cycle is amphibolic, serving in both catabolism and anabolism. Besides acetyl-CoA, any compound that gives rise to a four- or five-carbon intermediate of the


Bicarbonate
Adenosine
P
P
ATP
Biotinyl-lysine
Catalytic site 1
Catalytic site 2
Pyruvate carboxylase
①
ADP
Carboxyphosphate
②
P_i
③
H^+
Carboxybiotinyl-enzyme
④
⑤
Pyruvate
⑥
Pyruvate enolate
⑦
Oxaloacetate
Lys

Bicarbonate is activated by ATP, forming carboxyphosphate.

Carboxyphosphate breaks down to CO_2.

CO_2 reacts with biotin to form carboxybiotin.

Biotin transports the CO_2 from one active site to the other.

Biotin is decarboxylated. Pyruvate is converted to its enolate form.

Pyruvate enolate reacts with CO_2 to form oxaloacetate.

Oxaloacetate is released.

MECHANISM FIGURE 16-16 The role of biotin in the reaction catalyzed by pyruvate carboxylase. Biotin is attached to the enzyme through an amide bond with the ε-amino group of a Lys residue, forming biotinyl-enzyme. Biotin-mediated carboxylation reactions occur in two phases, generally catalyzed in separate active sites on the enzyme, as exemplified by the pyruvate carboxylase reaction. In the first phase (steps ① to ③), bicarbonate is converted to the more activated CO_2, and then used to carboxylate biotin. The biotin acts as a carrier to transport the CO_2 from one active site to another on an adjacent monomer of the tetrameric enzyme (step ④). In the second phase (steps ⑤ to ⑦), catalyzed in this second active site, the CO_2 reacts with pyruvate to form oxaloacetate.

---

citric acid cycle—for example, the breakdown products of many amino acids—can be oxidized by the cycle. Cycle intermediates can be drawn off and used as the starting material for a variety of biosynthetic products.

- When intermediates are shunted from the citric acid cycle to other pathways, they are replenished by several anaplerotic reactions, which produce four-carbon intermediates by carboxylation of three-carbon compounds; pyruvate carboxylase is a major anaplerotic enzyme.
- Enzymes that catalyze carboxylations commonly employ biotin to activate CO_2 and to carry it to acceptors such as pyruvate or phosphoenolpyruvate.

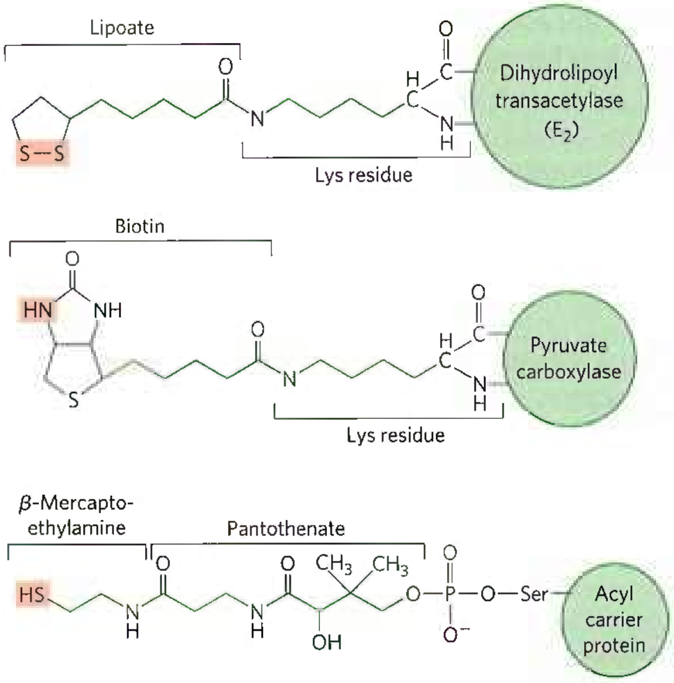

Lipoate
S—S
O
N
H
C
Dihydrolipoyl transacetylase (E_2)
Lys residue
Biotin
HN
NH
S
Pyruvate carboxylase
Lys residue
β-Mercapto-ethylamine
Pantothenate
HS
CH_3
CH_3
OH
O—P—O—Ser
O^-
Acyl carrier protein

FIGURE 16-17 Biological tethers. The cofactors lipoate, biotin, and the combination of β-mercaptoethylamine and pantothenate form long, flexible arms (green) on the enzymes to which they are covalently bound, acting as tethers that move intermediates from one active site to the next. The group shaded light red is, in each case, the point of attachment of the activated intermediate to the tether.

16.4 Regulation of the Citric Acid Cycle

P4 The central role of the citric acid cycle in metabolism requires stringent regulation to balance the supply of key intermediates with the demands of energy production and biosynthetic processes. Regulation occurs at several levels, including the oxidation of pyruvate to acetyl-CoA (catalyzed by the PDH complex) and the entry of acetyl-CoA into the cycle (the citrate synthase reaction). The transport of pyruvate into mitochondria by the mitochondrial pyruvate carrier (MPC) determines to some degree the fate of pyruvate produced by glycolysis. Most cells also produce acetyl-CoA from the oxidation of fatty acids (Chapter 17) and certain amino acids (Chapter 18), and the availability of intermediates from those other pathways is important in the regulation of pyruvate oxidation and of the citric acid cycle. There are therefore multiple points at which the metabolism of pyruvate and the citric acid cycle may be regulated.

Production of Acetyl-CoA by the PDH Complex Is Regulated by Allosteric and Covalent Mechanisms

The PDH complex of mammals is strongly inhibited allosterically by ATP and by acetyl-CoA and NADH, the products of the reaction catalyzed by the complex (**Fig. 16-18**). Long-chain fatty acids, which can be broken down to acetyl-CoA, are also inhibitory. AMP, CoA, and NAD^+, all of which accumulate when too little acetate flows into the citric acid cycle, allosterically activate the PDH complex. **P1** Thus, PDH complex activity is turned off when ample fuel is available in the form of fatty acids and acetyl-CoA and when the cell's [ATP]/[ADP] and [NADH]/[NAD^+] ratios are high; it is turned on again when energy demands are high and the cell requires greater flux of acetyl-CoA into the citric acid cycle.

In mammals, these allosteric regulatory mechanisms are complemented by a second level of regulation:

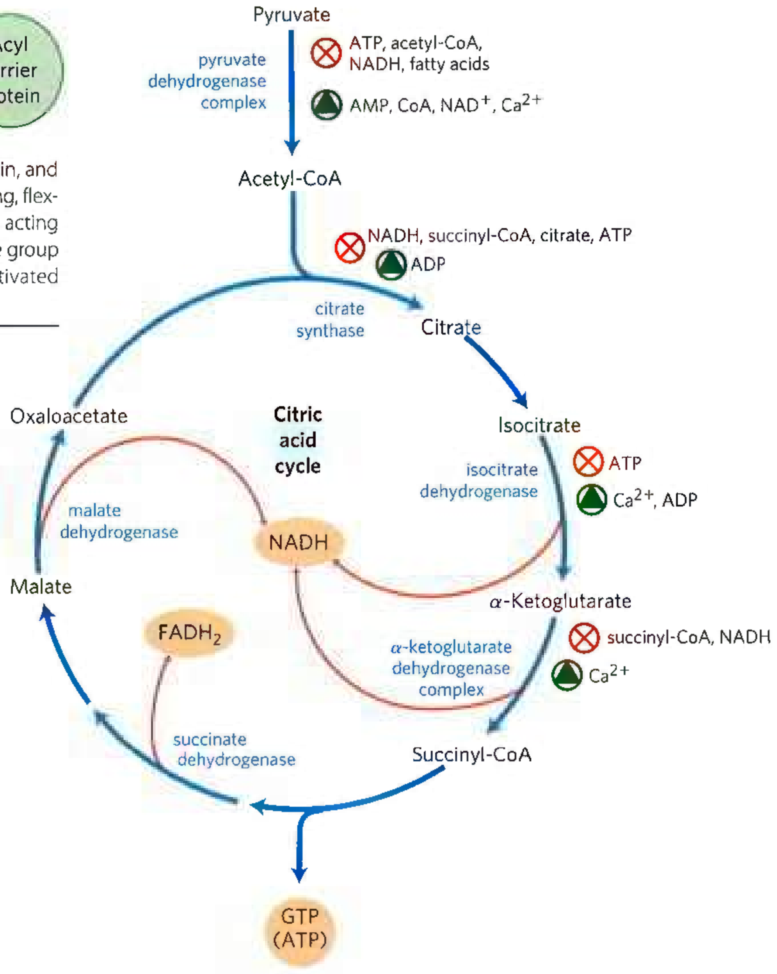
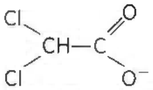

Pyruvate
pyruvate dehydrogenase complex
ATP, acetyl-CoA, NADH, fatty acids
AMP, CoA, NAD^+, Ca^{2+}
Acetyl-CoA
NADH, succinyl-CoA, citrate, ATP
ADP
citrate synthase
Citrate
Isocitrate
isocitrate dehydrogenase
ATP
Ca^{2+}, ADP
Oxaloacetate
Citric acid cycle
malate dehydrogenase
NADH
Malate
α-Ketoglutarate
succinyl-CoA, NADH
Ca^{2+}
α-ketoglutarate dehydrogenase complex
$FADH_2$
succinate dehydrogenase
Succinyl-CoA
GTP (ATP)

FIGURE 16-18 Regulation of metabolite flow from the PDH complex through the citric acid cycle in mammals. The PDH complex is allosterically inhibited when [ATP]/[ADP], [NADH]/[NAD^+], and [acetyl-CoA]/[CoA] ratios are high, all of which indicate an energy-sufficient metabolic state. When these ratios decrease, allosteric activation of pyruvate oxidation results. The rate of flow through the citric acid cycle can be limited by the availability of the citrate synthase substrates, oxaloacetate and acetyl-CoA, or of NAD^+, which is depleted by its conversion to NADH, slowing the three NAD^+-dependent oxidation steps. Feedback inhibition by succinyl-CoA, citrate, and ATP also slows the cycle by inhibiting early steps. In muscle tissue, Ca^{2+} stimulates contraction and, as shown here, stimulates energy-yielding metabolism to replace the ATP consumed by contraction.

phosphorylation/dephosphorylation. The PDH complex is inhibited by reversible phosphorylation of Ser residues on E_1 by **PDH kinase**, which is an intrinsic part of the PDH complex. PDH kinase is allosterically activated by the products of the PDH complex (ATP, NADH, and acetyl-CoA), and it is inhibited by the substrates for the PDH complex (ADP, NAD^+, and pyruvate) (**Fig. 16-19**). The complex also contains **PDH phosphatase**, which reverses the inhibition by PDH kinase. **P1** Together, the kinase and phosphatase exert strong control over the entry of acetyl-CoA from pyruvate into the citric acid cycle. Higher concentrations of ATP, NADH, or acetyl-CoA lead to inactivation of the PDH complex by phosphorylation of PDH. When the concentrations of ADP, NAD^+, or pyruvate rise, kinase activity decreases and pyruvate dehydrogenase phosphatase removes the phosphoryl group, reactivating the PDH complex and thereby stimulating the citric acid cycle.

The simple compound dichloroacetate (DCA), a structural analog of acetate, inhibits PDH kinase in the laboratory and so relieves the inhibition of the PDH complex. This stimulates pyruvate oxidation via the citric acid cycle (Fig. 16-19) and thus may have a use in directing metabolism in tumor cells away from aerobic glycolysis (see Box 14-1). The increase in mitochondrial oxidation also stimulates apoptosis (Chapter 19), thereby suppressing tumor growth. Phase III clinical trials of DCA began in 2019.



Dichloroacetate

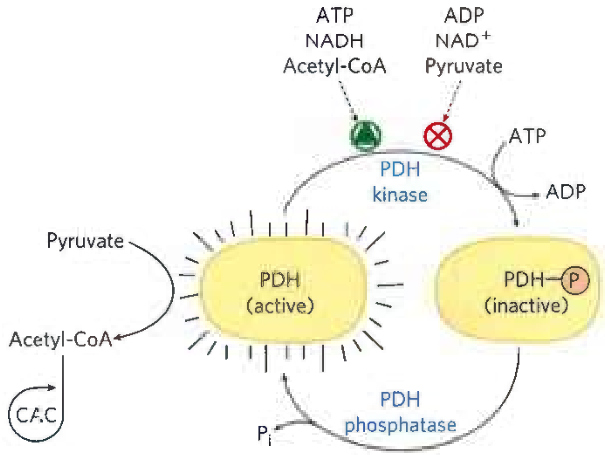


FIGURE 16-19 Pyruvate dehydrogenase is inactivated by phosphorylation catalyzed by pyruvate dehydrogenase kinase. The kinase is regulated by metabolites that signal the energetic state of the cell. Metabolites that accumulate in an energy-sufficient state activate PDH kinase, which phosphorylates and inactivates PDH. Pyruvate is then diverted away from the energy-yielding citric acid cycle (CAC). Metabolites indicating energy need or pyruvate accumulation have the opposite effect, keeping PDH active and sending acetyl-CoA into the CAC.

The Citric Acid Cycle Is Also Regulated at Three Exergonic Steps

Each of the three strongly exergonic steps in the cycle—those catalyzed by citrate synthase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase (Fig. 16-18)—can become the rate-limiting step under some circumstances. The availability of the substrates for citrate synthase (acetyl-CoA and oxaloacetate) varies with the metabolic state of the cell and sometimes limits the rate of citrate formation. NADH, a product of isocitrate and α-ketoglutarate oxidation, accumulates under some conditions, and at high $[NADH]/[NAD^+]$ both dehydrogenase reactions are severely inhibited by mass action. Similarly, the malate dehydrogenase reaction is essentially at equilibrium in the cell (that is, it is substrate-limited), and when $[NADH]/[NAD^+]$ is high the concentration of oxaloacetate is low, slowing the first step in the cycle. Product accumulation inhibits all three limiting steps of the cycle: succinyl-CoA inhibits α-ketoglutarate dehydrogenase (and also citrate synthase); citrate blocks citrate synthase; and the end product, ATP, inhibits both citrate synthase and isocitrate dehydrogenase. The inhibition of citrate synthase by ATP is relieved by ADP, an allosteric activator of this enzyme. In vertebrate muscle, Ca^{2+}, the signal for contraction and for a concomitant increase in demand for ATP, activates both isocitrate dehydrogenase and α-ketoglutarate dehydrogenase, as well as the PDH complex. **P4** In short, the concentrations of substrates and intermediates in the citric acid cycle set the flux through this pathway at a rate that provides optimal concentrations of ATP and NADH.

Under normal conditions, the rates of glycolysis and of the citric acid cycle are integrated so that only as much glucose is metabolized to pyruvate as is needed to supply the citric acid cycle with fuel (acetyl-CoA). Both pathways are inhibited by high levels of ATP and NADH, but also by the concentration of citrate, the product of the first step of the citric acid cycle, and an important allosteric inhibitor of phosphofructokinase-1 of the glycolytic pathway (see Fig. 14-23).

Citric Acid Cycle Activity Changes in Tumors

The mitochondrial pyruvate carrier (MPC) is down-regulated in tumor cells, leading to pyruvate accumulation in the cytosol. Several other mitochondrial enzymes are also inactivated in tumor cells, including the PDH complex and succinate dehydrogenase. As a result, tumor cells accumulate lactate (from the pyruvate produced by glycolysis) and succinate. Both of these intermediates are **oncometabolites**; they stimulate tumor growth, acting through specific G protein–coupled receptors (GPCRs; see Chapter 12) in the plasma membrane. The membrane receptor for lactate is upregulated in most cancers. L-Lactate acting through its membrane receptor, lowers [cAMP] and raises $[Ca^{2+}]$, with downstream effects currently under investigation.

P4 Mutations in citric acid cycle enzymes are very rare in humans and other mammals, but those that do occur are devastating. Genetic defects in succinate dehydrogenase lead to tumors of the adrenal gland (pheochromocytomas), and mutations in the fumarase gene lead to tumors of smooth muscle (leiomyomas) and kidney. Their activities thus define both enzymes as tumor suppressors (p. 451).

Another remarkable connection between citric acid cycle intermediates and cancer is the finding that in many glial cell tumors (gliomas), the NADPH-dependent isocitrate dehydrogenase has an unusual genetic defect. The mutant enzyme loses its normal activity (converting isocitrate to α-ketoglutarate) but *gains* a new activity: it converts α-ketoglutarate to 2-hydroxyglutarate (**Fig. 16-20**), which accumulates in the tumor cells. α-Ketoglutarate and Fe^{3+} are essential cofactors for a family of histone demethylases that alter gene expression. They do so by removing methyl groups from Arg and Lys residues in the histones that organize nuclear DNA. By competing with α-ketoglutarate for binding to the histone demethylases, 2-hydroxyglutarate inhibits their activity. Inhibition of the histone demethylases interferes with normal gene regulation, leading to unrestricted glial cell growth. The family of more than 60 dioxygenases that use α-ketoglutarate and Fe^{3+} as cofactors are also competitively inhibited by 2-hydroxyglutarate. Inhibition of one or more of these enzymes could interfere with normal regulation of cell division and thus produce a tumor. ■

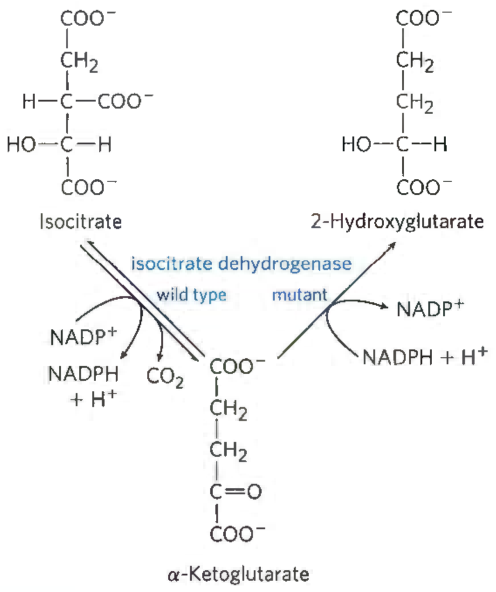
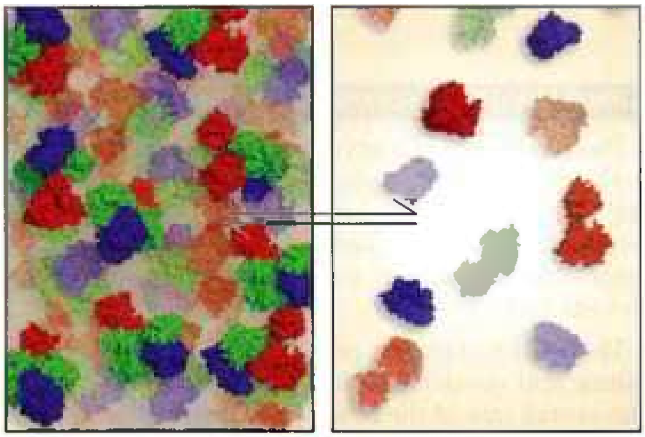


FIGURE 16-20 A mutant isocitrate dehydrogenase acquires a new activity. Wild-type isocitrate dehydrogenase catalyzes the conversion of isocitrate to α-ketoglutarate, but mutations that alter the binding site for isocitrate cause loss of the normal enzymatic activity and gain of a new activity: conversion of α-ketoglutarate to 2-hydroxyglutarate. Accumulation of this product inhibits histone demethylase, altering gene regulation and leading to glial cell tumors in the brain.

Certain Intermediates Are Channeled through Metabolons

We saw an example of substrate channeling in the five-reaction sequence of the PDH complex. Many other reactions occur in similar multienzyme complexes that ensure efficient passage of the product of one enzyme reaction to the next enzyme in the pathway. Such integrated multienzyme complexes, **metabolons**, are held together by noncovalent interactions and are not easily extracted intact from the cell. In the classic approach of enzymology—purification of individual proteins from extracts of broken cells—once cells are broken open, their contents, including enzymes, are diluted 100- or 1,000-fold (**Fig. 16-21**), which favors dissociation of noncovalent complexes such as metabolons.

The enzymes of the citric acid cycle are usually described as *soluble* components of the mitochondrial matrix (except for succinate dehydrogenase, which is membrane-bound), but there is evidence that at least three sequential enzymes of the citric acid cycle (malate dehydrogenase, citrate synthase, and aconitase) constitute a metabolon (**Fig. 16-22**). We will see more examples of substrate channeling in some amino acid biosynthesis pathways in Chapter 22. As cryo-EM increases our understanding of larger complexes and "—somes" (apoptosomes, respirasomes, and replisomes, for example), it seems likely that many more enzymes once thought to function as individual soluble proteins will be found to act in multienzyme complexes that facilitate the channeling of substrates.


In intact cell

In cell extract

FIGURE 16-21 Effect of protein concentration on complex stability. Dilution of a solution containing a noncovalently bound protein complex—such as a metabolon consisting of three enzymes (illustrated here in red, blue, and green)—favors dissociation of the complex into its constituents.

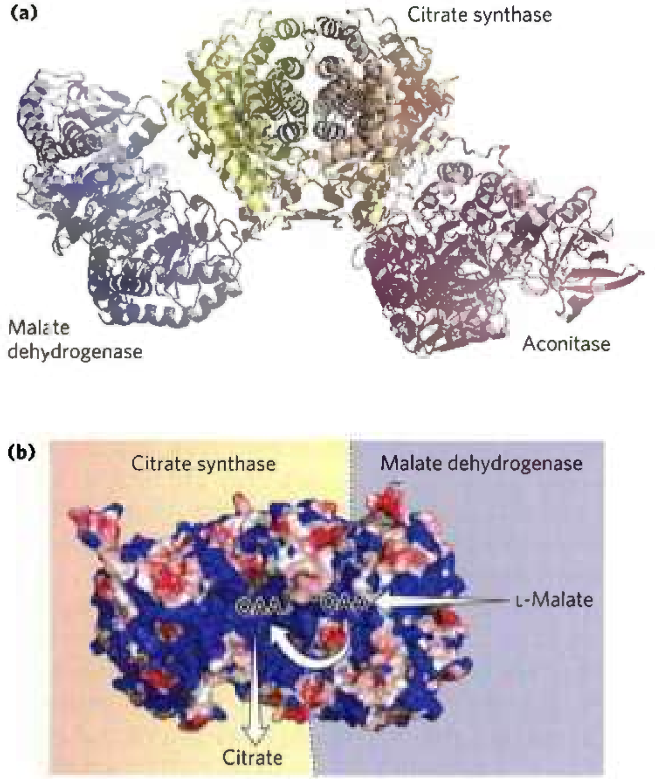


FIGURE 16-22 A three-enzyme metabolon of the citric acid cycle (a) Purified porcine enzymes malate dehydrogenase (MDH), citrate synthase (CS), and aconitase form a metabolon when combined in vitro. (b) Electrostatic modeling shows that a broad path of positive potential along the surface of a MDH-CS complex connects the active sites of MDH and CS. This path provides a channel for the passage of the negatively charged oxaloacetate (OAA) from the active site of MDH, where it is formed from L-malate, to the active site of CS, where it condenses with acetyl-CoA to form citrate. Engineered mutations that replaced a positively charged Arg residue along this path with a negatively charged Asp residue greatly reduced the rate of substrate channeling through the complex, providing evidence that the functional unit is a metabolon. [Information from B. Bulutoglu et al., *ACS Chem. Biol.* 1 :2847, 2016. Data from PDB ID 1MLD, W. B. Gleason et al., *Biochemistry* 33:2078, 1994; PDB ID 1CTS, S. Remington et al., *J. Mol. Biol.* 158:111, 1982; PDB ID 7ACN, H. Lauble et al., *Biochemistry* 31:2735, 1992.]

SUMMARY 16.4 *Regulation of the Citric Acid Cycle*

- The production of acetyl-CoA for the citric acid cycle by the PDH complex is inhibited allosterically by metabolites that signal a sufficiency of metabolic energy (ATP, acetyl-CoA, NADH, and fatty acids) and is stimulated by metabolites that indicate a reduced energy supply (AMP, CoA-SH, NAD^+).
- The PDH complex is regulated by allosteric mechanisms and covalent modification (phosphorylation). The overall rate of the citric acid cycle is controlled by the rate of conversion of pyruvate to acetyl-CoA and by the flux through citrate synthase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase. These fluxes are affected by the concentrations of substrates and products: the end products ATP and NADH are inhibitory, and the substrates NAD^+ and ADP are stimulatory. Long-chain fatty acids, which can break down to acetyl-CoA, are also inhibitory.
- Some mutations that affect the PDH complex or citric acid cycle enzymes are oncogenic: they occur very commonly in certain types of cancer.
- Complexes of consecutive enzymes in a pathway (metabolons) allow substrate channeling and more efficient passage of substrates through the reaction sequences.

KEY TERMS

Terms in bold are defined in the glossary.

cellular respiration	574
citric acid cycle	574
tricarboxylic acid (TCA) cycle	574
Krebs cycle	574
thioester	575
mitochondrial pyruvate carrier (MPC)	575
pyruvate dehydrogenase (PDH) complex	575
oxidative decarboxylation	575
lipoate	576
substrate channeling	577
iron-sulfur center	582
α-ketoglutarate dehydrogenase complex	582
moonlighting enzymes	584
nucleoside diphosphate kinase	586
prochiral molecule	588
amphibolic pathway	590
glyoxylate cycle	590
anaplerotic reaction	590
biotin	590
oncometabolite	594
metabolon	595

PROBLEMS

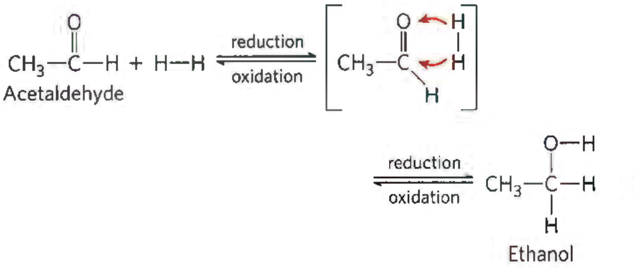
1. **Balance Sheet for the Citric Acid Cycle** The citric acid cycle has eight enzymes: citrate synthase, aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, fumarase, and malate dehydrogenase.

(a) Write a balanced equation for the reaction catalyzed by each enzyme.
(b) Name the cofactor(s) required by each enzyme reaction.
(c) For each enzyme, determine which of the following describes the type of reaction(s) catalyzed: condensation (carbon–carbon bond formation); dehydration (loss of water); hydration (addition of water); decarboxylation (loss of CO_2); oxidation-reduction; substrate-level phosphorylation; isomerization.
(d) Write a balanced net equation for the catabolism of acetyl-CoA to CO_2.

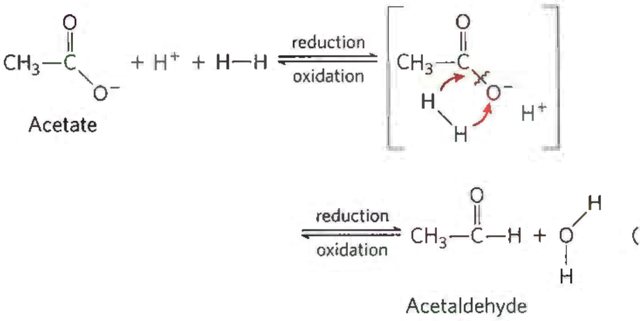
2. **Net Equation for Glycolysis and the Citric Acid Cycle** Write the net biochemical equation for the metabolism of a molecule of glucose by glycolysis and the citric acid cycle, including all cofactors.

3. **Recognizing Oxidation and Reduction Reactions** One biochemical strategy of many living organisms is the stepwise oxidation of organic compounds to CO_2 and H_2O and the conservation of a major part of the energy thus produced in the form of ATP. It is important to be able to recognize oxidation-reduction processes in metabolism. Reduction of an organic molecule results from the hydrogenation of a double bond (Eqn 1, below) or of a single bond with accompanying cleavage (Eqn 2). Conversely, oxidation results from dehydrogenation.

In biochemical redox reactions, the coenzymes NAD and FAD dehydrogenate/hydrogenate organic molecules in the presence of the proper enzymes.


Acetaldehyde
reduction
oxidation
reduction
oxidation
Ethanol

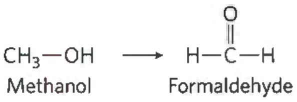
(1)


Acetate
reduction
oxidation
reduction
oxidation
Acetaldehyde

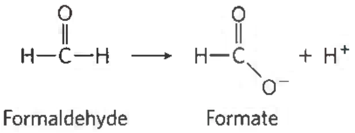
(2)

For each of the metabolic transformations (a) through (h), determine whether the compound on the left has undergone oxidation or reduction. Balance each transformation by inserting H—H and, where necessary, H_2O.

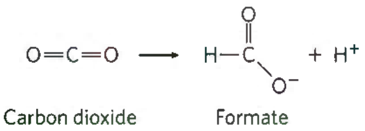
(a)


Methanol
Formaldehyde

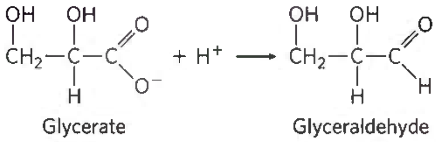
(b)


Formaldehyde
Formate

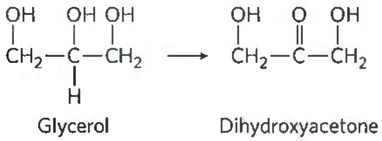
(c)


Carbon dioxide
Formate

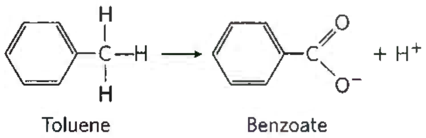
(d)


Glycerate
Glyceraldehyde

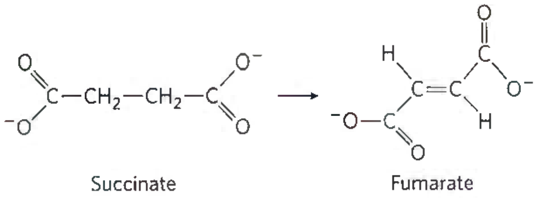
(e)


Glycerol
Dihydroxyacetone

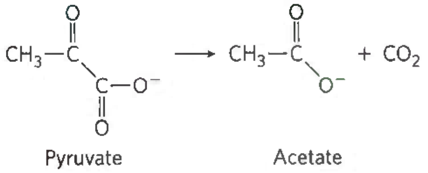
(f)


Toluene
Benzoate

(g)


Succinate
Fumarate

(h)


Pyruvate
Acetate

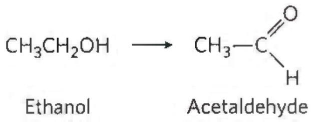
4. Relationship between Energy Release and the Oxidation State of Carbon A eukaryotic cell can use glucose ($C_6H_{12}O_6$) and hexanoate ($C_6H_{11}O_2$) as fuels for cellular respiration. On the basis of their structural formulas, which substance releases more energy per gram on complete combustion to CO_2 and H_2O?

5. Nicotinamide Coenzymes as Reversible Redox Carriers The nicotinamide coenzymes (see Fig. 13-24) can undergo reversible oxidation-reduction reactions with specific substrates in the presence of the appropriate dehydrogenase. In these reactions, NADH + H^+ serves as the hydrogen source, as described in Problem 3. Whenever the coenzyme is oxidized, a substrate must be simultaneously reduced:

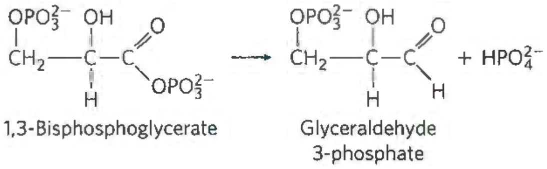
$$\underset{\text{Oxidized}}{\text{Substrate}} + \underset{\text{Reduced}}{\text{NADH}} + H^+ \rightleftharpoons \underset{\text{Reduced}}{\text{product}} + \underset{\text{Oxidized}}{\text{NAD}^+}$$

For each of the reactions in (a) through (f) shown below, determine whether the substrate has been oxidized or reduced or is unchanged in oxidation state (see Problem 3). If a redox change has occurred, balance the reaction with the necessary amount of NAD^+, NADH, H^+, and H_2O. The objective is to recognize when a redox coenzyme is necessary in a metabolic reaction.

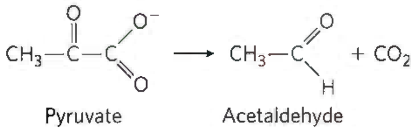
(a)


Ethanol
Acetaldehyde

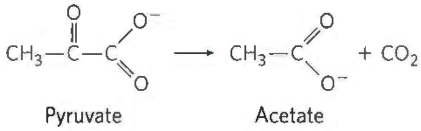
(b)


1,3-Bisphosphoglycerate
Glyceraldehyde 3-phosphate

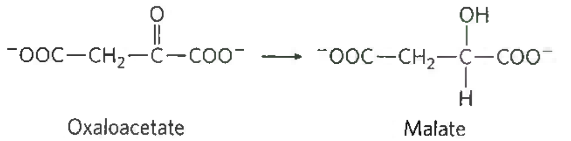
(c)


Pyruvate
Acetaldehyde

(d)

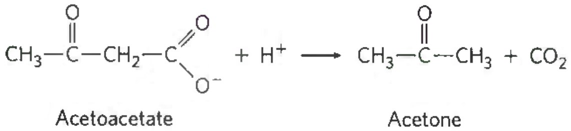

Pyruvate
Acetate

(e)



$$^{-}OOC{-}CH_2{-}\overset{\overset{O}{\|}}{C}{-}COO^{-} \longrightarrow {}^{-}OOC{-}CH_2{-}\underset{\underset{H}{|}}{\overset{\overset{OH}{|}}{C}}{-}COO^{-}$$

(f)



$$CH_3{-}\overset{\overset{O}{\|}}{C}{-}CH_2{-}COO^{-} + H^+ \longrightarrow CH_3{-}\overset{\overset{O}{\|}}{C}{-}CH_3 + CO_2$$

6. Pyruvate Dehydrogenase Cofactors and Mechanism Describe the role of each cofactor involved in the reaction catalyzed by the pyruvate dehydrogenase complex.

7. Thiamine Deficiency Individuals with a thiamine-deficient diet have relatively high levels of pyruvate in their blood. Explain this in biochemical terms.

8. Isocitrate Dehydrogenase Reaction What type of chemical reaction is involved in the conversion of isocitrate to α-ketoglutarate? Name and describe the role of any cofactors. What other reaction(s) of the citric acid cycle are of this same type?

9. Stimulation of Oxygen Consumption by Oxaloacetate and Malate In the early 1930s, Albert Szent-Györgyi reported the interesting observation that the addition of small amounts of oxaloacetate or malate to suspensions of minced pigeon breast muscle stimulated the oxygen consumption of the preparation. Surprisingly, the amount of oxygen consumed was about seven times more than the amount necessary for complete oxidation (to CO_2 and H_2O) of the added oxaloacetate or malate. Why did the addition of oxaloacetate or malate stimulate oxygen consumption? Why was the amount of oxygen consumed so much greater than the amount necessary to completely oxidize the added oxaloacetate or malate?

10. Formation of Oxaloacetate in a Mitochondrion In the last reaction of the citric acid cycle, malate is dehydrogenated to regenerate the oxaloacetate necessary for the entry of acetyl-CoA into the cycle:

$$\text{L-Malate} + \text{NAD}^+ \longrightarrow \text{oxaloacetate} + \text{NADH} + \text{H}^+ \qquad \Delta G'^{\circ} = 30.0 \text{ kJ/mol}$$

(a) Calculate the equilibrium constant for this reaction at 25 °C.
(b) Because $\Delta G'^{\circ}$ assumes a standard pH of 7, the equilibrium constant calculated in (a) corresponds to

$$K'_{\text{eq}} = \frac{[\text{oxaloacetate}][\text{NADH}]}{[\text{L-malate}][\text{NAD}^+]}$$

The measured concentration of L-malate in rat liver mitochondria is about 0.20 mM when $[\text{NAD}^+]/[\text{NADH}]$ is 10. Calculate the concentration of oxaloacetate at pH 7 in these mitochondria.
(c) To appreciate the magnitude of the mitochondrial oxaloacetate concentration, calculate the number of oxaloacetate molecules in a single rat liver mitochondrion. Assume the mitochondrion is a sphere of diameter 2.0 μm.

11. Cofactors for the Citric Acid Cycle Suppose you have prepared a mitochondrial extract that contains all the soluble enzymes of the matrix but has lost (by dialysis) all the low molecular weight cofactors. What must you add to the extract so that the preparation will oxidize acetyl-CoA to CO_2?

12. Riboflavin Deficiency How would a riboflavin deficiency affect the functioning of the citric acid cycle? Explain your answer.

13. Oxaloacetate Pool What factors might decrease the pool of oxaloacetate available for the activity of the citric acid cycle? How can the pool of oxaloacetate be replenished?

14. Energy Yield from the Citric Acid Cycle The reaction catalyzed by succinyl-CoA synthetase produces the high-energy compound GTP. How is the free energy contained in GTP incorporated into the cellular ATP pool?

15. Respiration Studies in Isolated Mitochondria Cellular respiration can be studied in isolated mitochondria by measuring oxygen consumption under different conditions. If 0.01 M sodium malonate is added to actively respiring mitochondria that are using pyruvate as fuel, respiration soon stops and a metabolic intermediate accumulates.
(a) What is the structure of this intermediate?
(b) Explain why it accumulates.
(c) Explain why oxygen consumption stops.
(d) Aside from removal of the malonate, what can overcome this inhibition of respiration? Explain.

16. Labeling Studies in Isolated Mitochondria Biochemists have often delineated the metabolic pathways of organic compounds by using a radioactively labeled substrate and following the fate of the label.
(a) How can you determine whether a suspension of isolated mitochondria metabolizes added glucose to CO_2 and H_2O?
(b) Suppose you add a brief pulse of [3-^{14}C]pyruvate (labeled in the methyl position) to the mitochondria. After one turn of the citric acid cycle, what is the location of the ^{14}C in the oxaloacetate? Explain by tracing the ^{14}C label through the pathway. How many turns of the cycle are required to release all the [3-^{14}C]pyruvate as CO_2?

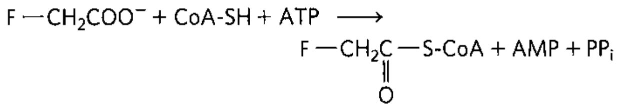
17. [1-^{14}C]Glucose Catabolism An investigator briefly incubates an actively respiring bacterial culture with [1-^{14}C]glucose and isolates the glycolytic and citric acid cycle intermediates. Where is the ^{14}C located in each of the intermediates listed? Consider only the initial incorporation of ^{14}C in the first pass of labeled glucose through the pathways.
(a) Fructose 1,6-bisphosphate
(b) Glyceraldehyde 3-phosphate
(c) Phosphoenolpyruvate
(d) Acetyl-CoA
(e) Citrate
(f) α-Ketoglutarate
(g) Oxaloacetate

18. Role of the Vitamin Thiamine People with beriberi, a disease caused by thiamine deficiency, have elevated levels of blood pyruvate and α-ketoglutarate, especially after consuming a meal rich in glucose. How are these effects related to a deficiency of thiamine?

19. Synthesis of Oxaloacetate by the Citric Acid Cycle In the last step of the citric acid cycle, NAD$^+$-dependent oxidation of L-malate forms oxaloacetate. Can a net synthesis of oxaloacetate from acetyl-CoA occur using only the enzymes and cofactors of the citric acid cycle, without depleting the intermediates of the cycle? Explain. How do cells replenish the oxaloacetate that is lost from the cycle to biosynthetic reactions?

20. Oxaloacetate Depletion Mammalian liver can carry out gluconeogenesis using oxaloacetate as the starting material (Chapter 14). Would the extensive use of oxaloacetate for gluconeogenesis affect the operation of the citric acid cycle? Explain your answer.

21. Mode of Action of the Rodenticide Fluoroacetate Fluoroacetate, prepared commercially for rodent control, is also produced by a South African plant. After entering a cell, fluoroacetate is converted to fluoroacetyl-CoA in a reaction catalyzed by the enzyme acetate thiokinase:



$$\text{F—CH}_2\text{COO}^- + \text{CoA-SH} + \text{ATP} \longrightarrow \text{F—CH}_2\underset{\underset{\text{O}}{\|}}{\text{C}}\text{—S-CoA} + \text{AMP} + \text{PP}_i$$

You perform a perfusion experiment to study the toxic effect of fluoroacetate using intact isolated rat heart. After perfusing the heart with 0.22 mM fluoroacetate, you see a decrease in the measured rate of glucose uptake and glycolysis as well as an accumulation of glucose 6-phosphate and fructose 6-phosphate. Examination of the citric acid cycle intermediates reveals that their concentrations are below normal, except for citrate, which has a concentration 10 times higher than normal.

(a) Where did the block in the citric acid cycle occur? What caused citrate to accumulate and the other cycle intermediates to be depleted?

(b) Fluoroacetyl-CoA is enzymatically transformed in the citric acid cycle. What is the structure of the end product of fluoroacetate metabolism? Why does it block the citric acid cycle? How might the inhibition be overcome?

(c) In the heart perfusion experiments, why did glucose uptake and glycolysis decrease? Why did hexose monophosphates accumulate?

(d) Why is fluoroacetate poisoning fatal?

22. Synthesis of L-Malate in Wine Making The tartness of some wines is due to high concentrations of L-malate. Write a sequence of reactions showing how yeast cells synthesize L-malate from glucose under anaerobic conditions in the presence of dissolved CO_2 (HCO_3^-). Note that the overall reaction for this fermentation cannot involve the consumption of nicotinamide coenzymes or citric acid cycle intermediates.

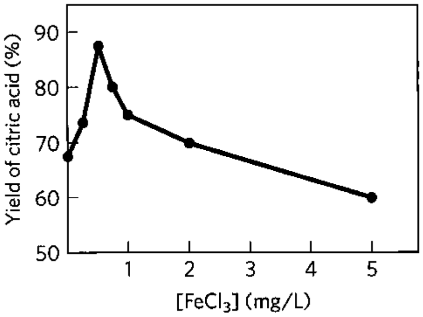
23. Net Synthesis of α-Ketoglutarate α-Ketoglutarate plays a central role in the biosynthesis of several amino acids. Write a sequence of enzymatic reactions that could result in the net synthesis of α-ketoglutarate from pyruvate. Your proposed sequence must not involve the net consumption of other citric acid cycle intermediates. Write an equation for the overall reaction.

24. Amphibolic Pathways Explain, giving examples, what is meant by the statement that the citric acid cycle is amphibolic.

25. Regulation of the Pyruvate Dehydrogenase Complex In animal tissues, the ratio of active, unphosphorylated to inactive, phosphorylated PDH complex regulates the rate of conversion of pyruvate to acetyl-CoA. Determine what happens to the rate of this reaction when a preparation of rabbit muscle mitochondria containing the PDH complex is treated with **(a)** pyruvate dehydrogenase kinase, ATP, and NADH; **(b)** pyruvate dehydrogenase phosphatase and Ca^{2+}; **(c)** malonate.

26. Commercial Synthesis of Citric Acid Manufacturers use citric acid as a flavoring agent in soft drinks, fruit juices, and many other foods. Worldwide, the market for citric acid is valued at hundreds of millions of dollars per year. Commercial production uses the mold *Aspergillus niger*, which metabolizes sucrose under carefully controlled conditions.

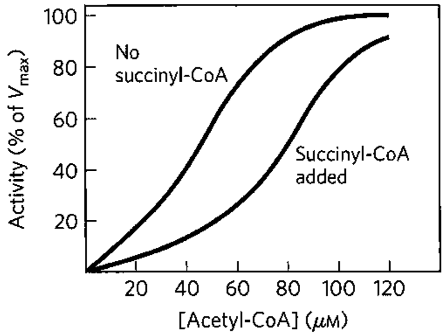
(a) The yield of citric acid strongly depends on the concentration of $FeCl_3$ in the culture medium, as indicated in the graph. Why does the yield decrease when the concentration of Fe^{3+} is above or below the optimal value of 0.5 mg/L?



(b) Write the sequence of reactions by which *A. niger* synthesizes citric acid from sucrose. Write an equation for the overall reaction.

(c) Does the commercial process require the culture medium to be aerated—that is, is this a fermentation or an aerobic process? Explain.

27. Regulation of Citrate Synthase In the presence of saturating amounts of oxaloacetate, the activity of citrate synthase from pig heart tissue shows a sigmoid dependence on the concentration of acetyl-CoA, as shown in the graph. Adding succinyl-CoA shifts the curve to the right and makes the sigmoid dependence more pronounced.



On the basis of these observations, suggest how succinyl-CoA regulates the activity of citrate synthase. (Hint: See Fig. 6-37.) Why is succinyl-CoA an appropriate signal for regulation of the citric acid cycle? How does the regulation of citrate synthase control the rate of cellular respiration in pig heart tissue?

28. Regulation of Pyruvate Carboxylase The carboxylation of pyruvate by pyruvate carboxylase occurs at a very low rate unless acetyl-CoA, a positive allosteric modulator, is present. If you have just eaten a meal rich in fatty acids (triacylglycerols) but low in carbohydrates (glucose), how does this regulatory property shut down the oxidation of glucose to CO_2 and H_2O but increase the oxidation of acetyl-CoA derived from fatty acids?

29. Relationship between Respiration and the Citric Acid Cycle Although oxygen does not participate directly in the citric acid cycle, the cycle operates only when O_2 is present. Why?

30. Effect of [NADH]/[NAD$^+$] on the Citric Acid Cycle How would you expect the operation of the citric acid cycle to respond to a rapid increase in the [NADH]/[NAD$^+$] ratio in the mitochondrial matrix? Why?

31. Thermodynamics of Citrate Synthase Reaction in Cells Citrate is formed by the condensation of acetyl-CoA with oxaloacetate, catalyzed by citrate synthase:

$$\text{Oxaloacetate} + \text{acetyl-CoA} + H_2O \rightleftharpoons \text{citrate} + \text{CoA} + H^+$$

In rat heart mitochondria at pH 7.0 and 25 °C, the concentrations of reactants and products are oxaloacetate, 1 μM; acetyl-CoA, 1 μM; citrate, 220 μM; and CoA, 65 μM. The standard free-energy change for the citrate synthase reaction is –32.2 kJ/mol. What is the direction of metabolite flow through the citrate synthase reaction in rat heart cells? Explain.

32. Reactions of the Pyruvate Dehydrogenase Complex Two of the steps in the oxidative decarboxylation of pyruvate (steps ④ and ⑤ in Fig. 16-6) do not involve any of the three carbons of pyruvate, yet are essential to the operation of the PDH complex. Explain.

33. Pyruvate Transport into Mitochondria The mitochondrial pyruvate carrier (MPC) is a heterodimer of the proteins MPC1 and MPC2. In a high proportion (80%) of certain cancers, including gliomas (tumors of the glial cells of the brain), the gene for one of these proteins is mutated such that pyruvate cannot enter the mitochondrial matrix. Name three metabolic effects that you would expect to see if cytosolic pyruvate could not gain access to the machinery of the citric acid cycle. (Hint: Box 14-1 may be helpful.)

34. Citric Acid Cycle Mutants There are many cases of human disease in which one or another enzyme activity is lacking due to genetic mutation. Why are cases in which individuals lack one of the enzymes of the citric acid cycle extremely rare?

DATA ANALYSIS PROBLEM

35. How the Citric Acid Cycle Was Discovered The detailed biochemistry of the citric acid cycle was determined by several researchers over a period of decades. In a 1937 article, Krebs and Johnson summarized their work and the work of others in the first published description of this pathway.

The methods used by these researchers were very different from those of modern biochemistry. Radioactive tracers were not commonly available until the 1940s, so Krebs and other researchers had to use nontracer techniques to work out the pathway. Using freshly prepared samples of pigeon breast muscle, they determined oxygen consumption by suspending minced muscle in buffer in a sealed flask and measuring the volume (in μL) of oxygen consumed under different conditions. They measured levels of substrates (intermediates) by treating samples with acid to remove contaminating proteins, then assaying the quantities of various small organic molecules. The two key observations that led Krebs and colleagues to propose a citric acid *cycle* as opposed to a *linear pathway* (like that of glycolysis) were made in the following experiments.

Experiment I: They incubated 460 mg of minced muscle in 3 mL of buffer at 40 °C for 150 minutes. Addition of *citrate* increased O_2 consumption by 893 μL compared with samples without added citrate. They calculated, based on the O_2 consumed during respiration of other carbon-containing compounds, that the expected O_2 consumption for complete respiration of this quantity of citrate was only 302 μL.

Experiment II: They measured O_2 consumption by 460 mg of minced muscle in 3 mL of buffer when incubated with *citrate* and/or with *1-phosphoglycerol* (glycerol 1-phosphate; this was known to be readily oxidized by cellular respiration) at 40 °C for 140 minutes. The results are shown in the table.

Sample	Substrate(s) added	μL O_2 absorbed
1	No extra	342
2	0.3 mL 0.2 M 1-phosphoglycerol	757
3	0.15 mL 0.02 M citrate	431
4	0.3 mL 0.2 M 1-phosphoglycerol and 0.15 mL 0.02 M citrate	1,385

(a) Why is O_2 consumption a good measure of cellular respiration?
(b) Why does sample 1 (unsupplemented muscle tissue) consume some oxygen?
(c) Based on the results for samples 2 and 3, can you conclude that 1-phosphoglycerol and citrate serve as substrates for cellular respiration in this system? Explain your reasoning.
(d) Krebs and colleagues used the results from these experiments to argue that citrate was "catalytic"—that it helped the muscle tissue samples metabolize 1-phosphoglycerol more completely. How would you use their data to make this argument?
(e) Krebs and colleagues further argued that citrate was not simply consumed by these reactions, but had to be *regenerated*. Therefore, the reactions had to be a *cycle* rather than a linear pathway. How would you make this argument?

Other researchers had found that *arsenate* (AsO_4^{3-}) inhibits α-ketoglutarate dehydrogenase and that *malonate* inhibits succinate dehydrogenase.

(f) Krebs and coworkers found that muscle tissue samples treated with arsenate and citrate would consume citrate only in the presence of oxygen; under these conditions, oxygen was consumed. Based on the pathway in Figure 16-7, what was the citrate converted to in this experiment, and why did the samples consume oxygen?

In their article, Krebs and Johnson further reported the following: (1) In the presence of arsenate, 5.48 mmol of citrate was converted to 5.07 mmol of α-ketoglutarate. (2) In the presence of malonate, citrate was quantitatively converted to large amounts of succinate and small amounts of α-ketoglutarate. (3) Addition of oxaloacetate in the absence of oxygen led to production of a large amount of citrate; the amount was increased if glucose was also added.

Other workers had found the following pathway in similar muscle tissue preparations:

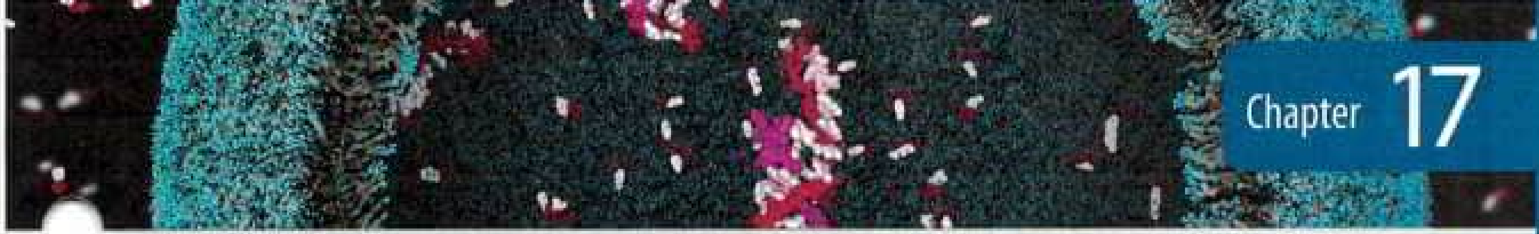
$$\text{Succinate} \longrightarrow \text{fumarate} \longrightarrow \text{malate} \longrightarrow \text{oxaloacetate} \longrightarrow \text{pyruvate}$$

(g) Based only on the data presented in this problem, what is the order of the intermediates in the citric acid cycle? How does this compare with Figure 16-7? Explain your reasoning.
(h) Why was it important to show the *quantitative* conversion of citrate to α-ketoglutarate?

The Krebs and Johnson article also contains other data that filled in most of the missing components of the cycle. The only component left unresolved was the molecule that reacted with oxaloacetate to form citrate.

Reference

Krebs, H.A., and W.A. Johnson. 1937. The role of citric acid in intermediate metabolism in animal tissues. *Enzymologia* 4:148–156. Reprinted in *FEBS Lett.* 117(Suppl.):K2–K10, 1980.



Chapter 17

FATTY ACID CATABOLISM

17.1 Digestion, Mobilization, and Transport of Fats *602*

17.2 Oxidation of Fatty Acids *606*

17.3 Ketone Bodies *619*

The oxidation of long-chain fatty acids to acetyl-CoA is a central energy-yielding pathway in many organisms and tissues. In mammalian heart and liver, for example, it provides as much as 80% of the energetic needs under all physiological circumstances. The electrons removed from fatty acids during oxidation pass through the respiratory chain, driving ATP synthesis; the acetyl-CoA produced from the fatty acids may be completely oxidized to CO_2 in the citric acid cycle, resulting in further energy conservation.

In Chapter 10 we described the properties of triacylglycerols (also called triglycerides or neutral fats) that make them especially suitable as storage fuels. The long alkyl chains of their constituent fatty acids are essentially hydrocarbons, highly reduced structures with an energy of complete oxidation (~38 kJ/g) more than twice that for the same weight of carbohydrate or protein. This advantage is compounded by the extreme insolubility of lipids in water; cellular triacylglycerols aggregate in lipid droplets, which do not raise the osmolarity of the cytosol, and they are unsolvated. (In storage polysaccharides, by contrast, water of solvation can account for two-thirds of the overall weight of the stored molecules.) And because of their relative chemical inertness, triacylglycerols can be stored in large quantity in cells without the risk of undesired chemical reactions with other cellular constituents.

The properties that make triacylglycerols good storage compounds, however, present problems in their role as fuels. Because they are insoluble in water, ingested triacylglycerols must be emulsified before they can be digested by water-soluble enzymes in the intestine, and triacylglycerols absorbed in the intestine or mobilized from storage tissues must be carried in the blood bound to proteins that counteract their insolubility. Also, to overcome the relative stability of the C—C bonds in a fatty acid, the carboxyl group at C-1 is activated by attachment to coenzyme A, which allows stepwise oxidation of the fatty acyl group at the C-3, or β, position—hence the name **β oxidation**.

The principles we emphasize in this chapter are not new. They apply to the catabolic pathways of carbohydrates that we just studied.

P1 **Metabolites of diverse origin funnel into a few central pathways.** Fatty acid catabolism and glycolysis convert quite different starting materials into the same product (acetyl-CoA). The electrons from the oxidative reactions of these pathways and of the citric acid cycle are carried by common cofactors (NAD and FAD) to the mitochondrial respiratory chain leading to oxygen, providing the energy for ATP synthesis by oxidative phosphorylation.

P2 **Evolution selects for chemical mechanisms that make useful reactions more energetically favorable, and those same mechanisms are used in different pathways.** In the breakdown of fatty acids we see the activation of a carboxylic acid by its conversion to a thioester, as we saw with acetyl-CoA in the citric acid cycle. To break the C—C bonds in the long chain of relatively inert —CH_2—CH_2— groups in fatty acids, a carbonyl group is created adjacent to the —CH_2— group, as we saw in the reactions of the citric acid cycle.

P3 **Allosteric mechanisms and posttranslational regulation (protein phosphorylation) coordinate metabolic processes within a cell. Hormones and growth factors coordinate metabolic activities among tissues and organs.** Reciprocal regulation of catabolic and anabolic pathways prevents the inefficiency of futile cycling.

P4 **When a process lacks a critical component—an enzyme, a cofactor, or a regulatory agent—the resulting loss of homeostasis may cause disease across a spectrum of severity.** Defects in fatty acid breakdown are no exception.

P5 **The liver plays a unique role in whole-body metabolism.** When glucose is unavailable, the liver makes glucose by gluconeogenesis and releases it to the blood for distribution to other tissues, including the brain. During starvation, the liver processes fatty acids into ketone bodies, which, unlike fatty acids, can cross the blood brain barrier and fuel the brain.

17.1 Digestion, Mobilization, and Transport of Fats

Cells can obtain fatty acid fuels from four sources: fats consumed in the diet, fats stored in cells as lipid droplets, fats synthesized in one organ for export to another, and fats obtained by autophagy (which degrades the cell's own organelles). Some species use all four sources under various circumstances; others use one or two. Vertebrates, for example, obtain fats in the diet; mobilize fats stored in specialized tissue (adipose tissue, consisting of cells called adipocytes); and, in the liver, convert excess dietary carbohydrates to fats for export to other tissues. During starvation, they can recycle lipids by autophagy. On average, 40% or more of the daily energy requirement of humans in highly industrialized countries is supplied by dietary triacylglycerols. Triacylglycerols provide more than half the energy requirements of some organs, particularly the liver, heart, and resting skeletal muscle. Stored triacylglycerols are virtually the sole source of energy in hibernating animals and migrating birds. Vascular plants mobilize fats stored in seeds during germination, but do not otherwise depend on fats for energy.

Dietary Fats Are Absorbed in the Small Intestine

In vertebrates, before ingested triacylglycerols can be absorbed through the intestinal wall they must be converted from insoluble macroscopic fat particles to finely dispersed microscopic micelles. This solubilization is carried out by bile salts, such as taurocholic acid (p. 352), which are synthesized from cholesterol in the liver, stored in the gallbladder, and released into the small intestine after ingestion of a fatty meal. Bile salts are amphipathic compounds that act as biological detergents, emulsifying dietary fats into mixed micelles of bile salts and triacylglycerols (**Fig. 17-1**, step ①). Micelle formation enormously increases the fraction of lipid molecules accessible to the action of water-soluble lipases in the intestine, and lipase action converts triacylglycerols to monoacylglycerols (monoglycerides) and diacylglycerols (diglycerides) and free fatty acids (step ②). These products of lipase action diffuse or are transported into the epithelial cells lining the intestinal surface (the intestinal mucosa) (step ③), where they are reconverted to triacylglycerols and packaged with dietary cholesterol and specific **apolipoproteins** (from the Greek *apo*, meaning "detached" or "separate," designating the protein in its lipid-free form) into lipoprotein aggregates

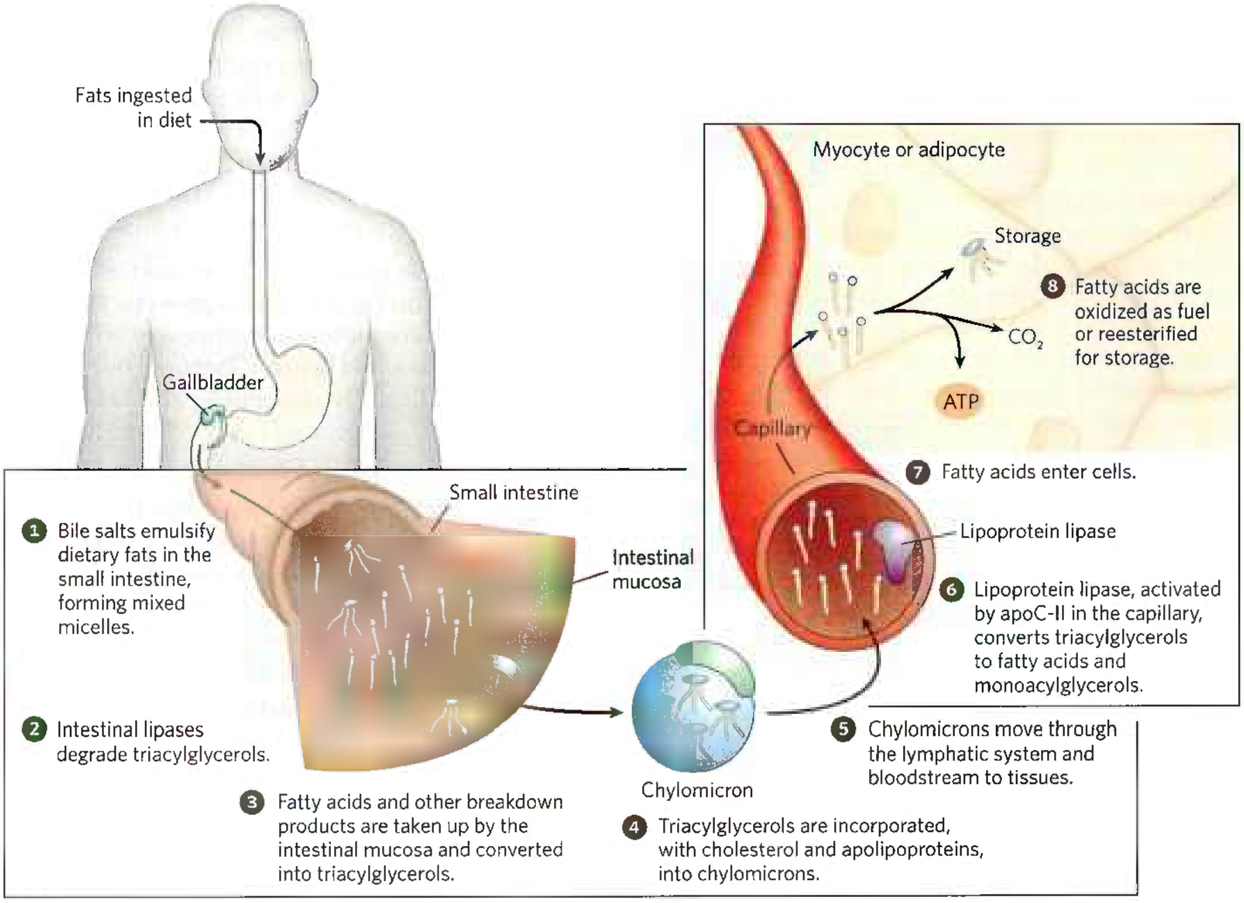

Fats ingested in diet
Gallbladder
Small intestine
Intestinal mucosa
Myocyte or adipocyte
Storage
⑧ Fatty acids are oxidized as fuel or reesterified for storage.
CO_2
ATP
Capillary
⑦ Fatty acids enter cells.
Lipoprotein lipase
⑥ Lipoprotein lipase, activated by apoC-II in the capillary, converts triacylglycerols to fatty acids and monoacylglycerols.
① Bile salts emulsify dietary fats in the small intestine, forming mixed micelles.
② Intestinal lipases degrade triacylglycerols.
③ Fatty acids and other breakdown products are taken up by the intestinal mucosa and converted into triacylglycerols.
Chylomicron
④ Triacylglycerols are incorporated, with cholesterol and apolipoproteins, into chylomicrons.
⑤ Chylomicrons move through the lymphatic system and bloodstream to tissues.

FIGURE 17-1 Processing of dietary lipids in vertebrates. Digestion and absorption of dietary lipids occur in the small intestine, and the fatty acids released from triacylglycerols are packaged and delivered to muscle and adipose tissues. The eight steps are discussed in the text.

called **chylomicrons** (step ❹). It is the protein moieties that target triacylglycerols, phospholipids, cholesterol, and cholesteryl esters for transport between organs.

Apolipoproteins combine with lipids to form several classes of **lipoprotein particles**, spherical aggregates with hydrophobic lipids at the core and hydrophilic protein side chains and lipid head groups at the surface. Various combinations of lipid and protein produce particles of different densities, ranging from chylomicrons and very-low-density lipoproteins (VLDL) to very-high-density lipoproteins (VHDL). These particles can be separated by ultracentrifugation. The structures of these lipoprotein particles and their roles in lipid transport are detailed in Chapter 21 (Fig. 21-39).

Apolipoprotein B-48 (apoB-48) is the primary protein component of chylomicrons. In lipid uptake from the intestine, chylomicrons move from the intestinal mucosa into the lymphatic system, and then enter the blood, where they can exchange apolipoproteins with other types of circulating lipoprotein. In the blood, chylomicrons pick up **apolipoprotein C-II (apoC-II)** from **high-density lipoprotein (HDL)** particles and are carried to muscle and adipose tissue (Fig. 17-1, step ❺). In the capillaries of these tissues, the extracellular enzyme **lipoprotein lipase**, activated by apoC-II, hydrolyzes triacylglycerols to free fatty acids and monoacylglycerols (step ❻), which are taken up by specific transporters in the plasma membranes of cells in the target tissues (step ❼). In muscle, the fatty acids are oxidized for energy; in adipose tissue, they are reesterified for storage as triacylglycerols (step ❽).

The remnants of chylomicrons, depleted of most of their triacylglycerols but still containing cholesterol and apolipoproteins, travel in the blood to the liver, where they are taken up by endocytosis mediated by receptors for their apolipoproteins. Triacylglycerols that enter the liver by this route may be oxidized to provide energy or to provide precursors for the synthesis of ketone bodies, as described in Section 17.3.

When the diet contains more fatty acids than are needed immediately for fuel or as precursors, the liver converts them to triacylglycerols, which are packaged with specific apolipoproteins into VLDLs. These VLDLs are secreted by hepatocytes and transported in the blood to adipose tissue, where the triacylglycerols are removed and stored in lipid droplets within adipocytes.

Hormones Trigger Mobilization of Stored Triacylglycerols

Neutral lipids (triacylglycerols, sterols, and steroyl esters) are stored in adipocytes (and in steroid-synthesizing cells of the adrenal cortex, ovary, and testis) in **lipid droplets**, which have a core of triacylglycerols and sterol esters surrounded by a monolayer of phospholipids. The surface of these droplets is coated with **perilipins**, a family of proteins that restrict access to lipid droplets, preventing untimely lipid mobilization. **P1** When hormones signal the need for metabolic energy, triacylglycerols stored in adipose tissue are mobilized (brought out of storage) and transported to tissues (skeletal muscle, heart, and renal cortex) in which fatty acids can be oxidized for energy production. The hormones epinephrine and glucagon, secreted in response to low blood glucose levels or a fight-or-flight situation, stimulate the enzyme adenylyl cyclase in the adipocyte plasma membrane (**Fig. 17-2**), which produces the intracellular second messenger cyclic AMP (cAMP; see Fig. 12-4). Cyclic AMP–dependent protein kinase (PKA) triggers changes that open the lipid droplet to the action of three cytosolic lipases, which act on tri-, di-, and monoacylglycerols, releasing fatty acids and glycerol.

The fatty acids thus released (**free fatty acids, FFAs**) pass from the adipocyte into the blood, where they bind to the blood protein **serum albumin** (**Fig. 17-3**). This protein (M_r 66,000), which makes up about half of the total serum protein, noncovalently binds as many as seven fatty acids. Bound to this soluble protein, the otherwise insoluble fatty acids are carried to tissues such as skeletal muscle, heart, and renal cortex. In these target tissues, fatty acids dissociate from albumin and are moved by plasma membrane transporters into cells to serve as fuel.

About 95% of the biologically available energy of triacylglycerols resides in their three long-chain fatty acids; only 5% is contributed by the glycerol moiety. The glycerol released by lipase action is phosphorylated by **glycerol kinase** (**Fig. 17-4**), and the resulting glycerol 3-phosphate is oxidized to dihydroxyacetone phosphate. The glycolytic enzyme triose phosphate isomerase converts this compound to glyceraldehyde 3-phosphate, which is oxidized via glycolysis.

Fatty Acids Are Activated and Transported into Mitochondria

The enzymes of fatty acid oxidation in animal cells are located in the mitochondrial matrix, as demonstrated in 1948 by Eugene P. Kennedy and Albert Lehninger. Short- and medium-chain fatty acids, those with chain lengths of 12 or fewer carbons, enter mitochondria without the help of membrane transporters. Long-chain fatty acids, those with 14 or more carbons, which constitute the majority of the FFAs obtained in the diet or released from adipose tissue, cannot pass directly through the mitochondrial membranes: they must be transported through the **carnitine shuttle**. First, the fatty acid must be activated by a **fatty acyl–CoA synthetase** isozyme specific for long-chain fatty acids. The isozymes are present in the outer mitochondrial membrane, where they promote the general reaction

$$\text{Fatty acid} + \text{CoA} + \text{ATP} \rightleftharpoons \text{fatty acyl–CoA} + \text{AMP} + \text{PP}_i$$

P2 Fatty acyl–CoA synthetases catalyze the formation of a thioester linkage between the fatty acid carboxyl group and the thiol group of coenzyme A to yield a

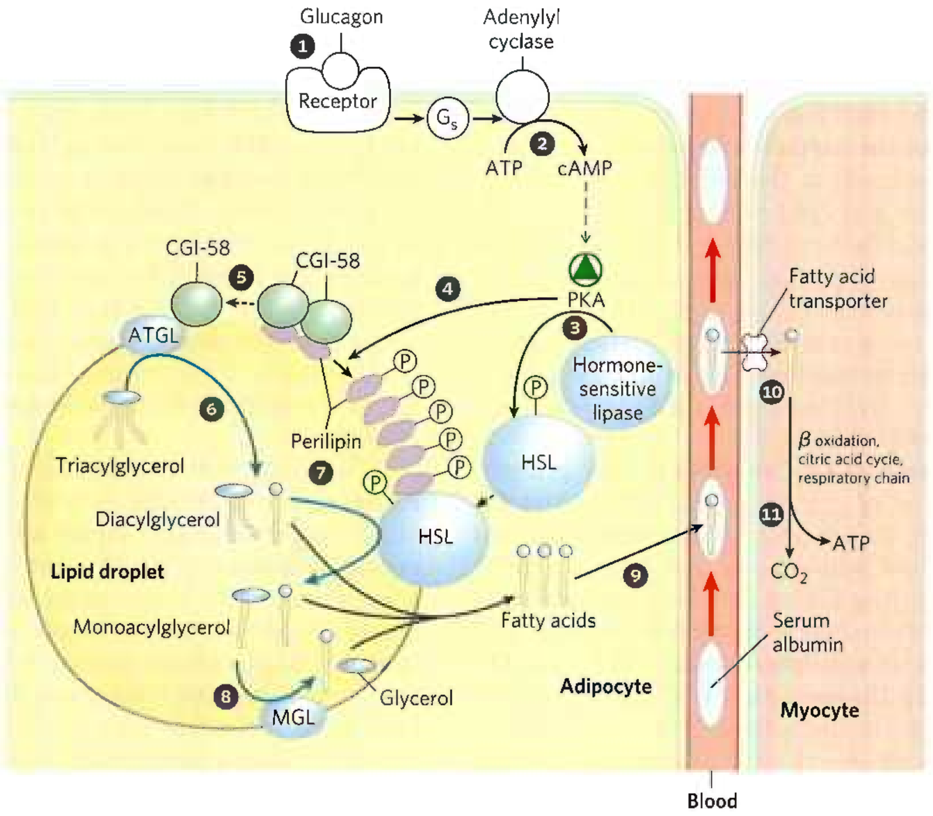

Glucagon
①
Receptor
Adenylyl cyclase
G_s
②
ATP
cAMP
PKA
③
Hormone-sensitive lipase
Ⓟ
HSL
④
CGI-58
CGI-58
⑤
ATGL
⑥
Triacylglycerol
Perilipin
⑦
Diacylglycerol
HSL
Lipid droplet
Monoacylglycerol
⑧
MGL
Glycerol
Fatty acids
⑨
Adipocyte
Fatty acid transporter
⑩
β oxidation, citric acid cycle, respiratory chain
⑪
ATP
CO_2
Serum albumin
Myocyte
Blood

FIGURE 17-2 Mobilization of triacylglycerols stored in adipose tissue. When low levels of glucose in the blood trigger the release of glucagon, ① the hormone binds its receptor in the adipocyte membrane and thus ② stimulates adenylyl cyclase, via a G protein, to produce cAMP. This activates PKA, which phosphorylates ③ the hormone-sensitive lipase (HSL) and ④ perilipin molecules on the surface of the lipid droplet. Phosphorylation of perilipin causes ⑤ dissociation of the protein CGI-58 from perilipin. CGI-58 (*c*omparative *g*ene *i*dentification-58), a protein closely associated with lipid droplets, then recruits adipose triacylglycerol lipase (ATGL) to the droplet surface and stimulates its lipase activity. Active ATGL ⑥ converts triacylglycerols to diacylglycerols. The phosphorylated perilipin associates with phosphorylated HSL, allowing it access to the surface of the lipid droplet, where ⑦ it converts diacylglycerols to monoacylglycerols. ⑧ A third lipase, monoacylglycerol lipase (MGL), hydrolyzes monoacylglycerols. ⑨ Fatty acids leave the adipocyte, and are transported in the blood bound to serum albumin. They are released from the albumin and ⑩ enter a myocyte via a specific fatty acid transporter. ⑪ In the myocyte, fatty acids are oxidized to CO_2, and the energy of oxidation is conserved in ATP, which fuels muscle contraction and other energy-requiring metabolism in the myocyte.

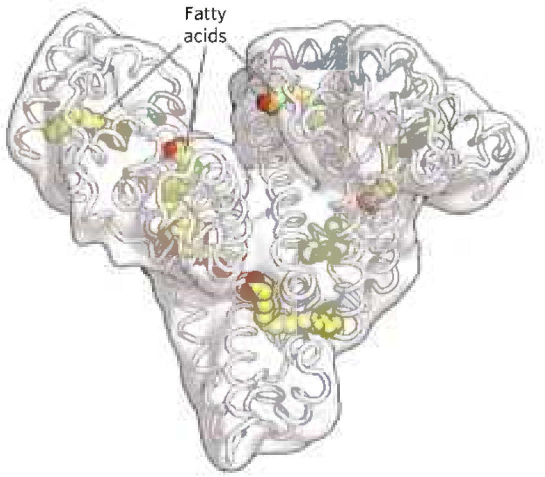

Fatty acids

FIGURE 17-3 Human serum albumin complexed with stearate. Each day the human liver releases 10–15 g of serum albumin into the bloodstream, where this workhorse protein transports many ligands, drugs, and particularly fatty acids through the blood. The structure has nooks and crannies that can carry up to seven fatty acids. Its passengers are both hydrophobic and hydrophilic, and include steroid hormones, the blood thinner warfarin, the antibiotic penicillin, the anti-inflammatory drug ibuprofen, and the anxiolytic diazepam.

fatty acyl–CoA, coupled to the cleavage of ATP to AMP and PP_i. (Recall the description of this reaction in Chapter 13, which illustrates how the free energy released by cleavage of phosphoanhydride bonds in ATP could be coupled to the formation of a high-energy compound; p. 485.) The reaction catalyzed by a fatty acyl–CoA synthetase occurs in two steps and involves a fatty acyl–adenylate intermediate (**Fig. 17-5**).

Fatty acyl–CoAs, like acetyl-CoA, are high-energy compounds; their hydrolysis to FFAs and CoA has a large, negative standard free-energy change. The formation of a fatty acyl–CoA is made more favorable by the hydrolysis of *two* high-energy bonds in ATP; the pyrophosphate formed in the activation reaction is immediately hydrolyzed by inorganic pyrophosphatase (left side of Fig. 17-5), which pulls the preceding activation reaction in the direction of fatty acyl–CoA formation. The overall reaction (three steps) is

$$\text{Fatty acid} + \text{CoA} + \text{ATP} \rightleftharpoons \text{fatty acyl–CoA} + \text{AMP} + 2\text{P}_i \quad (17\text{-}1)$$

$$\Delta G'^{\circ} = -34 \text{ kJ/mol}$$

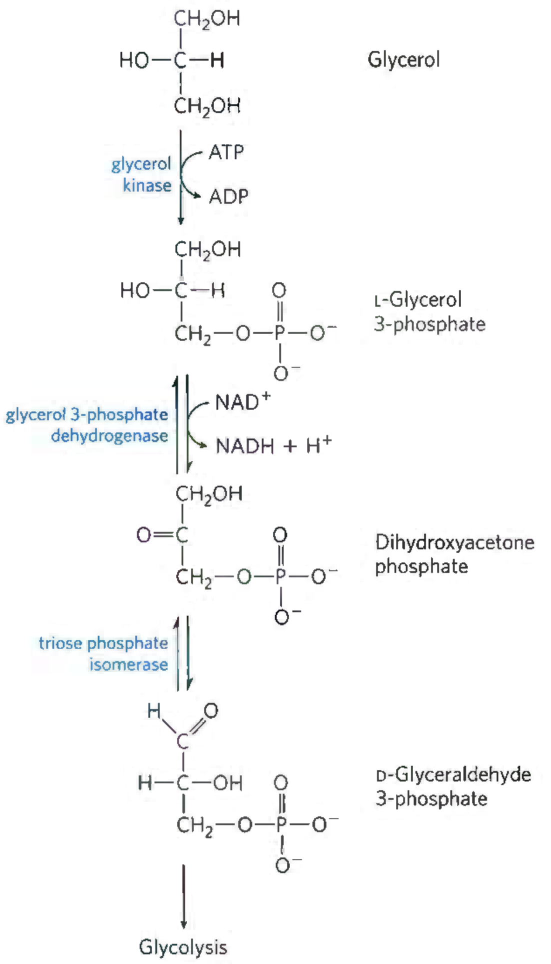
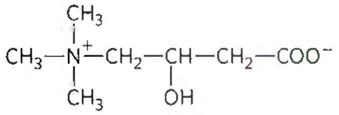

Glycerol
glycerol kinase
ATP
ADP
L-Glycerol 3-phosphate
glycerol 3-phosphate dehydrogenase
NAD+
NADH + H+
Dihydroxyacetone phosphate
triose phosphate isomerase
D-Glyceraldehyde 3-phosphate
Glycolysis

FIGURE 17-4 **Entry of glycerol into the glycolytic pathway.**

Fatty acyl–CoA esters formed on the cytosolic side of the outer mitochondrial membrane can be transported into the mitochondrion and oxidized to produce ATP, or they can be used in the cytosol to synthesize membrane lipids.

Fatty acyl–CoAs destined for mitochondrial oxidation must be attached to **carnitine** to be shuttled across the inner mitochondrial membrane.

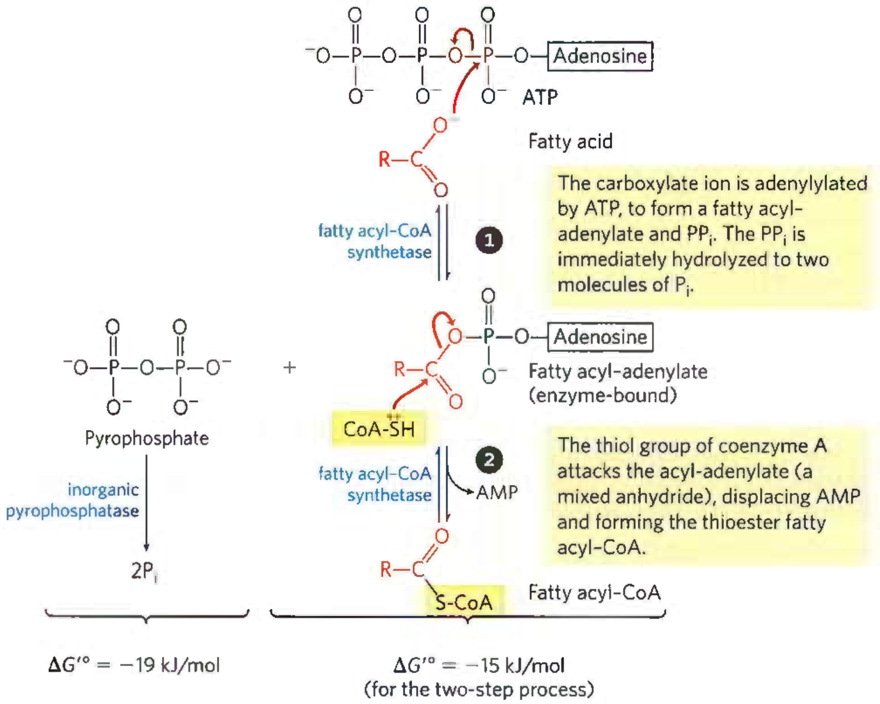


$CH_3-\overset{CH_3}{\underset{CH_3}{\overset{+}{N}}}-CH_2-\underset{OH}{CH}-CH_2-COO^-$

Carnitine

In a transesterification catalyzed by **carnitine acyltransferase 1, CAT1** (also called **carnitine palmitoyltransferase 1, CPT1**) in the outer mitochondrial membrane, the fatty acyl–CoA is transiently attached to the hydroxyl group of carnitine to form fatty acyl–carnitine (**Fig. 17-6**). The fatty acyl–carnitine ester then diffuses across the intermembrane space and enters the matrix by passive transport through the **acyl-carnitine/carnitine cotransporter** of the inner mitochondrial membrane. This cotransporter moves one molecule of carnitine from the matrix to the intermembrane space as one molecule of fatty acyl–carnitine moves into the matrix. Once inside the matrix, the fatty acyl group is transferred from carnitine back to coenzyme A from the intramitochondrial pool by **carnitine acyltransferase 2 (CAT2**, or **CPT2)**. This isozyme, located on the inner face of the inner mitochondrial membrane, regenerates fatty acyl–CoA and releases it, along with free carnitine, into the matrix. The carnitine is then available to be transferred back through

MECHANISM FIGURE 17-5 **Activation of a fatty acid by conversion to a fatty acyl-CoA.** Formation of the fatty acyl-CoA derivative occurs in two steps, catalyzed by fatty acyl-CoA synthetase. Hydrolysis of the pyrophosphate created in the first step of that reaction is catalyzed by inorganic pyrophosphatase. The overall reaction is highly exergonic.


Adenosine
ATP
Fatty acid
fatty acyl-CoA synthetase
1
Adenosine
Fatty acyl-adenylate (enzyme-bound)
Pyrophosphate
+
CoA-SH
2
fatty acyl-CoA synthetase
AMP
inorganic pyrophosphatase
2Pi
S-CoA
Fatty acyl-CoA

The carboxylate ion is adenylylated by ATP, to form a fatty acyl-adenylate and PP_i. The PP_i is immediately hydrolyzed to two molecules of P_i.

The thiol group of coenzyme A attacks the acyl-adenylate (a mixed anhydride), displacing AMP and forming the thioester fatty acyl-CoA.

$\Delta G'^\circ = -19$ kJ/mol

$\Delta G'^\circ = -15$ kJ/mol (for the two-step process)

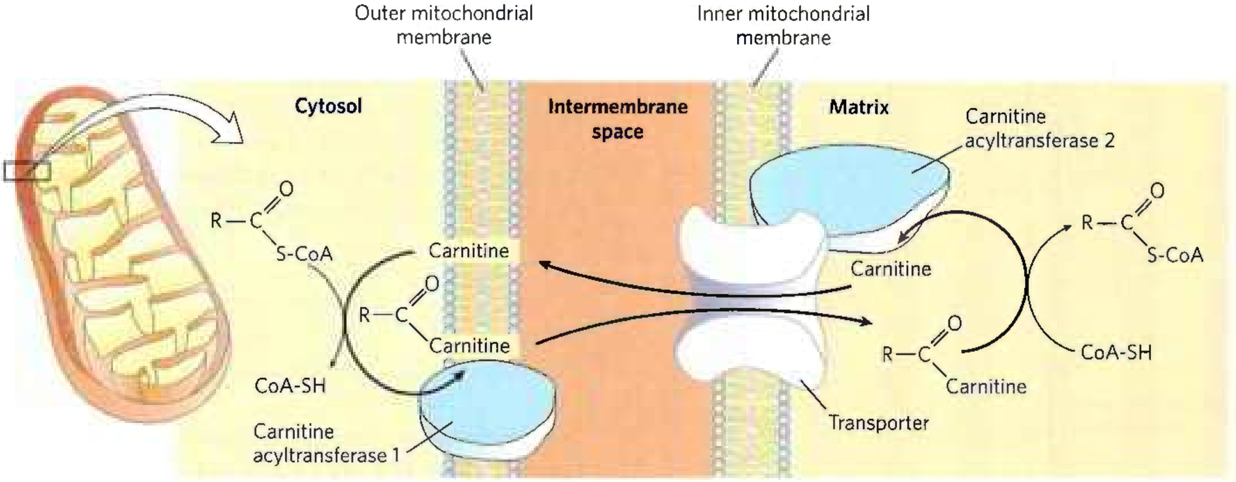

Outer mitochondrial membrane
Inner mitochondrial membrane
Cytosol
Intermembrane space
Matrix
Carnitine acyltransferase 2
R—C(=O)—S-CoA
Carnitine
R—C(=O)—Carnitine
CoA-SH
Carnitine acyltransferase 1
Transporter

FIGURE 17-6 Fatty acid entry into mitochondria via the acyl-carnitine/carnitine transporter. Fatty acyl–carnitine formed on the outer mitochondrial membrane moves into the matrix by passive cotransport through the inner membrane. In the matrix, the acyl group is transferred to mitochondrial coenzyme A, freeing carnitine to leave the matrix through the same transporter.

the acyl-carnitine/carnitine cotransporter to be used to shuttle the next fatty acid across. Once inside the mitochondrion, the fatty acyl–CoA is acted upon by a set of enzymes in the matrix.

This process for transferring fatty acids into the mitochondrion—esterification to CoA, transesterification to carnitine, translocation across the inner membrane, and transesterification back to CoA—links two separate pools of coenzyme A and of fatty acyl–CoA, one in the cytosol, the other in mitochondria. These pools have different functions. Coenzyme A in the mitochondrial matrix is largely used in oxidative degradation of pyruvate, fatty acids, and some amino acids, whereas cytosolic coenzyme A is used in the biosynthesis of fatty acids (see Fig. 21-10). Fatty acyl–CoA in the cytosolic pool can be used there for membrane lipid synthesis or can be moved into the mitochondrial matrix for oxidation and ATP production. Conversion to the carnitine ester commits the fatty acyl moiety to the oxidative fate. **P3** The carnitine-mediated entry process is the rate-limiting step for oxidation of fatty acids in mitochondria and, as discussed later, is a control point. Carnitine acyltransferase 1 is inhibited by malonyl-CoA, the first intermediate in fatty acid synthesis (see Fig. 21-1). This inhibition prevents the simultaneous synthesis and degradation of fatty acids, a wasteful futile cycle.

SUMMARY 17.1 *Digestion, Mobilization, and Transport of Fats*

- Dietary triacylglycerols are emulsified in the small intestine by bile salts, hydrolyzed by intestinal lipases, absorbed by intestinal epithelial cells, and reconverted into triacylglycerols. They are combined with specific apolipoproteins for passage through the blood and lymph to adipose tissue, where they are stored as lipid droplets.
- Triacylglycerols stored in adipose tissue are mobilized by a hormone-sensitive triacylglycerol lipase. The released fatty acids bind to serum albumin and are carried in the blood to the heart, skeletal muscle, and other tissues that use fatty acids for fuel.
- Once inside cells, fatty acids are activated at the outer mitochondrial membrane by conversion to fatty acyl–CoA thioesters. Fatty acyl–CoA that is to be oxidized enters mitochondria via the carnitine shuttle, which is a major control point. Malonyl-CoA, the first intermediate in fatty acid synthesis, inhibits carnitine acyltransferase 1, assuring that fatty acid oxidation and fatty acid synthesis do not occur simultaneously.

17.2 Oxidation of Fatty Acids

As noted earlier, mitochondrial oxidation of fatty acids takes place in three stages (**Fig. 17-7**). In the first stage—*β* oxidation—fatty acids undergo oxidative removal of successive two-carbon units in the form of acetyl-CoA, starting from the carboxyl end of the fatty acyl chain. For example, the 16-carbon palmitic acid (palmitate at pH 7) undergoes seven passes through the oxidative sequence, in each pass losing two carbons as acetyl-CoA. At the end of seven cycles, the last two carbons of palmitate (originally C-15 and C-16) remain as acetyl-CoA. The overall result is the conversion of the 16-carbon chain of palmitate to eight two-carbon acetyl groups of acetyl-CoA molecules. Formation of each acetyl-CoA requires removal of four hydrogen atoms (two pairs of electrons and four H^+) from the fatty acyl moiety by dehydrogenases.

In the second stage of fatty acid oxidation, the acetyl groups of acetyl-CoA are oxidized to CO_2 in the citric acid cycle, which also takes place in the mitochondrial matrix. Acetyl-CoA derived from fatty

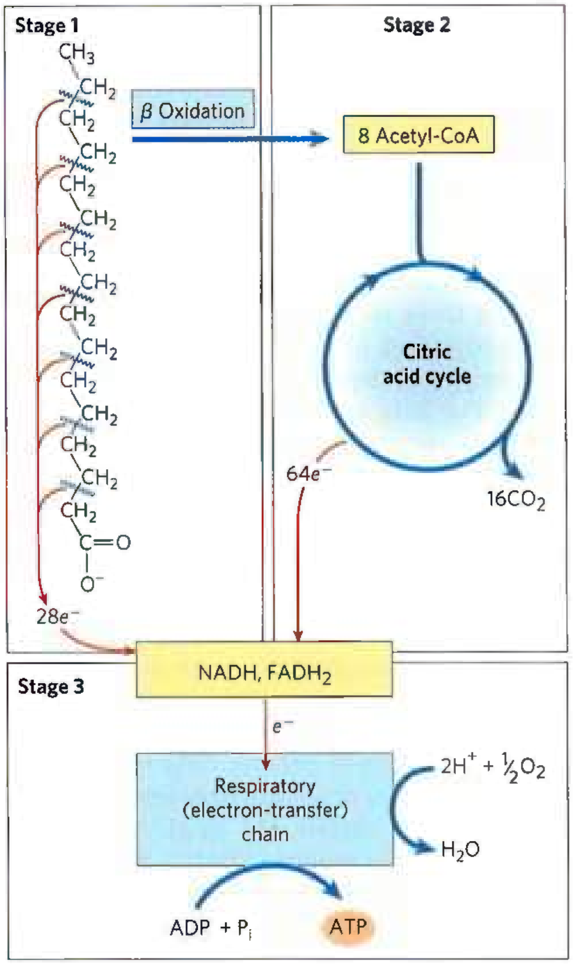


FIGURE 17-7 Stages of fatty acid oxidation. Stage 1: A long-chain fatty acid is oxidized to yield acetyl residues in the form of acetyl-CoA. This process is called β oxidation. Stage 2: The acetyl groups are oxidized to CO_2 via the citric acid cycle. Stage 3: Electrons derived from the oxidations of stages 1 and 2 pass to O_2 via the mitochondrial respiratory chain, providing the energy for ATP synthesis by oxidative phosphorylation.

acids thus enters a final common pathway of oxidation with the acetyl-CoA derived from glucose via glycolysis and pyruvate oxidation (see Fig. 16-1). The first two stages of fatty acid oxidation produce the reduced electron carriers NADH and $FADH_2$, which in the third stage donate electrons to the mitochondrial respiratory chain, through which the electrons pass to oxygen with the concomitant phosphorylation of ADP to ATP (Fig. 17-7). The energy released by fatty acid oxidation is thus conserved as ATP.

We now take a closer look at the first stage of fatty acid oxidation, beginning with the simple case of a saturated fatty acyl chain with an even number of carbons, then turning to the slightly more complicated cases of unsaturated and odd-number chains. We also consider the regulation of fatty acid oxidation, the β-oxidative processes as they occur in organelles other than mitochondria, and, finally, a less-common mode of fatty acid catabolism—α oxidation.

The β Oxidation of Saturated Fatty Acids Has Four Basic Steps

Four enzyme-catalyzed reactions make up the first stage of fatty acid oxidation (**Fig. 17-8a**). First, dehydrogenation of fatty acyl–CoA produces a double bond between the α and β carbon atoms (C-2 and C-3), yielding a ***trans*-Δ^2-enoyl-CoA** (the symbol Δ^2 designates the position of the double bond; you may want to review fatty acid nomenclature, p. 342). Note that the new double bond has the trans configuration, whereas the double bonds in naturally occurring unsaturated fatty acids are normally in the cis configuration. We consider the significance of this difference later.

This first step is catalyzed by three isozymes of **acyl-CoA dehydrogenase**, each specific for a range of fatty-acyl chain lengths: very-long-chain acyl-CoA dehydrogenase (VLCAD), acting on fatty acids of 12 to 18 carbons; medium-chain (MCAD), acting on fatty acids of 4 to 14 carbons; and short-chain (SCAD), acting on fatty acids of 4 to 8 carbons. VLCAD is in the inner mitochondrial membrane; MCAD and SCAD are in the matrix. All three isozymes are flavoproteins with tightly bound FAD (see Fig. 13-27) as a prosthetic group. The electrons removed from the fatty acyl–CoA are transferred to FAD, and the reduced form of the dehydrogenase immediately donates its electrons to an electron carrier, the **electron transfer flavoprotein (ETF)** (see Fig. 19-15). Electrons move from ETF to a second flavoprotein, **ETF:ubiquinone oxidoreductase**, and through ubiquinone into the mitochondrial respiratory chain. P2 The oxidation catalyzed by an acyl-CoA dehydrogenase is analogous to succinate dehydrogenation in the citric acid cycle (p. 586); in both reactions the enzyme is bound to the inner membrane, a double bond is introduced into a carboxylic acid between the α and β carbons, FAD is the electron acceptor, and electrons from the reaction ultimately enter the respiratory chain and pass to O_2, with the concomitant synthesis of about 1.5 ATP molecules per electron pair.

In the second step of the β-oxidation cycle (Fig. 17-8a), water is added to the double bond of the *trans*-Δ^2-enoyl-CoA to form the L stereoisomer of **β-hydroxyacyl-CoA (3-hydroxyacyl-CoA)**. This reaction, catalyzed by **enoyl-CoA hydratase**, is formally analogous to the fumarase reaction in the citric acid cycle, in which H_2O adds across an α–β double bond (p. 587).

In the third step, L-β-hydroxyacyl-CoA is dehydrogenated to form **β-ketoacyl-CoA**, by the action of **β-hydroxyacyl-CoA dehydrogenase**; NAD^+ is the electron acceptor. This enzyme is absolutely specific for the L stereoisomer of hydroxyacyl-CoA. The NADH formed in the reaction donates its electrons to **NADH dehydrogenase (Complex I)**, an electron carrier of the respiratory chain (see Fig. 19-15), and ATP is formed from ADP as the electrons pass to O_2. P2 The reaction catalyzed by β-hydroxyacyl-CoA dehydrogenase is closely analogous to the malate dehydrogenase reaction of the citric acid cycle (p. 587).

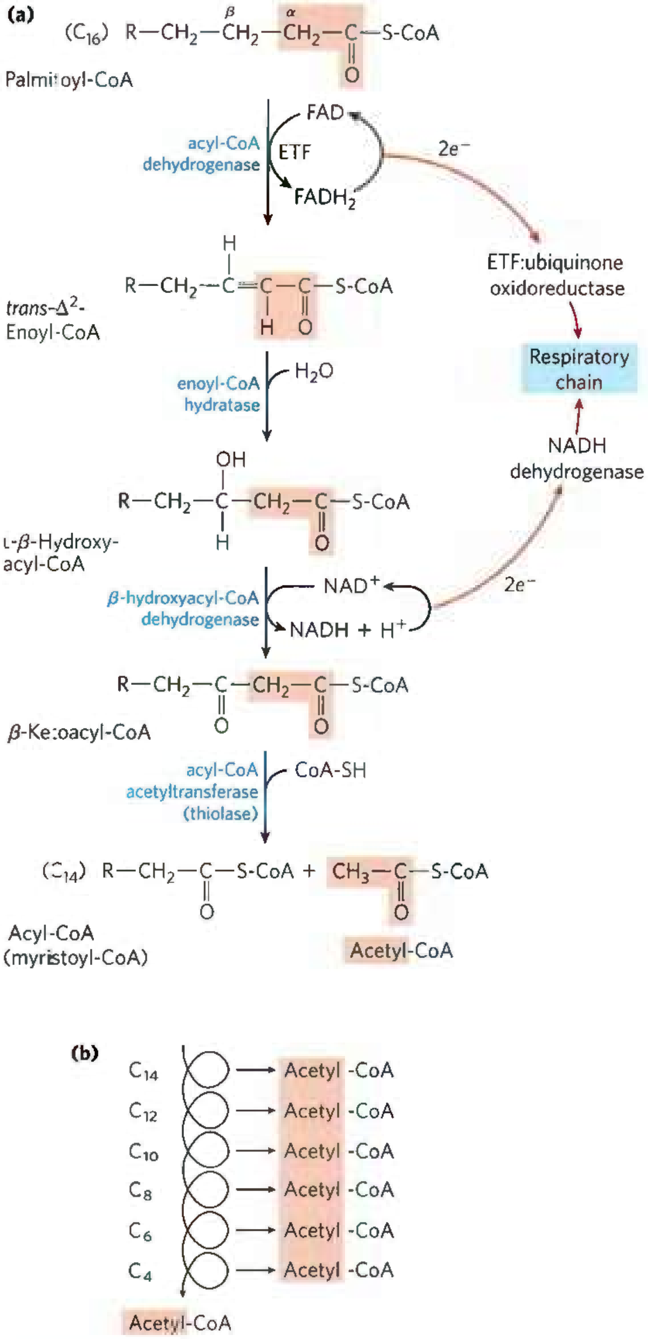


FIGURE 17-8 The β-oxidation pathway. (a) In each pass through this four-step sequence, one acetyl residue (shaded in light red) is removed in the form of acetyl-CoA from the carboxyl end of the fatty acyl chain—in this example palmitate (C_{16}), which enters as palmitoyl-CoA. Electrons from the first oxidation pass through electron transfer flavoprotein (ETF), and then through a second flavoprotein (ETF:ubiquinone oxidoreductase), into the respiratory chain. Electrons from the second oxidation enter the respiratory chain through NADH dehydrogenase. (b) Six more passes through the β-oxidation pathway yield seven more molecules of acetyl-CoA, the seventh arising from the last two carbon atoms of the 16-carbon chain. Eight molecules of acetyl-CoA are formed in all. The acetyl-CoA may be oxidized in the citric acid cycle, donating more electrons to the respiratory chain.

The fourth and last step of the β-oxidation cycle is catalyzed by **acyl-CoA acetyltransferase**, more commonly called **thiolase**, which promotes reaction of β-ketoacyl-CoA with a molecule of free coenzyme A to split off the carboxyl-terminal two-carbon fragment of the original fatty acid as acetyl-CoA. The other product is the coenzyme A thioester of the fatty acid, now shortened by two carbon atoms (Fig. 17-8a). This reaction is called thiolysis, by analogy with the process of hydrolysis, because the β-ketoacyl-CoA is cleaved by reaction with the thiol group of coenzyme A. The thiolase reaction is a reverse Claisen condensation (see Fig. 13-4).

The last three steps of this four-step sequence are catalyzed by either of two sets of enzymes, with the enzymes employed depending on the length of the fatty acyl chain. For fatty acyl chains of 12 or more carbons, the reactions are catalyzed by a multienzyme complex associated with the inner mitochondrial membrane, the **trifunctional protein (TFP)**. TFP is a heterooctamer of $\alpha_4\beta_4$ subunits. Each α subunit contains two activities, the enoyl-CoA hydratase and the β-hydroxyacyl-CoA dehydrogenase; the β subunits contain the thiolase activity. This tight association of three enzymes allows efficient substrate channeling from one active site to the next, without diffusion of the intermediates away from the enzyme surface. When TFP has shortened the fatty acyl chain to 12 or fewer carbons, further oxidations are catalyzed by a set of four soluble enzymes in the matrix.

P2 As noted earlier, the single bond between methylene (—CH_2—) groups in fatty acids is relatively stable. The β-oxidation sequence is an elegant mechanism for destabilizing and breaking these bonds. The first three reactions of β oxidation create a much less stable C—C bond, in which the α carbon (C-2) is bonded to *two* carbonyl carbons (the β-ketoacyl-CoA intermediate). The ketone function on the β carbon (C-3) makes it a good target for nucleophilic attack by the —SH of coenzyme A, catalyzed by thiolase. The acidity of the α hydrogen and the resonance stabilization of the carbanion generated by the departure of this hydrogen make the terminal —CH_2—CO—S-CoA a good leaving group, facilitating breakage of the α–β bond.

We have already seen a reaction sequence nearly identical with these four steps of fatty acid oxidation, in the citric acid cycle reaction steps between succinate and oxaloacetate (see Fig. 16-7). A nearly identical reaction sequence occurs again in the pathways by which the branched-chain amino acids (isoleucine, leucine, and valine) are oxidized as fuels (see Fig. 18-28). **Figure 17-9** shows the common features of these three sequences, almost certainly an example of the conservation of a mechanism by gene duplication and evolution of a new specificity in the enzyme products of the duplicated genes.

The Four β-Oxidation Steps Are Repeated to Yield Acetyl-CoA and ATP

In one pass through the β-oxidation sequence, one molecule of acetyl-CoA, two pairs of electrons, and four protons (H^+) are removed from the long-chain fatty

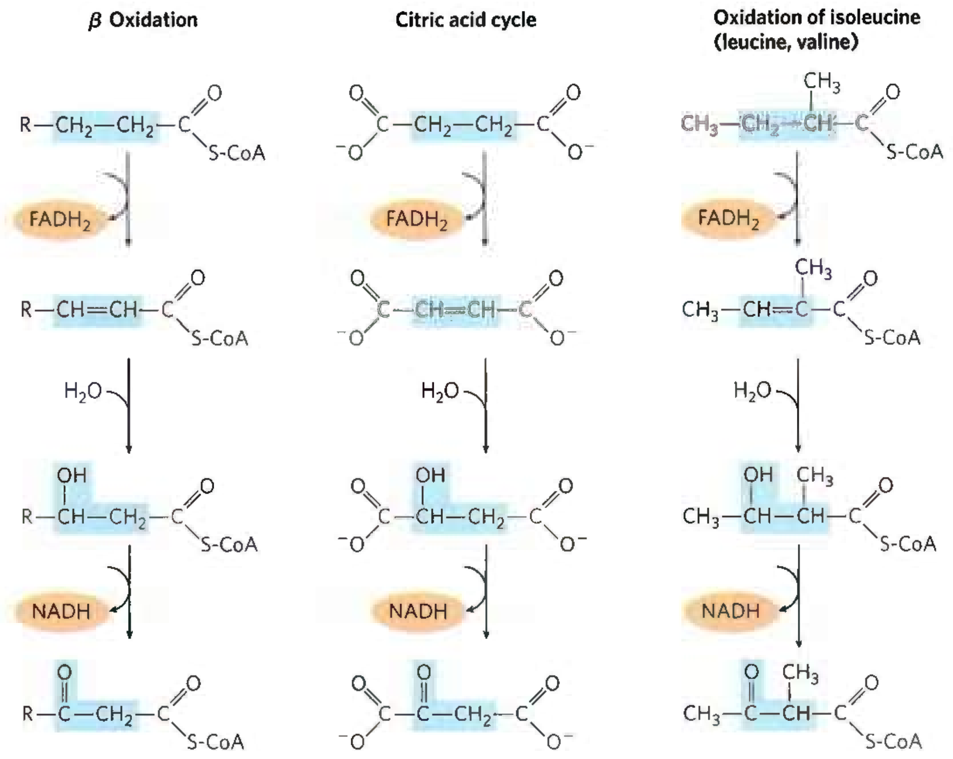


FIGURE 17-9 A conserved reaction sequence to introduce a carbonyl function on the carbon β to a carboxyl group. The β-oxidation pathway for fatty acyl–CoAs, the pathway from succinate to oxaloacetate in the citric acid cycle, and the pathway by which the deaminated carbon skeletons from isoleucine, leucine, and valine are oxidized as fuels—all use the same reaction sequence.

acyl–CoA, shortening it by two carbon atoms. The equation for one pass, beginning with the coenzyme A ester of our example, palmitate, is

$$\text{Palmitoyl-CoA} + \text{CoA} + \text{FAD} + \text{NAD}^+ + \text{H}_2\text{O} \longrightarrow \text{myristoyl-CoA} + \text{acetyl-CoA} + \text{FADH}_2 + \text{NADH} + \text{H}^+ \qquad (17\text{-}2)$$

Following removal of one acetyl-CoA unit from palmitoyl-CoA, the coenzyme A thioester of the shortened fatty acid (now the 14-carbon myristate) remains. The myristoyl-CoA can now go through another set of four β-oxidation reactions, exactly analogous to the first, to yield a second molecule of acetyl-CoA and lauroyl-CoA, the coenzyme A thioester of the 12-carbon laurate. Altogether, seven passes through the β-oxidation sequence are required to oxidize one molecule of palmitoyl-CoA to eight molecules of acetyl-CoA (Fig. 17-8b). The overall equation is

$$\text{Palmitoyl-CoA} + 7\text{CoA} + 7\text{FAD} + 7\text{NAD}^+ + 7\text{H}_2\text{O} \longrightarrow 8\ \text{acetyl-CoA} + 7\text{FADH}_2 + 7\text{NADH} + 7\text{H}^+ \qquad (17\text{-}3)$$

P1 Each molecule of $FADH_2$ formed during oxidation of the fatty acid donates a pair of electrons to ETF of the respiratory chain, and about 1.5 molecules of ATP are generated during the ensuing transfer of each electron pair to O_2. Similarly, each molecule of NADH formed delivers a pair of electrons to the mitochondrial NADH dehydrogenase, and the subsequent transfer of each pair of electrons to O_2 results in formation of about 2.5 molecules of ATP. Thus four molecules of ATP are formed for each two-carbon unit removed in one pass through the sequence.

Note that water is also produced in this process. Each pair of electrons transferred from NADH or $FADH_2$ to O_2 yields one H_2O, referred to as "metabolic water." Reduction of O_2 by NADH also consumes one H^+ per NADH molecule: $\text{NADH} + \text{H}^+ + \frac{1}{2}\text{O}_2 \longrightarrow \text{NAD}^+ + \text{H}_2\text{O}$. In hibernating animals, fatty acid oxidation provides metabolic energy, heat, and water—all essential for survival of an animal that neither eats nor drinks for long periods (**Box 17-1**). Camels obtain water to supplement the meager supply available in their natural environment by oxidation of fats stored in their hump.

The overall equation for the oxidation of palmitoyl-CoA to eight molecules of acetyl-CoA, including the electron transfers and oxidative phosphorylations, is

$$\text{Palmitoyl-CoA} + 7\text{CoA} + 7\text{O}_2 + 28\text{P}_\text{i} + 28\text{ADP} \longrightarrow 8\ \text{acetyl-CoA} + 28\text{ATP} + 7\text{H}_2\text{O} \qquad (17\text{-}4)$$

Acetyl-CoA Can Be Further Oxidized in the Citric Acid Cycle

P1 The acetyl-CoA produced from the oxidation of fatty acids can be oxidized to CO_2 and H_2O by the citric acid cycle. The following equation represents the

BOX 17-1

A Long Winter's Nap: Oxidizing Fats during Hibernation

Many animals depend on fat stores for energy during hibernation, during migratory periods, and in other situations involving radical metabolic adjustments. One of the most pronounced adjustments of fat metabolism occurs in hibernating grizzly bears. These animals remain in a continuous state of dormancy for as long as seven months. Unlike most hibernating species, the bear maintains a body temperature of about 31 °C, close to its normal (nonhibernating) temperature near 40 °C. Although expending about 25,000 kJ/day (6,000 kcal/day) while hibernating, the bear does not eat, drink, urinate, or defecate for months at a time. Its heart rate drops from 90 to 8 beats per minute, and its respiration rate drops from 6 to 10 breaths to approximately 1 breath per minute.

As we shall see in Chapter 19, mitochondrial electron transfer can be uncoupled from ATP production so that all of the energy of fuel oxidation is dissipated as heat, to maintain a body temperature near normal in the face of much lower ambient temperatures. One form of fat tissue, **brown adipose tissue**, is especially important in thermogenesis; we discuss it in more detail in Chapter 23.

Experimental studies have shown that hibernating grizzly bears use body fat as their sole fuel. Fat oxidation yields sufficient energy to maintain body temperature, synthesize amino acids and proteins, and carry out other energy-requiring activities, such as membrane transport. Fat oxidation also releases large amounts of water, as described in the text, which replenishes water lost in breathing. The glycerol released by degradation of triacylglycerols is converted into blood glucose by gluconeogenesis. Urea formed during breakdown of amino acids is reabsorbed in the kidneys and recycled, with the amino groups reused to make new amino acids for maintaining body proteins.

Bears store an enormous amount of body fat in preparation for their long sleep. An adult grizzly consumes about 38,000 kJ/day during the late spring and summer, but as winter approaches it feeds for 20 hours a day, consuming up to 84,000 kJ daily. This increase in feeding is a response to a seasonal change in hormone secretion. Large amounts of triacylglycerols are formed from the huge intake of carbohydrates during the fattening-up period. The bear will emerge from hibernation having lost 15% to 40% of its maximum body weight.

The winter sleep of bears is sometimes called torpor, and it differs in important ways from the hibernation behavior of a group of smaller animals that undergo alternating periods of high and low body temperature. In these animals, body temperature approaches ambient temperature, close to 0 °C, for much of the time during hibernation, but rises to almost prehibernation level during brief periods of wakefulness. During these periods, the animals eat, drink, and defecate. In the Arctic ground squirrel (*Urocitellus parryii*), for example, body temperature (37 °C prehibernation) drops to 0 °C during hibernation, and respiration drops to less than 10% of its prehibernation rate.

Studies of hibernation mechanisms may yield insight into several problems in human medicine; for example, slowing the metabolism of organs donated for transplantation might extend the period of their viability. And if humans are to make long trips into space, inducing a torporlike state might relieve the monotony of long missions and conserve onboard resources such as food and oxygen.

A grizzly bear prepares its hibernation nest near the McNeil River in Canada.
[Stouffer Productions/Animals Animals]

balance sheet for the second stage in the oxidation of palmitoyl-CoA, together with the coupled phosphorylations of the third stage:

$$\text{8 Acetyl-CoA} + 16O_2 + 80P_i + 80\text{ADP} \longrightarrow \text{8CoA} + 80\text{ATP} + 16CO_2 + 16H_2O \qquad (17\text{-}5)$$

Combining Equations 17-4 and 17-5, we obtain the overall equation for the complete oxidation of palmitoyl-CoA to carbon dioxide and water:

$$\text{Palmitoyl-CoA} + 23O_2 + 108P_i + 108\text{ADP} \longrightarrow \text{CoA} + 108\text{ATP} + 16CO_2 + 23H_2O \qquad (17\text{-}6)$$

Table 17-1 summarizes the yields of NADH, $FADH_2$, and ATP in the successive steps of palmitoyl-CoA oxidation. Note that because the activation of palmitate to palmitoyl-CoA breaks both phosphoanhydride bonds in ATP (Fig. 17-5), the energetic cost of activating a fatty acid is equivalent to two ATP, and the net gain per molecule of palmitate is 106 ATP. The standard free-energy change for the oxidation of palmitate to CO_2 and H_2O is about 9,800 kJ/mol. Under standard conditions, the energy recovered as the phosphate bond energy of ATP is 106 × 30.5 kJ/mol = 3,230 kJ/mol, about 33% of the theoretical maximum. However, when the free-energy changes are calculated from actual concentrations of reactants and products under intracellular conditions, the free-energy recovery is more than 60%; the energy conservation is remarkably efficient (see Worked Example 13-2, p. 480).

Oxidation of Unsaturated Fatty Acids Requires Two Additional Reactions

The fatty acid oxidation sequence just described is typical when the incoming fatty acid is saturated (that is, has only single bonds in its carbon chain). However, most of the fatty acids in the triacylglycerols and phospholipids of animals and plants are unsaturated, having one or more double bonds. These bonds are in the cis configuration and cannot be acted upon by enoyl-CoA hydratase, the enzyme catalyzing the addition of H_2O to the trans double bond of the Δ^2-enoyl-CoA generated during β oxidation. P1 Two auxiliary enzymes—an isomerase and a reductase—must act on the common unsaturated fatty acids to transform them into substrates for the β-oxidation pathway. We illustrate these auxiliary reactions with two examples.

Oleate is an abundant 18-carbon monounsaturated fatty acid with a cis double bond between C-9 and C-10 (denoted Δ^9). In the first step of oxidation, oleate is converted to oleoyl-CoA and, like the saturated fatty acids, enters the mitochondrial matrix via the carnitine shuttle (Fig. 17-6). Oleoyl-CoA then undergoes three passes through the fatty acid oxidation cycle to yield three molecules of acetyl-CoA and the coenzyme A ester of a Δ^3, 12-carbon unsaturated fatty acid, *cis*-Δ^3-dodecenoyl-CoA (**Fig. 17-10**). This product cannot serve as a substrate for enoyl-CoA hydratase, which acts only on trans double bonds. The auxiliary enzyme **Δ^3,Δ^2-enoyl-CoA isomerase** isomerizes the *cis*-Δ^3-enoyl-CoA to the *trans*-Δ^2-enoyl-CoA, which is converted by enoyl-CoA hydratase into the corresponding L-β-hydroxyacyl-CoA (*trans*-Δ^2-dodecenoyl-CoA). This intermediate is now acted upon by the remaining enzymes of β oxidation to yield acetyl-CoA and the coenzyme A ester of a 10-carbon saturated fatty acid, decanoyl-CoA. The latter undergoes four more passes through the β-oxidation pathway to yield five more molecules of acetyl-CoA. Altogether, nine acetyl-CoAs are produced from one molecule of the 18-carbon oleate.

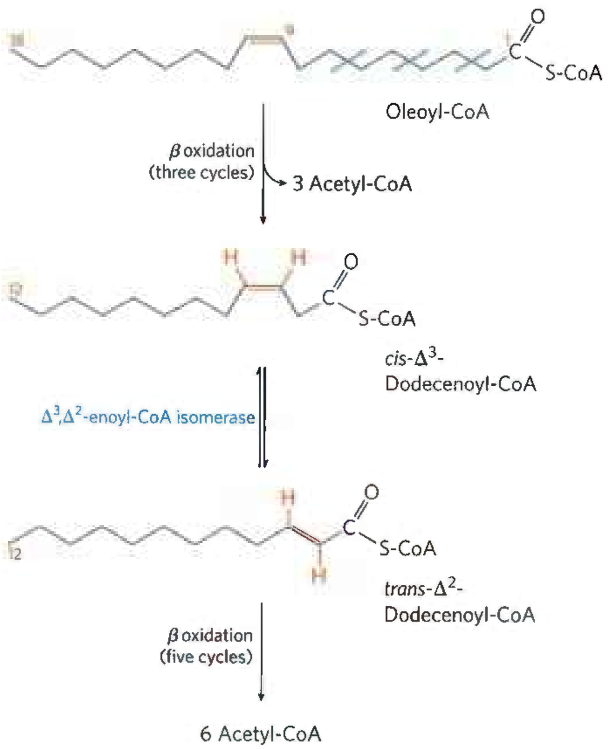
The other auxiliary enzyme (a reductase) is required for oxidation of polyunsaturated fatty acids—for example,

TABLE 17.1 Yield of ATP during Oxidation of One Molecule of Palmitoyl-CoA to CO_2 and H_2O

Enzyme catalyzing the oxidation step	Number of NADH or $FADH_2$ formed	Number of ATP ultimately formed[a]
β Oxidation		
Acyl-CoA dehydrogenase	7 $FADH_2$	10.5
β-Hydroxyacyl-CoA dehydrogenase	7 NADH	17.5
Citric acid cycle		
Isocitrate dehydrogenase	8 NADH	20
α-Ketoglutarate dehydrogenase	8 NADH	20
Succinyl-CoA synthetase		8[b]
Succinate dehydrogenase	8 $FADH_2$	12
Malate dehydrogenase	8 NADH	20
Total		108

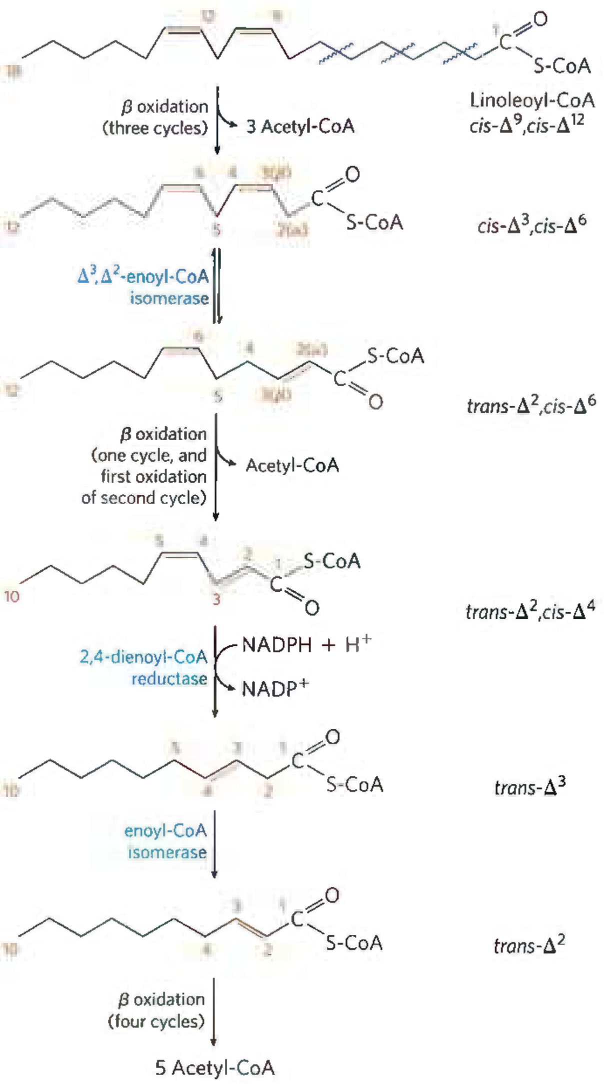
[a] These calculations assume that mitochondrial oxidative phosphorylation produces 1.5 ATP per $FADH_2$ oxidized and 2.5 ATP per NADH oxidized.

[b] GTP produced directly in this step yields ATP in the reaction catalyzed by nucleoside diphosphate kinase (p. 487).



18
9
1
O
C
S-CoA
Oleoyl-CoA
β oxidation (three cycles)
3 Acetyl-CoA
H
H
12
cis-Δ3-Dodecenoyl-CoA
Δ3,Δ2-enoyl-CoA isomerase
trans-Δ2-Dodecenoyl-CoA
β oxidation (five cycles)
6 Acetyl-CoA

FIGURE 17-10 Oxidation of a monounsaturated fatty acid. Oleic acid, as oleoyl-CoA (Δ^9), is the example used here. Oxidation requires an additional enzyme, enoyl-CoA isomerase, to reposition the double bond, converting the cis isomer to a trans isomer, an intermediate in β oxidation.



18
12
9
1
O
C
S-CoA
Linoleoyl-CoA cis-Δ9,cis-Δ12
β oxidation (three cycles)
3 Acetyl-CoA
cis-Δ3,cis-Δ6
Δ3,Δ2-enoyl-CoA isomerase
trans-Δ2,cis-Δ6
β oxidation (one cycle, and first oxidation of second cycle)
Acetyl-CoA
trans-Δ2,cis-Δ4
2,4-dienoyl-CoA reductase
NADPH + H+
NADP+
trans-Δ3
enoyl-CoA isomerase
trans-Δ2
β oxidation (four cycles)
5 Acetyl-CoA

FIGURE 17-11 Oxidation of a polyunsaturated fatty acid. The example here is linoleic acid, as linoleoyl-CoA ($\Delta^{9,12}$). Oxidation requires a second auxiliary enzyme in addition to enoyl-CoA isomerase: NADPH-dependent 2,4-dienoyl-CoA reductase. The combined action of these two enzymes converts a *trans*-Δ^2,*cis*-Δ^4-dienoyl-CoA intermediate to the *trans*-Δ^2-enoyl-CoA substrate necessary for β oxidation.

the 18-carbon linoleate, which has a *cis*-Δ^9,*cis*-Δ^{12} configuration (**Fig. 17-11**). Linoleoyl-CoA undergoes three passes through the β-oxidation sequence to yield three molecules of acetyl-CoA and the coenzyme A ester of a 12-carbon unsaturated fatty acid with a *cis*-Δ^3,*cis*-Δ^6 configuration. This intermediate cannot be used by the enzymes of the β-oxidation pathway: its double bonds are in the wrong position and have the wrong configuration (cis, not trans). However, the combined action of enoyl-CoA isomerase and **2,4-dienoyl-CoA reductase**, as shown in Figure 17-11, transforms this intermediate into one that can enter the β-oxidation pathway and be degraded to six acetyl-CoAs. The overall result is conversion of linoleate to nine molecules of acetyl-CoA.

Complete Oxidation of Odd-Number Fatty Acids Requires Three Extra Reactions

Although most naturally occurring lipids contain fatty acids with an even number of carbon atoms, fatty acids with an odd number of carbons are common in the lipids of many plants and some marine organisms. Cattle and other ruminant animals form large amounts of the three-carbon **propionate** ($CH_3—CH_2—COO^-$) during fermentation of carbohydrates in the rumen. The propionate is absorbed into the blood and oxidized by the liver and other tissues.

Long-chain odd-number fatty acids are oxidized in the same pathway as the even-number acids, beginning at the carboxyl end of the chain. However, the substrate for the last pass through the β-oxidation sequence is a fatty acyl–CoA with a five-carbon fatty acid. When this is oxidized and cleaved, the products are acetyl-CoA and **propionyl-CoA**. The acetyl-CoA can be oxidized in the citric acid cycle, of course, but propionyl-CoA enters a different pathway, having three enzymes.

Propionyl-CoA is first carboxylated to form the D stereoisomer of **methylmalonyl-CoA** by **propionyl-CoA carboxylase**, which contains the cofactor biotin (**Fig. 17-12**). In this enzymatic reaction, as in the pyruvate carboxylase reaction (see Fig. 16-16), bicarbonate ion (HCO_3^-) is activated by attachment to biotin before its transfer to the substrate, in this case the propionate moiety. Formation of the carboxybiotin intermediate requires energy, which is provided by ATP. The D-methylmalonyl-CoA thus

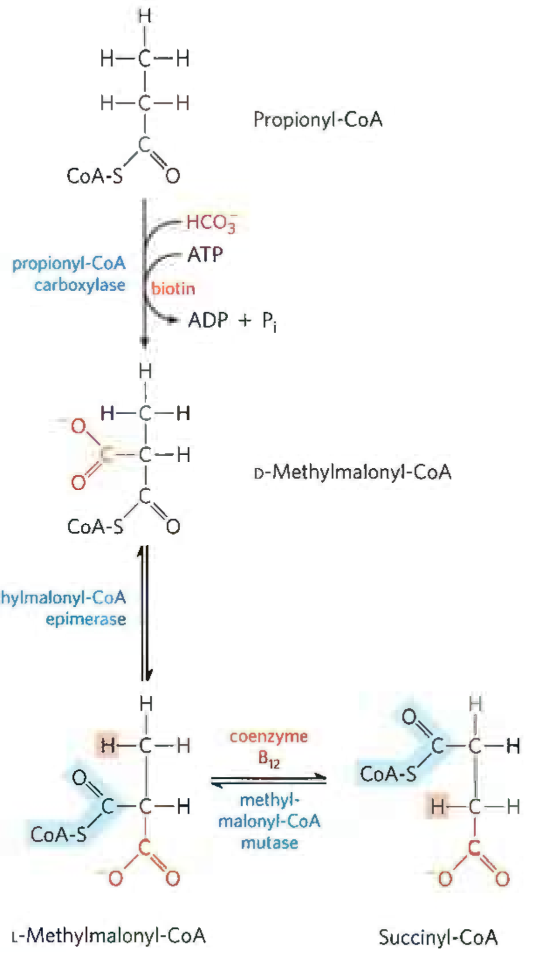


FIGURE 17-12 Oxidation of propionyl-CoA produced by β oxidation of odd-number fatty acids. The sequence involves the carboxylation of propionyl-CoA to D-methylmalonyl-CoA and conversion of the latter to succinyl-CoA. This conversion requires epimerization of D- to L-methylmalonyl-CoA, followed by a remarkable reaction in which substituents on adjacent carbon atoms exchange positions (see Box 17-2).

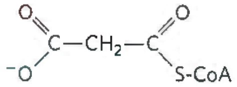
formed is enzymatically epimerized to its L stereoisomer by **methylmalonyl-CoA epimerase**. The L-methylmalonyl-CoA then undergoes an intramolecular rearrangement to form succinyl-CoA, which can enter the citric acid cycle. This rearrangement is catalyzed by **methylmalonyl-CoA mutase**, which requires as its coenzyme **5′-deoxyadenosylcobalamin**, or **coenzyme B_{12}**, which is derived from vitamin B_{12} (cobalamin). **Box 17-2** describes the role of coenzyme B_{12} in this remarkable exchange reaction.

About 1 in 100,000 babies are born with a genetic deficiency in propionyl-CoA carboxylase, making them unable to metabolize the propionyl-CoA that results from catabolism of odd-number fatty acids, fatty acids with methyl branches, and the amino acids isoleucine, valine, threonine, and methionine. **P4** The absence of functional propionyl-CoA carboxylase leads to an accumulation of propionyl-CoA in mitochondria, depleting the available supply of coenzyme A for continuing β-oxidation and other metabolism. The propionyl-CoA is esterified to carnitine, transported out of the mitochondria through the carnitine shuttle, and eventually released to the blood as propionate, which severely acidifies the blood and urine, a condition called propionic acidemia. Symptoms generally manifest in the first few days of life, with vomiting, low blood glucose, seizures, and neurologic abnormalities. The condition is diagnosed by detection of propionate and its metabolites in the blood and urine. Treatments include severe restriction of dietary protein, supplying carnitine, and the use of antibiotics targeted at gut bacteria that produce odd-chain and branched-chain fatty acids. Some individuals who are defective in the incorporation of biotin into the enzyme benefit from treatment with high doses of biotin. ■

Fatty Acid Oxidation Is Tightly Regulated

P3 Oxidation of fatty acids consumes a precious fuel, and it is regulated so as to occur only when the organism's need for energy requires it. In the liver, fatty acyl–CoA formed in the cytosol has two major pathways open to it: (1) β oxidation by enzymes in mitochondria or (2) conversion into triacylglycerols and phospholipids by enzymes in the cytosol. The pathway taken depends on the rate of transfer of long-chain fatty acyl–CoA into mitochondria. The three-step carnitine shuttle by which fatty acyl groups are carried into the mitochondrial matrix as fatty acyl–carnitine (Fig. 17-6) is rate-limiting for fatty acid oxidation and is an important point of regulation. Once fatty acyl groups have entered the mitochondrion, they are committed to oxidation to acetyl-CoA.

Figure 17-13 illustrates, with numbered steps described below, the coordinated down-regulation of β oxidation when carbohydrate levels are sufficient. Ingestion of a high-carbohydrate meal raises the blood glucose level and thus ① triggers the release of insulin. ② Insulin-dependent protein phosphatase dephosphorylates the enzyme acetyl-CoA carboxylase (ACC), activating it. ③ ACC catalyzes the formation of **malonyl-CoA** (the first intermediate of cytosolic fatty acid synthesis; see Figs 21-1 and 21-2), and ④ malonyl-CoA inhibits carnitine acyltransferase 1, thereby preventing fatty acid entry into the mitochondrial matrix through the carnitine shuttle. The inhibition of carnitine acyltransferase 1 by malonyl-CoA ensures that the oxidation of fatty acids is inhibited whenever the liver is amply supplied with glucose as fuel and is actively making triacylglycerols from excess glucose. Two of the enzymes of β oxidation (⑧) are also regulated by metabolites that signal energy sufficiency. When the [NADH]/[NAD^+] ratio is high, β-hydroxyacyl-CoA dehydrogenase is inhibited; in addition, high concentrations of acetyl-CoA inhibit thiolase.



Malonyl-CoA

BOX 17-2

Coenzyme B_{12}: A Radical Solution to a Perplexing Problem

In the methylmalonyl-CoA mutase reaction (see Fig. 17-12), the group —CO—S-CoA at C-2 of the original propionate exchanges position with a hydrogen atom at C-3 of the original propionate (Fig. 1a). Coenzyme B_{12} is the cofactor for this general type of reaction (Fig. 1b). These coenzyme B_{12}–dependent processes are among the very few enzymatic reactions in biology in which there is an exchange of an alkyl or substituted alkyl group (X) with a hydrogen atom on an adjacent carbon, *with no mixing of the transferred hydrogen atom with the hydrogen of the solvent,* H_2O. How can the hydrogen atom move between two carbons without mixing with the enormous excess of hydrogen atoms in the solvent?

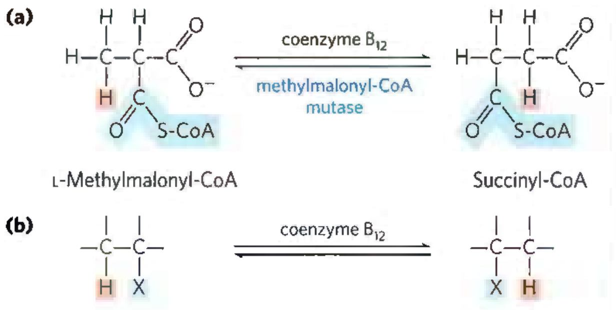


FIGURE 1

Coenzyme B_{12} is the cofactor form of vitamin B_{12}, which is unique among all the vitamins in that it contains an essential trace element, cobalt. The complex **corrin ring system** of vitamin B_{12} (colored blue in Fig. 2), to which cobalt (as Co^{3+}) is coordinated, is chemically related to the porphyrin ring system of heme (see Fig. 5-1). A fifth coordination position of cobalt is filled by dimethylbenzimidazole ribonucleotide (shaded yellow), bound covalently by its 3′-phosphate group to a side chain of the corrin ring, through aminoisopropanol. The formation of this complex cofactor occurs in one of only two known reactions in which triphosphate is cleaved from ATP (Fig. 3); the other reaction is the formation of *S*-adenosylmethionine from ATP and methionine (see Fig. 18-18).

Dorothy Crowfoot Hodgkin, 1910–1994 [© Godfrey Argent Studio/The Royal Society]

Vitamin B_{12} is usually isolated as **cyanocobalamin**, because it contains a cyano group (picked up during purification) attached to cobalt in the sixth coordination position. In **5′-deoxyadenosylcobalamin**, the cofactor for methylmalonyl-CoA mutase, the cyano group is replaced by the **5′-deoxyadenosyl** group (red in Fig. 2), covalently bound through C-5′ to the cobalt. The three-dimensional structure of the cofactor was determined by Dorothy Crowfoot Hodgkin in 1956, using x-ray crystallography.

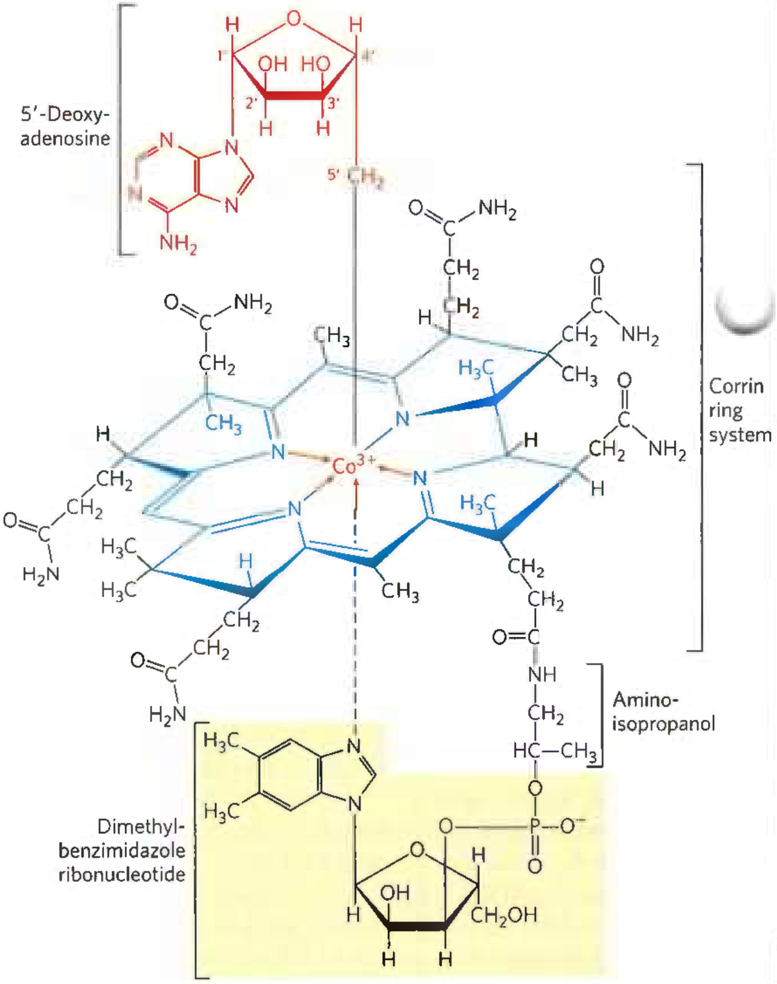
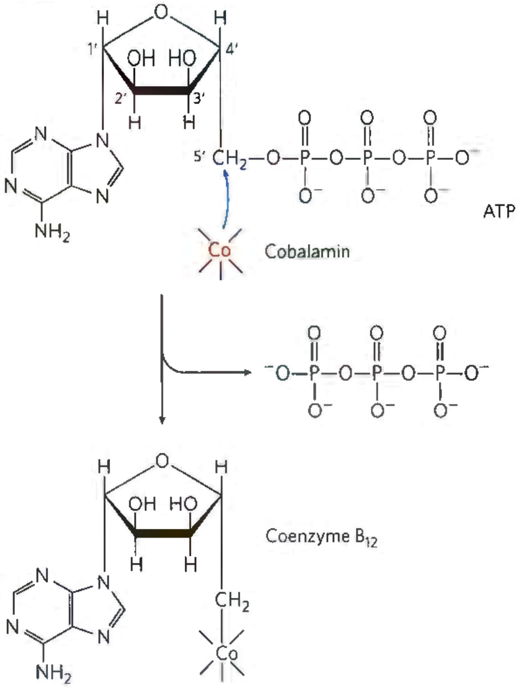


FIGURE 2

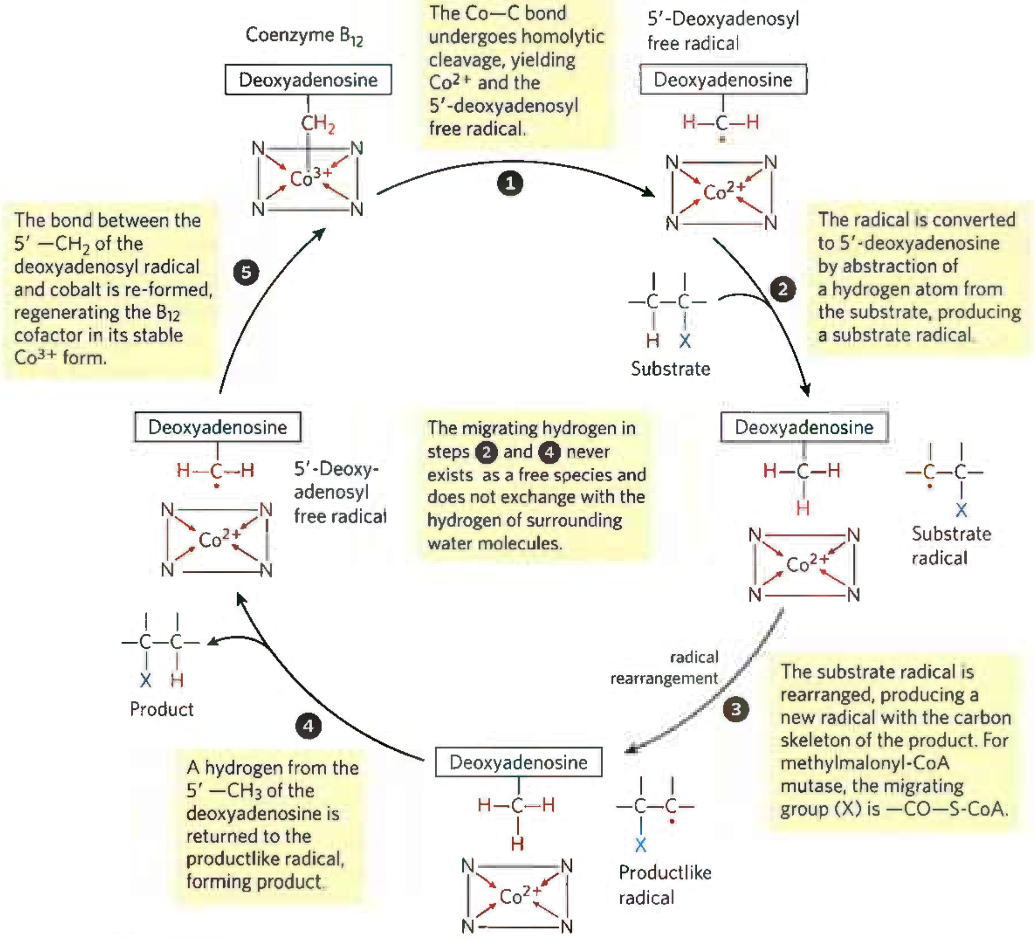
The key to understanding how coenzyme B_{12} catalyzes hydrogen exchange lies in the properties of the covalent bond between cobalt and C-5′ of the deoxyadenosyl group (Fig. 2). This is a relatively weak bond; merely illuminating the compound with visible light is enough to break this Co—C bond. (This extreme photolability probably accounts for the absence of vitamin B_{12} in plants.) Dissociation produces a 5′-deoxyadenosyl radical and the Co^{2+} form of the vitamin. By generating free radicals in this way, 5′-deoxyadenosylcobalamin initiates a series of transformations such as that illustrated in Figure 4—a postulated mechanism for the reaction catalyzed by methylmalonyl-CoA mutase and several other coenzyme B_{12}–dependent transformations. In this postulated mechanism, the migrating hydrogen atom never exists as a free species and is thus never free to exchange with the hydrogen of surrounding water molecules.

Vitamin B_{12} is not made by plants or animals and can be synthesized only by a few species of microorganisms. It is required by healthy people in only minute amounts, about 3 μg/day. Vitamin B_{12} deficiency results in **pernicious anemia**, caused by a failure to absorb vitamin B_{12} efficiently from the intestine, where it is synthesized by intestinal bacteria or obtained from digestion of meat. Individuals with this disease do not produce sufficient amounts of **intrinsic factor**, a glycoprotein essential to vitamin B_{12} absorption. The pathology in pernicious anemia includes reduced production of erythrocytes, reduced levels of hemoglobin, and severe, progressive impairment of the central nervous system. Administration of large doses of vitamin B_{12} alleviates these symptoms in at least some cases.



H
O
H
1′
4′
OH HO
2′
3′
N
NH2
5′ CH2—O—P—O—P—O—P—O⁻
ATP
Co
Cobalamin
⁻O—P—O—P—O—P—O⁻
CH2
Coenzyme B12

FIGURE 3



Coenzyme B12
Deoxyadenosine
CH2
Co3+
N
1
5′-Deoxyadenosyl free radical
H—C—H
Co2+
2
Substrate
H X
Substrate radical
3
radical rearrangement
Productlike radical
4
Product
X H
5′-Deoxyadenosyl free radical
5

The Co—C bond undergoes homolytic cleavage, yielding Co^{2+} and the 5′-deoxyadenosyl free radical.

The radical is converted to 5′-deoxyadenosine by abstraction of a hydrogen atom from the substrate, producing a substrate radical.

The migrating hydrogen in steps ② and ④ never exists as a free species and does not exchange with the hydrogen of surrounding water molecules.

The substrate radical is rearranged, producing a new radical with the carbon skeleton of the product. For methylmalonyl-CoA mutase, the migrating group (X) is —CO—S-CoA.

A hydrogen from the 5′ —CH_3 of the deoxyadenosine is returned to the productlike radical, forming product.

The bond between the 5′ —CH_2 of the deoxyadenosyl radical and cobalt is re-formed, regenerating the B_{12} cofactor in its stable Co^{3+} form.

MECHANISM FIGURE 4

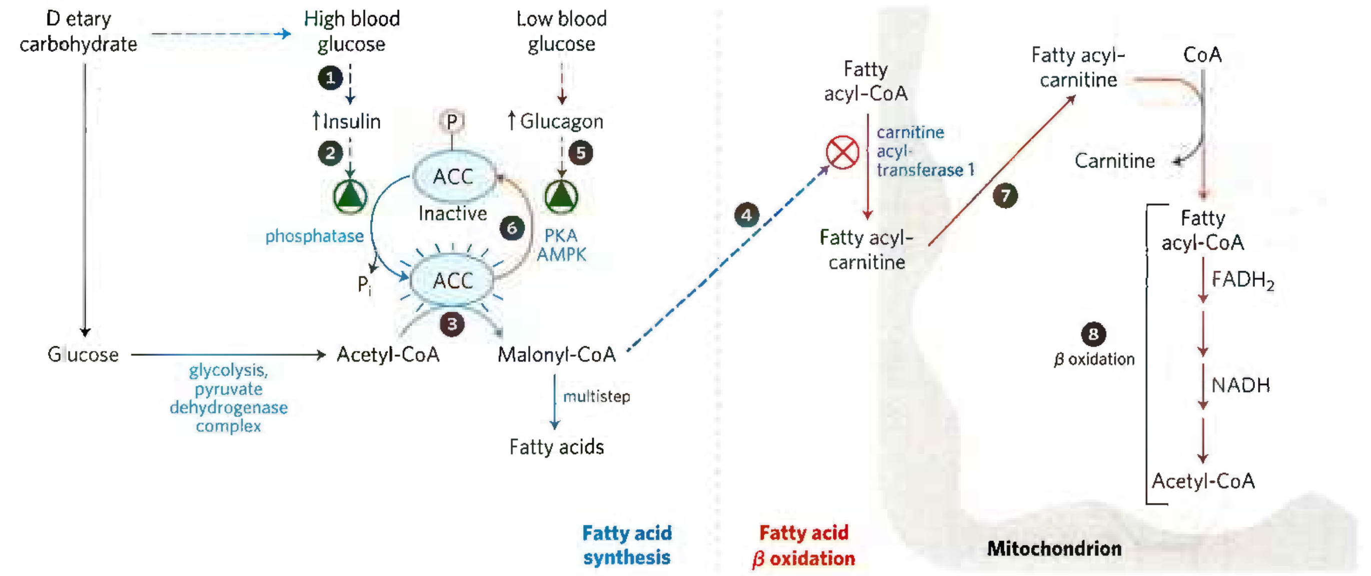

Dietary carbohydrate
High blood glucose
Low blood glucose
1
↑Insulin
P
↑Glucagon
2
5
ACC
Inactive
phosphatase
6
PKA
AMPK
P_i
ACC
3
Glucose
glycolysis, pyruvate dehydrogenase complex
Acetyl-CoA
Malonyl-CoA
multistep
Fatty acids
4
Fatty acyl-CoA
carnitine acyl-transferase 1
Fatty acyl-carnitine
7
Fatty acyl-carnitine
CoA
Carnitine
8
β oxidation
Fatty acyl-CoA
$FADH_2$
NADH
Acetyl-CoA
Fatty acid synthesis
Fatty acid β oxidation
Mitochondrion

FIGURE 17-13 Coordinated regulation of fatty acid synthesis and breakdown. The steps are explained in the text.

Figure 17-13 also shows that conversely, when blood glucose levels drop between meals, ❺ glucagon release activates protein kinase A (PKA), which ❻ phosphorylates and inactivates ACC. Recall from Chapter 14 that in a low-energy state, [AMP] rises relative to [ATP], activating AMP kinase (AMPK). Activated AMPK also phosphorylates and inactivates ACC. The concentration of malonyl-CoA falls, and the inhibition of fatty acid entry into mitochondria is relieved. Fatty acids ❼ enter the mitochondrial matrix, ❽ allowing *β* oxidation to replenish the supply of ATP. Because glucagon also triggers the mobilization of fatty acids in adipose tissue, a supply of fatty acids begins arriving in the blood.

Transcription Factors Turn on the Synthesis of Proteins for Lipid Catabolism

P3 In addition to the various short-term regulatory mechanisms that modulate the activity of existing enzymes, transcriptional regulation can change the number of molecules of the enzymes of fatty acid oxidation on a longer time scale—minutes to hours. The **PPAR** family of nuclear receptors are transcription factors that affect many metabolic processes in response to a variety of fatty acid–like ligands. (They were originally recognized as *p*eroxisome *p*roliferator-*a*ctivated *r*eceptors, then were found to function more broadly.) PPAR*α* acts in muscle, adipose tissue, and liver to turn on a set of genes essential for fatty acid oxidation, including the fatty acid transporter, carnitine acyltransferases 1 and 2; the fatty acyl–CoA dehydrogenases for short, medium, long, and very long acyl chains; and related enzymes. This response is triggered when a cell or an organism has an increased demand for energy from fat catabolism, such as during a fast between meals or under conditions of longer-term starvation. Glucagon, released in response to low blood glucose, can act through cAMP and the transcription factor CREB to turn on certain genes for lipid catabolism.

Another situation that is accompanied by major changes in the expression of the enzymes of fatty acid oxidation is the transition from fetal to neonatal metabolism in the heart. In the fetus, the principal fuels in heart muscle are glucose and lactate, but in the neonatal heart, fatty acids are the main fuel. At the time of this transition, PPAR*α* is activated and in turn activates the genes essential for fatty acid metabolism. As we will see in Chapter 23, two other transcription factors in the PPAR family also play crucial roles in determining the enzyme complements—and therefore the metabolic activities—of specific tissues at particular times (see Fig. 23-38).

The major sites of fatty acid oxidation, at rest and during exercise, are skeletal and heart muscle. Endurance training increases PPAR*α* expression in muscle, leading to increased levels of fatty acid–oxidizing enzymes and increased oxidative capacity of the muscle.

Genetic Defects in Fatty Acyl–CoA Dehydrogenases Cause Serious Disease

Stored triacylglycerols are typically the chief source of energy for muscle contraction, and an inability to oxidize fatty acids from triacylglycerols has serious consequences for health. **P4** The most common genetic defect in fatty acid catabolism in U.S. and northern European populations is due to a mutation in the gene encoding the **medium-chain acyl-CoA dehydrogenase (MCAD)**, mentioned earlier in the chapter. Among northern Europeans, the frequency of carriers (individuals with this recessive mutation on one of the two homologous chromosomes) is about 1 in 40, and about 1 individual in 10,000 has the disease—that is, has two copies of the mutant MCAD allele and is unable to oxidize fatty acids of 6 to 12 carbons. The disease is characterized by recurring episodes of a syndrome that includes fat accumulation in the liver, high blood levels of octanoic acid (8:0), low blood glucose (hypoglycemia), sleepiness, vomiting, and coma. Although individuals may have no symptoms between episodes, the episodes are very serious; mortality due to this disease is 25% to 60% in early childhood. If the genetic defect is detected shortly after birth, the infant can be started on a low-fat, high-carbohydrate diet. With early detection and careful management of the diet—including avoiding long intervals between meals, to prevent the body from turning to its fat reserves for energy—the prognosis for these individuals is good.

P4 More than 20 human genetic defects in fatty acid transport or oxidation have been documented, most much less common than the defect in MCAD. One of the most severe disorders results from loss of the long-chain β-hydroxyacyl-CoA dehydrogenase activity of the trifunctional protein, TFP. Other disorders include defects in the α or β subunits that affect all three activities of TFP and cause serious heart disease and abnormal skeletal muscle. ■

Peroxisomes Also Carry Out β Oxidation

The mitochondrial matrix is the major site of fatty acid oxidation in animal cells, but other compartments also contain enzymes capable of oxidizing fatty acids to acetyl-CoA, by a pathway similar but not identical to that in mitochondria. In plant cells, the major site of β oxidation is not mitochondria but **peroxisomes**. In peroxisomes, organelles also found in animal cells, the intermediates for β oxidation of fatty acids are coenzyme A derivatives, and the process consists of four steps, as in mitochondrial β oxidation (**Fig. 17-14**): (1) dehydrogenation, (2) addition of water to the resulting double bond, (3) oxidation of the β-hydroxyacyl-CoA to a ketone, and (4) thiolytic cleavage by coenzyme A. The identical reactions also occur in glyoxysomes, organelles found only in germinating seeds.

One difference between the peroxisomal and mitochondrial pathways is in the chemistry of the first step (Fig. 17-14). In peroxisomes, the flavoprotein acyl-CoA oxidase that introduces the double bond passes electrons directly to O_2, producing H_2O_2 (thus the name "peroxisomes"). This strong and potentially damaging oxidant is immediately cleaved to H_2O and O_2 by **catalase**. Recall that in mitochondria, the electrons removed in the first oxidation step pass through the respiratory chain to O_2 to produce H_2O, and this process is accompanied by ATP synthesis. In peroxisomes, the energy released in the first oxidative step of fatty acid breakdown is not conserved as ATP, but is dissipated as heat.

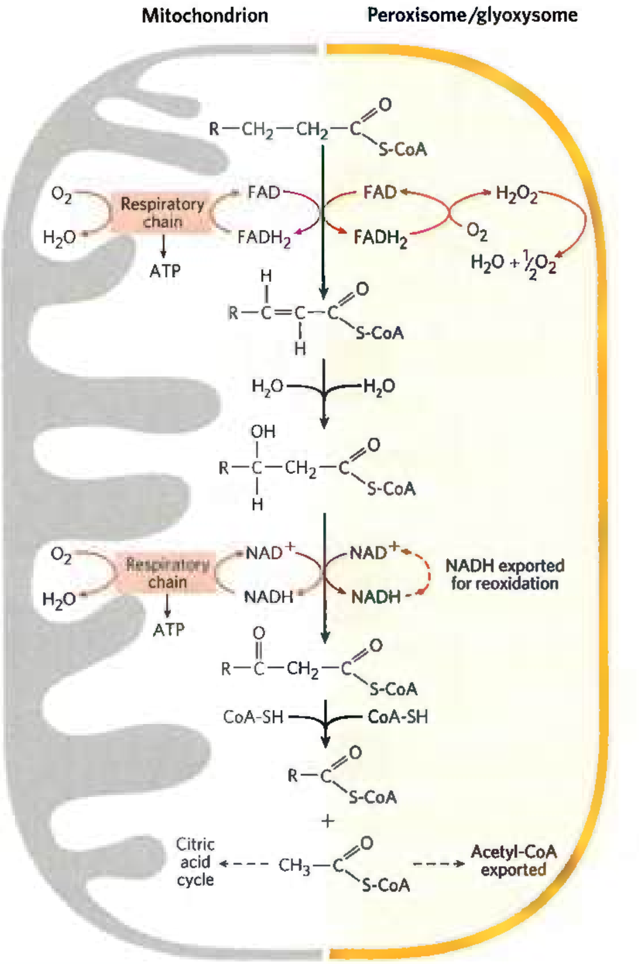


FIGURE 17-14 Comparison of β oxidation in mitochondria and in peroxisomes and glyoxysomes. The peroxisomal/glyoxysomal system differs from the mitochondrial system in three respects: (1) the peroxisomal system prefers very-long-chain fatty acids; (2) in the first oxidative step, electrons pass directly to O_2, generating H_2O_2; and (3) the NADH formed in the second oxidative step cannot be reoxidized in the peroxisome or glyoxysome, so reducing equivalents are exported to the cytosol, eventually entering mitochondria. The acetyl-CoA produced by peroxisomes and glyoxysomes is also exported; the acetate from glyoxysomes serves as a biosynthetic precursor. Acetyl-CoA produced in mitochondria is further oxidized in the citric acid cycle.

A second important difference between mitochondrial and peroxisomal β oxidation in mammals is in the specificity for fatty acyl–CoAs; the peroxisomal system is much more active on very-long-chain fatty acids such as hexacosanoic acid (26:0) and on branched-chain fatty acids such as phytanic acid and pristanic acid (see **Fig. 17-15**). These less-common fatty acids are obtained from dietary intake of dairy products, the fat of ruminant animals, meat, and fish. Their catabolism in the peroxisome involves several auxiliary enzymes unique to this organelle. P4 The inability to oxidize these compounds is responsible for several serious human genetic diseases. Individuals with **Zellweger syndrome** are unable to make peroxisomes and therefore lack all the metabolism unique to that organelle. In **X-linked adrenoleukodystrophy (XALD)**,

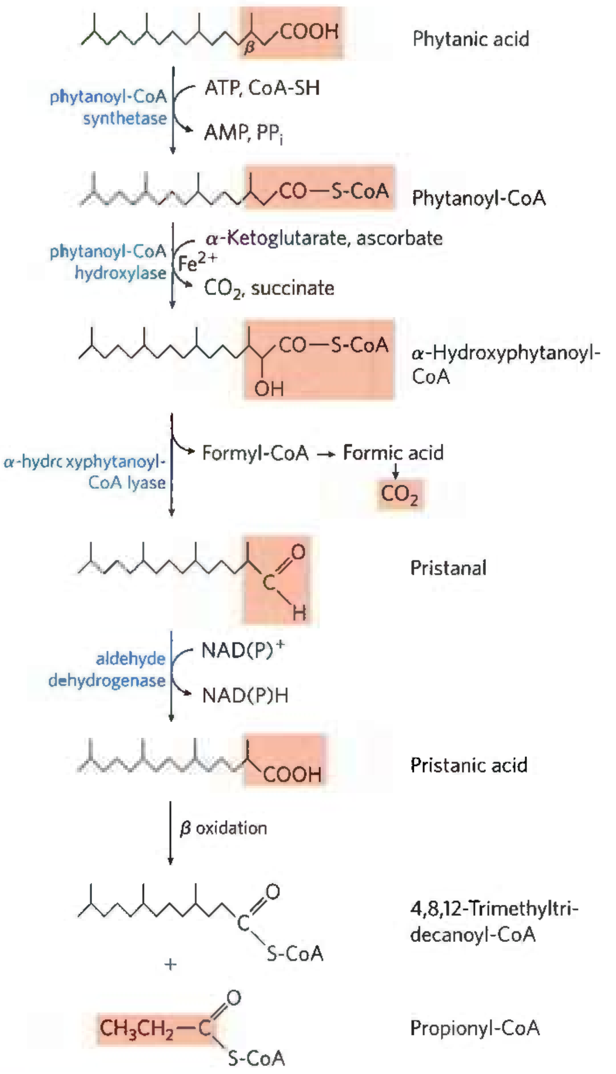


FIGURE 17-15 The α oxidation of a branched-chain fatty acid (phytanic acid) in peroxisomes. Phytanic acid has a methyl-substituted β carbon and therefore cannot undergo β oxidation. The combined action of the enzymes shown here removes the carboxyl carbon of phytanic acid to produce pristanic acid, in which the β carbon is unsubstituted, allowing β oxidation. Notice that β oxidation of pristanic acid releases propionyl-CoA, not acetyl-CoA. This is further catabolized as in Figure 17-12. (The details of the reaction that produces pristanal remain controversial.)

peroxisomes fail to oxidize very-long-chain fatty acids, apparently due to lack of a functional transporter for these fatty acids in the peroxisomal membrane. Both defects lead to accumulation in the blood of very-long-chain fatty acids, especially 26:0. XALD affects young boys before the age of 10 years, causing loss of vision, behavioral disturbances, and death within a few years. ■

In mammals, high concentrations of fats in the diet result in increased synthesis of the enzymes of peroxisomal β oxidation in the liver. Liver peroxisomes do not contain the enzymes of the citric acid cycle and cannot catalyze the oxidation of acetyl-CoA to CO$_2$. Instead, long-chain or branched fatty acids are catabolized in peroxisomes to shorter-chain products, such as hexanoyl-CoA, which are exported to mitochondria and completely oxidized there. As we will see in Chapter 20, the germinating seeds of plants can synthesize carbohydrates and many other metabolites from acetyl-CoA produced in peroxisomes, using a pathway (the glyoxylate cycle) not present in vertebrates.

Phytanic Acid Undergoes *α* Oxidation in Peroxisomes

Phytanic acid, a long-chain fatty acid with methyl branches, is derived from the phytol side chain of chlorophyll (see Fig. 20-5). The presence of a methyl group on the β carbon of this fatty acid prevents the formation of a β-keto intermediate, making its β oxidation impossible. Humans obtain phytanic acid in the diet, primarily from dairy products and from the fats of ruminant animals; microorganisms in the rumen of these animals produce phytanic acid as they digest plant chlorophyll. The typical western diet includes 50 to 100 mg of phytanic acid per day.

Phytanic acid is metabolized in peroxisomes by *α* **oxidation**, in which a single carbon is removed from the carboxyl end of the fatty acid (**Fig. 17-15**). Phytanoyl-CoA is first hydroxylated on its α carbon in a reaction that involves molecular oxygen. The product is decarboxylated to form an aldehyde one carbon shorter, and then oxidized to the corresponding carboxylic acid, which now has no substituent on the β carbon. Further β oxidation produces acetyl-CoA and then propionyl-CoA in successive oxidation cycles. **Refsum disease**, resulting from a genetic defect in phytanoyl-CoA hydroxylase, leads to the accumulation of very high blood levels of phytanic acid, causing (by unknown mechanisms) severe neurological deficits, including blindness and deafness. ■

SUMMARY 17.2 *Oxidation of Fatty Acids*

- In the first stage of β oxidation, four sequential reactions remove each acetyl-CoA unit, in turn, from the carboxyl end of a saturated fatty acyl–CoA: (1) dehydrogenation of the α and β carbons by acyl–CoA dehydrogenases; (2) hydration of the resulting *trans*-Δ^2 double bond by enoyl-CoA hydratase; (3) dehydrogenation of the resulting L-β-hydroxyacyl-CoA; and (4) cleavage of the resulting β-ketoacyl–CoA by thiolase, to form acetyl-CoA and a fatty acyl–CoA shortened by two carbons.

- The shortened fatty acyl–CoA then reenters the β oxidation sequence for removal of another, and then another, acetyl-CoA.

- In the second stage of fatty acid oxidation, the acetyl-CoA is oxidized to CO$_2$ in the citric acid cycle. Much of the free energy from fatty acid oxidation is recovered as ATP by oxidative phosphorylation, the final stage of the oxidative pathway.

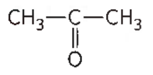
- Oxidation of unsaturated fatty acids requires two additional enzymes: enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase.

- Odd-number fatty acids are oxidized by the β-oxidation pathway to yield acetyl-CoA and a molecule of propionyl-CoA. The propionyl-CoA is carboxylated to methylmalonyl-CoA, which is isomerized to succinyl-CoA in a reaction catalyzed by methylmalonyl-CoA mutase, an enzyme requiring coenzyme B_{12}.

- To prevent futile cycling, entry of fatty acids into mitochondria is blocked by the first intermediate in fatty acid synthesis, malonyl-CoA.
- The transcription factor PPARα stimulates the synthesis of several enzymes required in β oxidation when glucose is not available as an energy source.
- Individuals who lack medium-chain acyl-CoA dehydrogenase experience fatty liver, high blood levels of octanoic acid, coma, and sometimes death.
- Peroxisomes, of plants and animals, and glyoxysomes, of plants, carry out β oxidation in four steps similar to those of the mitochondrial pathway. The first oxidation step, however, transfers electrons directly to O_2, generating H_2O_2. Peroxisomes of animal tissues specialize in the oxidation of very-long-chain fatty acids and branched fatty acids.
- The reactions of α oxidation convert branched fatty acids such as phytanic acid to products that can undergo β oxidation, yielding acetyl-CoA and propionyl-CoA.

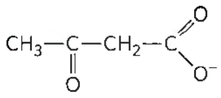
17.3 Ketone Bodies

In humans and most other mammals, acetyl-CoA formed in the liver during oxidation of fatty acids can either enter the citric acid cycle (stage 2 of Fig. 17-7) or undergo conversion to the "**ketone bodies**," **acetone**, **acetoacetate**, and **D-β-hydroxybutyrate**, for export to other tissues. (The term "bodies" is an historical artifact; the compounds are soluble in blood and urine, not particulate, and not all of them are ketones.)

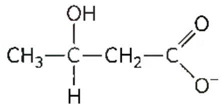


$$CH_3-\underset{\underset{O}{\|}}{C}-CH_3$$

Acetone



Acetoacetate



D-β-Hydroxybutyrate

Acetone, produced in smaller quantities than the other ketone bodies, is exhaled. **P5** Acetoacetate and D-β-hydroxybutyrate are transported by the blood to tissues other than the liver (extrahepatic tissues), where they are converted to acetyl-CoA and oxidized in the citric acid cycle, providing much of the energy required by tissues such as skeletal and heart muscle and the renal cortex. The brain, which preferentially uses glucose as fuel, can adapt to the use of acetoacetate and D-β-hydroxybutyrate under starvation conditions, when glucose is unavailable. The brain cannot use fatty acids as fuel, because they do not cross the blood-brain barrier. The production and export of ketone bodies from the liver to extrahepatic tissues allows continued oxidation of fatty acids in the liver when acetyl-CoA is not being oxidized in the citric acid cycle.

Ketone Bodies, Formed in the Liver, Are Exported to Other Organs as Fuel

The first step in the formation of acetoacetate, occurring in the liver (**Fig. 17-16**), is the enzymatic condensation of two molecules of acetyl-CoA, catalyzed by thiolase; this

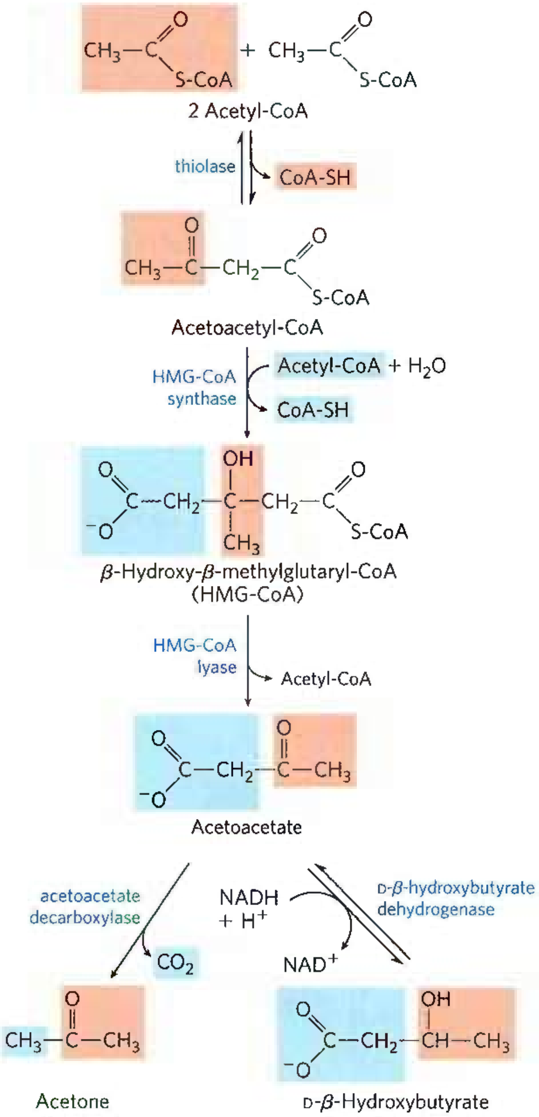


FIGURE 17-16 Formation of ketone bodies from acetyl-CoA. Healthy, well-nourished individuals produce ketone bodies at a relatively low rate. When acetyl-CoA accumulates (as in starvation or untreated diabetes, for example), thiolase catalyzes the condensation of two acetyl-CoA molecules to acetoacetyl-CoA, the parent compound of the three ketone bodies. The reactions of ketone body formation occur in the matrix of liver mitochondria. The six-carbon compound β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) is also an intermediate of sterol biosynthesis, but the enzyme that forms HMG-CoA in that pathway is cytosolic. HMG-CoA lyase is present only in the mitochondrial matrix.

is simply the reversal of the last step of β oxidation. The acetoacetyl-CoA then condenses with another molecule of acetyl-CoA to form **β-hydroxy-β-methylglutaryl-CoA (HMG-CoA)**, which is cleaved to free acetoacetate and acetyl-CoA. The acetoacetate is reversibly reduced by D-β-hydroxybutyrate dehydrogenase, a mitochondrial enzyme, to D-β-hydroxybutyrate. This enzyme is specific for the D stereoisomer; it does not act on L-β-hydroxyacyl-CoAs and is distinct from L-β-hydroxyacyl-CoA dehydrogenase of the β-oxidation pathway. This difference in stereospecificity of the two enzymes that use β-hydroxyacyl-CoAs as substrates in fatty acid breakdown and fatty acid synthesis means that the cell can maintain separate pools of β-hydroxyacyl-CoAs, earmarked for either breakdown or synthesis.

In extrahepatic tissues, D-β-hydroxybutyrate is oxidized to acetoacetate by D-β-hydroxybutyrate dehydrogenase (**Fig. 17-17**). The acetoacetate is activated to its coenzyme A ester by transfer of CoA from succinyl-CoA, an intermediate of the citric acid cycle (see Fig. 16-7), in a reaction catalyzed by **β-ketoacyl-CoA transferase**, also called thiophorase. The acetoacetyl-CoA is then cleaved by thiolase to yield two molecules of acetyl-CoA, which enter the citric acid cycle. Thus the ketone bodies are used as fuels in all tissues except liver, which lacks β-ketoacyl-CoA transferase. **P5** The liver is therefore a producer of ketone bodies for other tissues, but not a consumer.

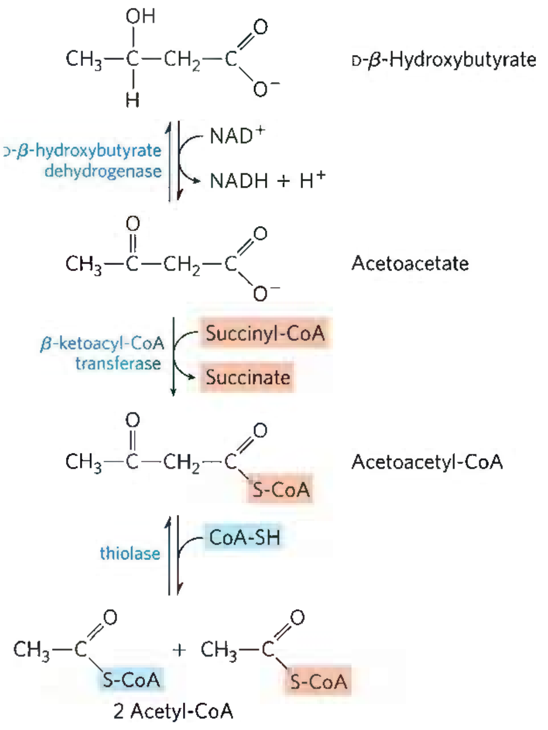

D-β-Hydroxybutyrate
D-β-hydroxybutyrate dehydrogenase
NAD+
NADH + H+
Acetoacetate
β-ketoacyl-CoA transferase
Succinyl-CoA
Succinate
Acetoacetyl-CoA
thiolase
CoA-SH
2 Acetyl-CoA

FIGURE 17-17 **D-β-Hydroxybutyrate as a fuel.** D-β-Hydroxybutyrate, synthesized in the liver, passes into the blood and thus to other tissues, where it is converted in three steps to acetyl-CoA. It is first oxidized to acetoacetate, which is activated with coenzyme A donated from succinyl-CoA, then split by thiolase. The acetyl-CoA thus formed enters the citric acid cycle.

The production and export of ketone bodies by the liver allows continued oxidation of fatty acids with only minimal oxidation of acetyl-CoA. When intermediates of the citric acid cycle are being siphoned off for glucose synthesis by gluconeogenesis, for example, oxidation of cycle intermediates slows—and so does acetyl-CoA oxidation. Moreover, the liver contains only a limited amount of coenzyme A, and when most of it is tied up in acetyl-CoA, β oxidation slows for want of the free coenzyme. The production and export of ketone bodies frees coenzyme A, allowing continued fatty acid oxidation.

Ketone Bodies Are Overproduced in Diabetes and during Starvation

Starvation (including very-low-calorie diets) and untreated diabetes mellitus lead to overproduction of ketone bodies in the liver, with several adverse effects on health. During starvation, gluconeogenesis depletes citric acid cycle intermediates, diverting acetyl-CoA from oxidation of mobilized stored fats to ketone body production (**Fig. 17-18**). In untreated diabetes, when the insulin level is insufficient, extrahepatic tissues cannot take up glucose efficiently from the blood, either for fuel or for conversion to fat. Under these conditions, levels of malonyl-CoA fall, inhibition of carnitine acyltransferase 1 is relieved, and fatty acids enter mitochondria to be degraded to acetyl-CoA (see Fig. 17-13). But this acetyl-CoA cannot pass through the citric acid cycle because cycle intermediates have been drawn off for use as substrates in gluconeogenesis. **P4** **P5** The resulting

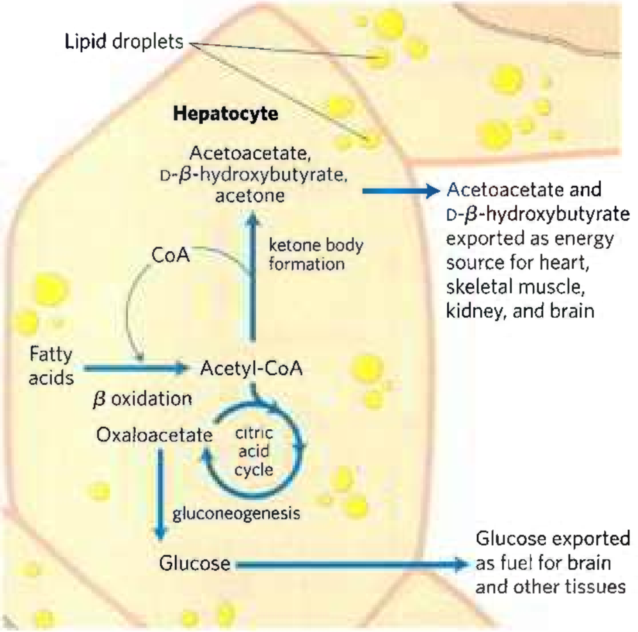

Lipid droplets
Hepatocyte
Acetoacetate, D-β-hydroxybutyrate, acetone
Acetoacetate and D-β-hydroxybutyrate exported as energy source for heart, skeletal muscle, kidney, and brain
CoA
ketone body formation
Fatty acids
Acetyl-CoA
β oxidation
Oxaloacetate
citric acid cycle
gluconeogenesis
Glucose
Glucose exported as fuel for brain and other tissues

FIGURE 17-18 **Ketone body formation and export from the liver.** Conditions that promote gluconeogenesis (untreated diabetes, severely reduced food intake) slow the citric acid cycle (by drawing off oxaloacetate) and enhance the conversion of acetyl-CoA to acetoacetate. The released coenzyme A allows continued β oxidation of fatty acids.

accumulation of acetyl-CoA accelerates the formation of ketone bodies and their release into the blood beyond the capacity of extrahepatic tissues to oxidize them. The increased blood levels of acetoacetate and D-β-hydroxybutyrate lower the blood pH, causing the condition known as **acidosis**. Extreme acidosis can lead to coma and in some cases death. Ketone bodies in the blood and urine of individuals with untreated diabetes can reach extraordinary levels—a blood concentration of 90 mg/100 mL (compared with a normal level of <3 mg/100 mL) and urinary excretion of 5,000 mg/24 h (compared with a normal rate of ≤125 mg/24 h). This condition is called **ketosis** or, when combined with acidosis, **ketoacidosis**.

In healthy people, acetone is formed in very small amounts from acetoacetate, which is easily decarboxylated, either spontaneously or by the action of **acetoacetate decarboxylase** (Fig. 17-16). Because individuals with untreated diabetes produce large quantities of acetoacetate, their blood contains significant amounts of acetone, which is toxic. Acetone is volatile and imparts a characteristic odor to the breath, which is sometimes useful in diagnosing diabetes. ■

SUMMARY 17.3 *Ketone Bodies*

- The ketone bodies—acetone, acetoacetate, and D-β-hydroxybutyrate—are formed in the liver when fatty acids are the principal fuel supporting whole-body metabolism. Acetoacetate and D-β-hydroxybutyrate serve as fuel molecules in extrahepatic tissues, including the brain, through oxidation to acetyl-CoA and entry into the citric acid cycle.
- Overproduction of ketone bodies in uncontrolled diabetes or severely reduced calorie intake can lead to life-threatening ketoacidosis, characterized by high concentrations of ketones in blood and urine and lower blood pH.

KEY TERMS

Terms in bold are defined in the glossary.

β oxidation 601
apolipoprotein 602
chylomicron 603
lipoprotein 603
perilipin 603
free fatty acids 603
serum albumin 603
carnitine shuttle 603
fatty acyl–CoA synthetase 603
fatty acyl–CoA 604
carnitine acyltransferase 1 (CAT1) 605
acyl-carnitine/carnitine cotransporter 605
carnitine acyltransferase 2 (CAT2) 605
electron-transfer flavoprotein (ETF) 607
NADH dehydrogenase (Complex I) 607
trifunctional protein (TFP) 608
methylmalonyl-CoA mutase 613
coenzyme B_{12} 613
malonyl-CoA 613
pernicious anemia 615
intrinsic factor 615
PPAR (peroxisome proliferator-activated receptor) 616
medium-chain acyl-CoA dehydrogenase (MCAD) 616
peroxisome 617
α oxidation 618
ketone bodies 619
acetone 619
acetoacetate 619
D-β-hydroxybutyrate 619
acidosis 621
ketosis 621
ketoacidosis 621

PROBLEMS

1. **Energy in Triacylglycerols** On a per-carbon basis, where does the largest amount of biologically available energy in triacylglycerols reside: in the fatty acid portions or in the glycerol portion? Indicate how knowledge of the chemical structure of triacylglycerols provides the answer.

2. **Effect of PDE Inhibitor on Adipocytes** How would the addition of a cAMP phosphodiesterase (PDE) inhibitor affect the response of an adipocyte to epinephrine? (Hint: See Fig. 12-4.)

3. **Compartmentation in β Oxidation** The activation of free palmitate to its coenzyme A derivative (palmitoyl-CoA) in the cytosol occurs before it can be oxidized in the mitochondrion. After adding palmitate and [^{14}C]coenzyme A to a liver homogenate, you find palmitoyl-CoA isolated from the cytosolic fraction is radioactive, but that isolated from the mitochondrial fraction is not. Explain.

4. **Mutant Carnitine Acyltransferase** What changes in metabolic pattern would result from a mutation in the muscle carnitine acyltransferase 1 in which the mutant protein has lost its affinity for malonyl-CoA but not its catalytic activity?

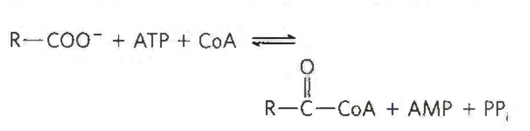
5. **Effect of Carnitine Deficiency** An individual developed a condition characterized by progressive muscular weakness and aching muscle cramps. The symptoms were aggravated by fasting, exercise, and a high-fat diet. An homogenate of a skeletal muscle specimen from the patient oxidized added oleate more slowly than did control homogenates consisting of muscle specimens from healthy individuals. When the pathologist added carnitine to the patient's muscle homogenate, the rate of oleate oxidation equaled that in the control homogenates. Based on these results, the attending physician diagnosed the patient as having a carnitine deficiency.
(a) Why did added carnitine increase the rate of oleate oxidation in the patient's muscle homogenate?
(b) Why did fasting, exercise, and a high-fat diet aggravate the patient's symptoms?
(c) Suggest two possible reasons for the deficiency of muscle carnitine in this individual.

6. **Fuel Reserves in Adipose Tissue** Triacylglycerols, with their hydrocarbon-like fatty acids, have the highest energy content of the major nutrients.
(a) If 15% of the body mass of a 70.0 kg adult consists of triacylglycerols, what is the total available fuel reserve, in both kilojoules and kilocalories, in the form of triacylglycerols? Recall that 1.00 kcal = 4.18 kJ.
(b) If the basal energy requirement is approximately 8,400 kJ/day (2,000 kcal/day), how long could this person survive if the oxidation of fatty acids stored as triacylglycerols were the only source of energy?
(c) What would be the weight loss in pounds per day under such starvation conditions (1 lb = 0.454 kg)?

7. Common Reaction Steps in the Fatty Acid Oxidation Cycle and Citric Acid Cycle Cells often use the same enzyme reaction pattern for analogous metabolic conversions. For example, the steps in the oxidation of pyruvate to acetyl-CoA and of α-ketoglutarate to succinyl-CoA, although catalyzed by different enzymes, are very similar. The first stage of fatty acid oxidation follows a reaction sequence closely resembling a sequence in the citric acid cycle. Use equations to show the analogous reaction sequences in the two pathways.

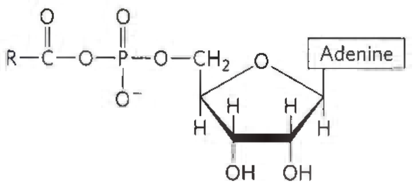
8. β Oxidation: How Many Cycles? How many cycles of β oxidation are required for the complete oxidation of activated oleic acid, 18:1(Δ^9)?

9. Chemistry of the Acyl-CoA Synthetase Reaction Fatty acids are converted to their coenzyme A esters in a reversible reaction catalyzed by acyl-CoA synthetase:



$$R{-}COO^- + ATP + CoA \rightleftharpoons R{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}CoA + AMP + PP_i$$

(a) The enzyme-bound intermediate in this reaction has been identified as the mixed anhydride of the fatty acid and adenosine monophosphate (AMP), acyl-AMP:



Write two equations corresponding to the two steps of the reaction catalyzed by acyl-CoA synthetase.
(b) The acyl-CoA synthetase reaction is readily reversible, with an equilibrium constant near 1. How can this reaction be made to favor formation of fatty acyl–CoA?

10. Intermediates in Oleic Acid Oxidation What is the structure of the partially oxidized fatty acyl group that is formed when oleic acid, 18:1(Δ^9), has undergone three cycles of β oxidation? What are the next two steps in the continued oxidation of this intermediate?

11. β Oxidation of an Odd-Number Fatty Acid What are the direct products of β oxidation of a fully saturated, straight-chain fatty acid of 11 carbons?

12. Oxidation of Tritiated Palmitate An investigator adds palmitate uniformly labeled with tritium (^{3}H) to a specific activity of 2.48×10^8 counts per minute (cpm) per micromole of palmitate to a mitochondrial preparation that oxidizes it to acetyl-CoA. She then isolates the acetyl-CoA and hydrolyzes it to acetate. The specific activity of the isolated acetate is 1.00×10^7 cpm/μmol. Is this result consistent with the β-oxidation pathway? Explain. What is the final fate of the removed tritium? (Note: Specific activity is a measure of the degree of labeling with a radioactive tracer expressed as radioactivity per unit mass. In a uniformly labeled compound, all atoms of a given type are labeled.)

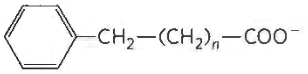
13. Comparative Biochemistry: Energy-Generating Pathways in Birds One indication of the relative importance of various ATP-producing pathways is the V_{max} of certain enzymes of these pathways. The values of V_{max} of several enzymes from the pectoral muscles (chest muscles used for flying) of pigeon and pheasant are listed below.

	V_{max} (μmol substrate/min/g tissue)	
Enzyme	**Pigeon**	**Pheasant**
Hexokinase	3.0	2.3
Glycogen phosphorylase	18.0	120.0
Phosphofructokinase-1	24.0	143.0
Citrate synthase	100.0	15.0
Triacylglycerol lipase	0.07	0.01

(a) Discuss the relative importance of glycogen metabolism and fat metabolism in generating ATP in the pectoral muscles of these birds.
(b) Compare oxygen consumption in the two birds.
(c) Judging from the data in the table, which bird is the long-distance flyer? Justify your answer.
(d) Why were these particular enzymes selected for comparison? Would the activities of triose phosphate isomerase and malate dehydrogenase be equally good bases for comparison? Explain.

14. Fatty Acids as a Source of Water Contrary to legend, camels do not store water in their humps, which actually consist of large fat deposits. How can these fat deposits serve as a source of water? Calculate the amount of water (in liters) that a camel can produce from 1.0 kg of fat. Assume for simplicity that the fat consists entirely of tripalmitoylglycerol.

15. Metabolism of a Straight-Chain Phenylated Fatty Acid Investigators isolate a crystalline metabolite from the urine of a rabbit that has been fed a straight-chain fatty acid containing a terminal phenyl group:



The addition of 22.2 mL of 0.100 M NaOH completely neutralized a 302 mg sample of the metabolite in aqueous solution.
(a) What is the probable molecular weight and structure of the metabolite?
(b) Did the straight-chain fatty acid contain an even or an odd number of methylene (—CH_2—) groups (i.e., is n even or odd)? Explain.

16. Fatty Acid Oxidation in Uncontrolled Diabetes When the acetyl-CoA produced during β oxidation in the liver exceeds the capacity of the citric acid cycle, the excess acetyl-CoA forms ketone bodies—acetone, acetoacetate, and D-β-hydroxybutyrate. This occurs in people with severe, uncontrolled diabetes; because their tissues cannot use glucose, they oxidize large amounts of fatty acids instead. Although acetyl-CoA is not toxic, the mitochondrion must divert the acetyl-CoA to ketone bodies. What problem would arise if acetyl-CoA were not converted to ketone bodies? How does the diversion to ketone bodies solve the problem?

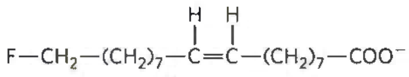
17. Consequences of a High-Fat Diet with No Carbohydrates Suppose you had to subsist on a diet of whale blubber and seal blubber, with little or no carbohydrate.

(a) What would be the effect of carbohydrate deprivation on the utilization of fats for energy?

(b) If your diet were totally devoid of carbohydrate, would it be better to consume odd- or even-number fatty acids? Explain.

18. Even- and Odd-Number Fatty Acids in the Diet In a laboratory experiment, investigators feed two groups of rats two different fatty acids as their sole source of carbon for a month. The first group gets heptanoic acid (7:0), and the second gets octanoic acid (8:0). After the experiment, those in the first group are healthy and have gained weight, whereas those in the second group are weak and have lost weight as a result of losing muscle mass. What is the biochemical basis for this difference?

19. Metabolic Consequences of Ingesting ω-Fluorooleate The shrub *Dichapetalum toxicarium,* native to Sierra Leone, produces ω-fluorooleate, which is highly toxic to warm-blooded animals.



$$F{-}CH_2{-}(CH_2)_7{-}\overset{H}{\overset{|}{C}}{=}\overset{H}{\overset{|}{C}}{-}(CH_2)_7{-}COO^-$$

ω-Fluorooleate

This substance has been used as an arrow poison, and powdered fruit from the plant is sometimes used as a rat poison (hence the plant's common name, ratsbane). Why is this substance so toxic? (Hint: Review Chapter 16, Problem 21.)

20. Mutant Acetyl-CoA Carboxylase What would be the consequences for fat metabolism of a mutation in acetyl-CoA carboxylase that replaced the Ser residue normally phosphorylated by AMPK with an Ala residue? What might happen if the same Ser were replaced by Asp? (Hint: Compare the structures of phosphoserine, alanine, aspartate; see Fig. 17-13.)

21. Role of FAD as Electron Acceptor Acyl-CoA dehydrogenase uses enzyme-bound FAD as a prosthetic group to dehydrogenate the α and β carbons of fatty acyl–CoA. What is the advantage of using FAD as an electron acceptor rather than NAD^+? Explain in terms of the standard reduction potentials for the Enz-FAD/$FADH_2$ ($E'^\circ = -0.219$ V) and NAD^+/NADH ($E'^\circ = -0.320$ V) half-reactions.

22. β Oxidation of Arachidic Acid How many turns of the fatty acid oxidation cycle are required for complete oxidation of arachidic acid (20:0) to acetyl-CoA?

23. Fate of Labeled Propionate Adding [3-^{14}C]propionate (^{14}C in the methyl group) to a liver homogenate leads to the rapid production of ^{14}C-labeled oxaloacetate. Draw a flowchart for the pathway by which propionate is transformed to oxaloacetate, and indicate the location of the ^{14}C in oxaloacetate.

24. Phytanic Acid Metabolism A mouse fed phytanic acid uniformly labeled with ^{14}C produces detectable levels of radioactive malate, a citric acid cycle intermediate, within minutes. Draw a metabolic pathway that could account for this. Which of the carbon atoms in malate would contain ^{14}C label?

25. Sources of H_2O Produced in β Oxidation The complete oxidation of palmitoyl-CoA to carbon dioxide and water is represented by the overall equation

$$\text{Palmitoyl-CoA} + 23O_2 + 108P_i + 108\text{ADP} \longrightarrow \text{CoA} + 16CO_2 + 108\text{ATP} + 23H_2O$$

Water also forms in the reaction

$$\text{ADP} + P_i \longrightarrow \text{ATP} + H_2O$$

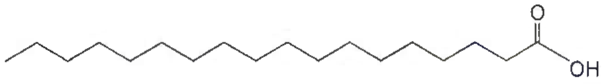
but is not included as a product in the overall equation. Why?

26. Biological Importance of Cobalt Cattle, deer, sheep, and other ruminant animals produce large amounts of propionate in the rumen through the bacterial fermentation of ingested plant matter. Propionate is the principal source of glucose for these animals, via the route propionate ⟶ oxaloacetate ⟶ glucose. In some areas of the world, notably Australia, ruminant animals sometimes show symptoms of anemia with concomitant loss of appetite and retarded growth, resulting from an inability to transform propionate to oxaloacetate. This condition is due to a cobalt deficiency caused by very low cobalt levels in the soil and thus in plant matter. Explain.

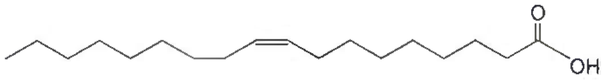
27. Fat Loss during Hibernation Bears expend about 25×10^6 J/day during periods of hibernation, which may last as long as seven months. The energy required to sustain life is obtained from fatty acid oxidation. How much weight (in kilograms) do bears lose after 7 months of hibernation? How could a bear's body minimize ketosis during hibernation? (Assume the oxidation of fat yields 38 kJ/g.)

DATA ANALYSIS PROBLEM

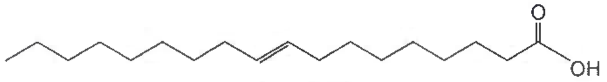
28. β Oxidation of Trans Fats Unsaturated fats with trans double bonds are commonly referred to as "trans fats." In their investigations of the effects of trans fatty acid metabolism on health, Yu and colleagues (2004) showed that a model trans fatty acid was processed differently from its cis isomer. They used three related 18-carbon fatty acids to explore the difference in β oxidation between cis and trans isomers of the same-size fatty acid.



Stearic acid
(octadecenoic acid)

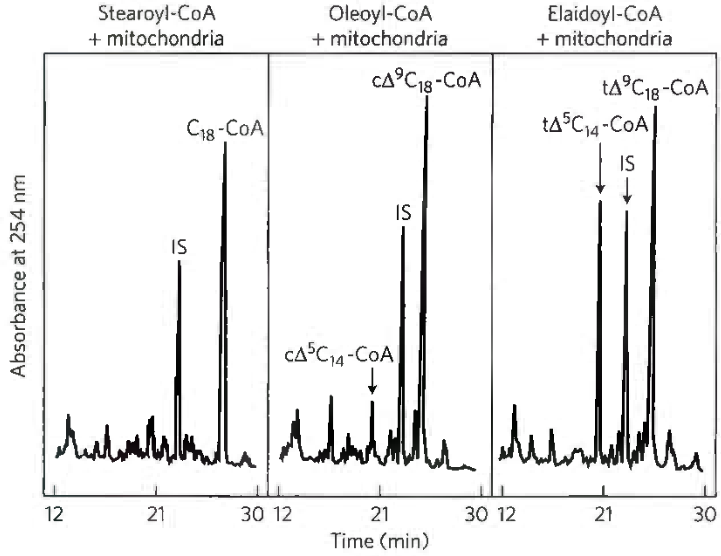


Oleic acid
(*cis*-Δ^9-octadecenoic acid)

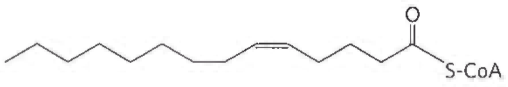


Elaidic acid
(*trans*-Δ^9-octadecenoic acid)

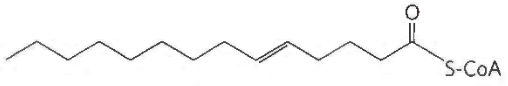
The researchers incubated the coenzyme A derivative of each acid with rat liver mitochondria for 5 minutes, then separated the remaining CoA derivatives in each mixture by HPLC (high-performance liquid chromatography). The results are shown below, with separate panels for the three experiments.



In the figure, IS indicates an internal standard (pentadecanoyl-CoA) added to the mixture after the reaction as a molecular marker. The researchers abbreviated the CoA derivatives as follows: stearoyl-CoA, C_{18}-CoA; *cis*-Δ^5-tetradecenoyl-CoA, $c\Delta^5C_{14}$-CoA; oleoyl-CoA, $c\Delta^9C_{18}$-CoA; *trans*-Δ^5-tetradecenoyl-CoA, $t\Delta^5C_{14}$-CoA; and elaidoyl-CoA, $t\Delta^9C_{18}$-CoA.



cis-Δ^5-Tetradecenoyl-CoA



trans-Δ^5-Tetradecenoyl-CoA

(a) Why did Yu and colleagues need to use CoA derivatives rather than the free fatty acids in these experiments?

(b) Why were no lower molecular weight CoA derivatives found in the reaction with stearoyl-CoA?

(c) How many rounds of β oxidation would be required to convert the oleoyl-CoA and the elaidoyl-CoA to *cis*-Δ^5-tetradecenoyl-CoA and *trans*-Δ^5-tetradecenoyl-CoA, respectively?

Yu and coworkers measured the kinetic parameters of two forms of the enzyme acyl-CoA dehydrogenase: long-chain acyl-CoA dehydrogenase (LCAD) and very-long-chain acyl-CoA dehydrogenase (VLCAD). They used the CoA derivatives of three fatty acids: tetradecanoyl-CoA (C_{14}-CoA), *cis*-Δ^5-tetradecenoyl-CoA ($c\Delta^5C_{14}$-CoA), and *trans*-Δ^5-tetradecenoyl-CoA ($t\Delta^5C_{14}$-CoA). The results are shown below. (See Chapter 6 for definitions of the kinetic parameters.)

	LCAD			**VLCAD**		
	C_{14}-CoA	**$c\Delta^5C_{14}$-CoA**	**$t\Delta^5C_{14}$-CoA**	**C_{14}-CoA**	**$c\Delta^5C_{14}$-CoA**	**$t\Delta^5C_{14}$-CoA**
V_{max}	3.3	3.0	2.9	1.4	0.32	0.88
K_m	0.41	0.40	1.6	0.57	0.44	0.97
k_{cat}	9.9	8.9	8.5	2.0	0.42	1.12
k_{cat}/K_m	24	22	5	4	1	1

(d) For LCAD, the K_m differs dramatically for the cis and trans substrates. Provide a plausible explanation for this observation in terms of the structures of the substrate molecules. (Hint: You may want to refer to Fig. 10-1.)

(e) The kinetic parameters of the two enzymes are relevant to the differential processing of these fatty acids *only* if the LCAD or VLCAD reaction (or both) is the rate-limiting step in the pathway. What evidence is there to support this assumption?

(f) How do these different kinetic parameters explain the different levels of the CoA derivatives found after incubation of rat liver mitochondria with stearoyl-CoA, oleoyl-CoA, and elaidoyl-CoA (shown in the three-panel figure)?

Yu and coworkers measured the substrate specificity of rat liver mitochondrial thioesterase, which hydrolyzes acyl-CoA to CoA and free fatty acid. This enzyme was approximately twice as active with C_{14}-CoA thioesters as with C_{18}-CoA thioesters.

(g) Other research has suggested that free fatty acids can pass through membranes. In their experiments, Yu and colleagues found *trans*-Δ^5-tetradecenoic acid outside (i.e., in the medium surrounding) mitochondria that had been incubated with elaidoyl-CoA. Describe the pathway that led to this extramitochondrial *trans*-Δ^5-tetradecenoic acid. Be sure to indicate where in the cell the various transformations take place, as well as the enzymes that catalyze the transformations.

(h) It is sometimes said in the popular press that "trans fats are not broken down by your cells and instead accumulate in your body." In what sense is this statement correct and in what sense is it an oversimplification?

Reference

Yu, W., X. Liang, R. Ensenauer, J. Vockley, L. Sweetman, and H. Schultz. 2004. Leaky β-oxidation of a *trans*-fatty acid. *J. Biol. Chem.* 279:52,160–52,167.

Chapter 18

AMINO ACID OXIDATION AND THE PRODUCTION OF UREA

18.1 Metabolic Fates of Amino Groups *626*

18.2 Nitrogen Excretion and the Urea Cycle *633*

18.3 Pathways of Amino Acid Degradation *639*

We now turn our attention to the amino acids, the final class of biomolecules that, through their oxidative degradation, make a significant contribution to the generation of metabolic energy. The fraction of metabolic energy obtained from amino acids, whether they are derived from dietary protein or from tissue protein, varies greatly with the type of organism and with metabolic conditions. Carnivores consume primarily protein and thus must obtain most of their energy from amino acids, whereas herbivores may fill only a small fraction of their energy needs by this route. Most microorganisms can scavenge amino acids from their environment and use them as fuel when required by metabolic conditions. Plants, however, rarely if ever oxidize amino acids to provide energy; the carbohydrate produced from CO_2 and H_2O in photosynthesis is generally their sole energy source. Amino acid concentrations in plant tissues are carefully regulated to just meet the requirements for biosynthesis of proteins, nucleic acids, and other molecules needed to support growth. Amino acid catabolism does occur in plants, but only to produce metabolites for other biosynthetic pathways.

Amino acid oxidation pathways can seem complex, and they are best understood in the context of five principles:

P1 **The many paths for amino acid catabolism have two broad parts, one involving the amino groups and the other involving the carbon skeletons.** All of the pathways for amino acid degradation include a key step, always involving a pyridoxal phosphate cofactor, in which the α-amino group is separated from the carbon skeleton and shunted into the pathways of amino group metabolism. The carbon skeletons are broken down to citric acid cycle intermediates (**Fig. 18-1**).

P2 **Four amino acids—alanine, glutamate, glutamine, and aspartate—play key roles in the transport and distribution of amino groups.** All are present in relatively high concentrations in one or many mammalian tissues. All are readily converted to key citric acid cycle intermediates.

P3 **Metabolic pathways are not distinct.** The various pathways for amino acid catabolism are elaborately intertwined with other catabolic and anabolic pathways.

P4 **Free ammonia is toxic.** Excess amino groups must be safely excreted. In mammals, the urea cycle serves this purpose.

P5 **Each amino acid has a different catabolic fate.** The varied carbon skeletons of amino acids are broken down via equally varied pathways. All can be oxidized to generate ATP. All but leucine and lysine can contribute to gluconeogenesis when needed.

In mammals, blood glucose must be supplemented by gluconeogenesis, often just a few hours after a meal. Amino acids, particularly alanine and glutamine, can make a significant contribution to the fuel for gluconeogenesis. Amino acids undergo oxidative degradation in three different metabolic circumstances:

1. Amino acids released during normal protein turnover are not needed for new protein synthesis.
2. Ingested amino acids exceed the body's needs for protein synthesis.
3. Cellular proteins are used as fuel because carbohydrates are either unavailable or not properly utilized due to starvation or uncontrolled diabetes mellitus.

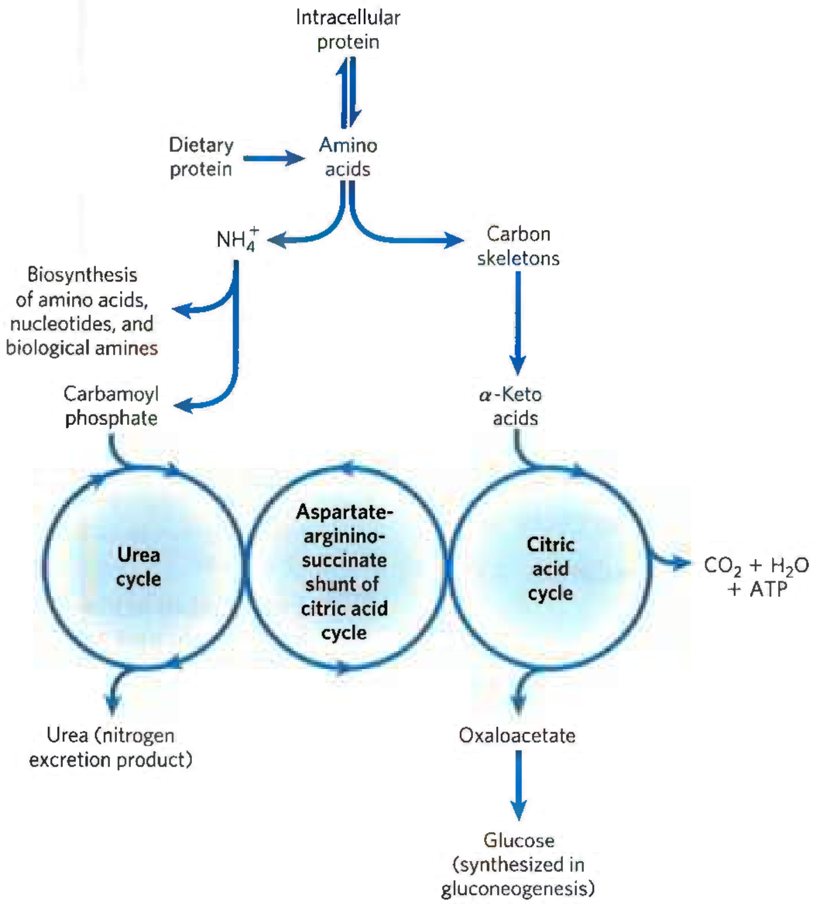

Intracellular protein
Dietary protein
Amino acids
NH_4^+
Carbon skeletons
Biosynthesis of amino acids, nucleotides, and biological amines
Carbamoyl phosphate
α-Keto acids
Urea cycle
Aspartate-argininosuccinate shunt of citric acid cycle
Citric acid cycle
$CO_2 + H_2O + ATP$
Urea (nitrogen excretion product)
Oxaloacetate
Glucose (synthesized in gluconeogenesis)

FIGURE 18-1 Overview of amino acid catabolism in mammals. The amino groups and the carbon skeleton take separate but interconnected pathways.

The pathways of amino acid catabolism are quite similar in most organisms. The focus of this chapter is on the pathways in vertebrates, because these have received the most research attention. As in carbohydrate and fatty acid catabolism, the processes of amino acid degradation converge on the central catabolic pathways, with the carbon skeletons of most amino acids finding their way to the citric acid cycle. In some cases, the reaction pathways of amino acid breakdown closely parallel steps in the catabolism of fatty acids (see Fig. 17-9). We begin with amino group metabolism and nitrogen excretion, before turning to the fate of the carbon skeletons derived from the amino acids; along the way we see how the pathways are interconnected.

18.1 Metabolic Fates of Amino Groups

Nitrogen, N_2, is abundant in the atmosphere but is too unreactive for use in most biochemical processes. Reduced nitrogen is essential for life but bioenergetically expensive. Only a few microorganisms can convert N_2 to biologically useful forms such as NH_3 (Chapter 22). Amino groups are thus used efficiently in biological systems, and the lack of reactive nitrogen can limit growth.

P4 If not reused for the synthesis of new amino acids or other nitrogenous products, amino groups are channeled into a single excretory end product (**Fig. 18-2**). Most aquatic species, such as the bony fishes, are **ammonotelic**, excreting amino nitrogen as ammonia. The toxic ammonia is simply diluted in the surrounding water. Terrestrial animals require pathways for nitrogen excretion that minimize toxicity and water loss. Most terrestrial animals are **ureotelic**, excreting amino nitrogen in the form of urea; birds and reptiles are **uricotelic**, excreting amino nitrogen as uric acid. (The pathway of uric acid synthesis is described in Fig. 22-48.) Plants recycle virtually all amino groups—they excrete nitrogen only under rare circumstances. Reactive nitrogen excreted in any form is rapidly assimilated into the global nitrogen web (see Fig. 22-1), metabolized by microorganisms that are ubiquitous in aqueous and soil environments.

Figure 18-2a provides an overview of the catabolic pathways of ammonia and amino groups in vertebrates. Glutamine, glutamate, and alanine are prominent in this scheme. Amino acids derived from dietary protein are the source of most amino groups. Most amino acids are metabolized in the liver. Some of the ammonia generated in this process is recycled and used in biosynthetic

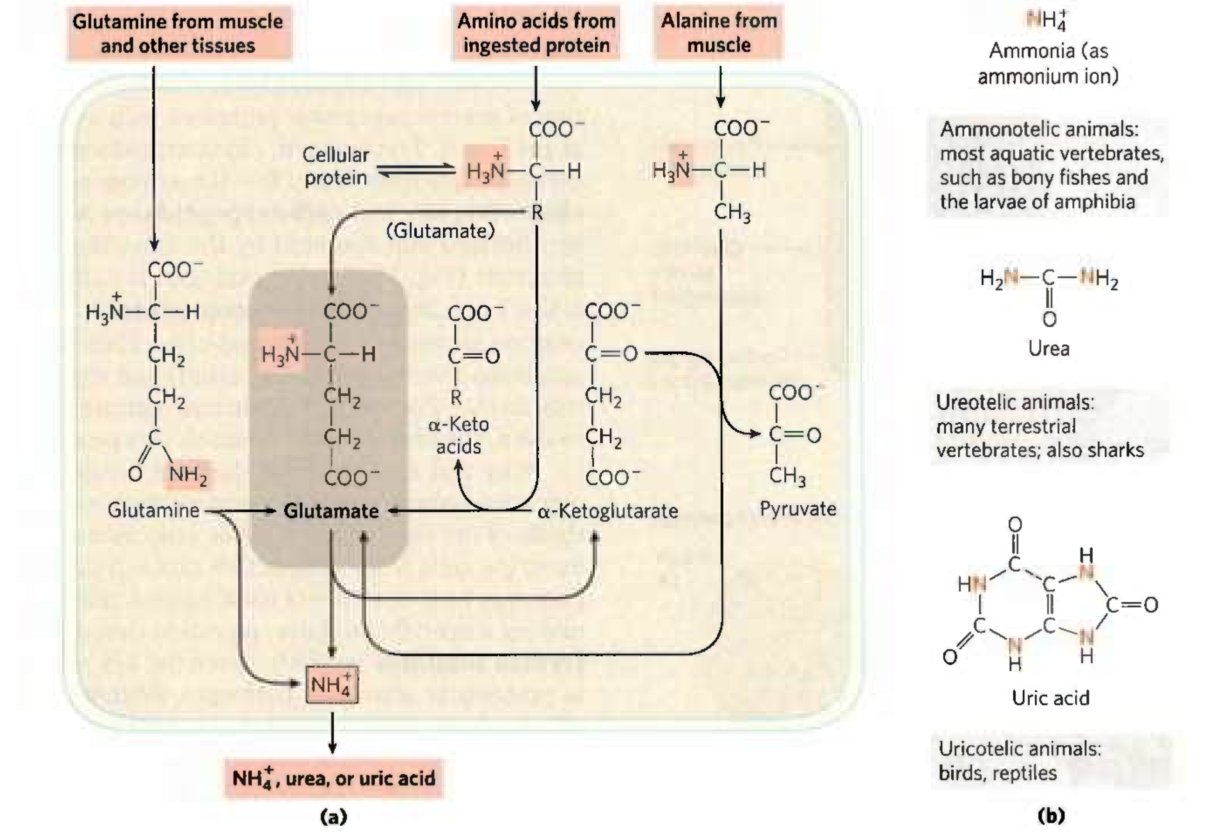

Glutamine from muscle and other tissues
Amino acids from ingested protein
Alanine from muscle
Cellular protein
(Glutamate)
α-Keto acids
Glutamine
Glutamate
α-Ketoglutarate
Pyruvate
NH_4^+
NH_4^+, urea, or uric acid
(a)
NH_4^+
Ammonia (as ammonium ion)
Ammonotelic animals: most aquatic vertebrates, such as bony fishes and the larvae of amphibia
Urea
Ureotelic animals: many terrestrial vertebrates; also sharks
Uric acid
Uricotelic animals: birds, reptiles
(b)

FIGURE 18-2 Amino group catabolism. (a) Overview of catabolism of amino groups (shaded) in vertebrate liver. (b) Excretory forms of nitrogen. Excess NH_4^+ is excreted as ammonia, urea, or uric acid. Notice that the carbon atoms of urea and uric acid are highly oxidized; the organism discards carbon only after extracting most of its available energy of oxidation.

pathways where glutamine, glutamate, and aspartate play major roles (Chapter 22). Excess amino groups are either excreted directly or converted to urea or uric acid for excretion, depending on the organism (Fig. 18-2b). In mammals, including marsupials, most excess ammonia generated in other (extrahepatic) tissues travels to the liver for conversion to urea.

P2 The special place of glutamate, glutamine, alanine, and aspartate in nitrogen metabolism is not an evolutionary accident. These particular amino acids are the ones most easily converted into citric acid cycle intermediates: glutamate and glutamine to α-ketoglutarate, alanine to pyruvate, and aspartate to oxaloacetate. Glutamate and glutamine are especially important, acting as general collection points for amino groups. In the cytosol of liver cells (hepatocytes), amino groups from most amino acids are transferred to α-ketoglutarate to form glutamate, which enters mitochondria and gives up its amino group to form NH_4^+. Excess ammonia generated in most other tissues is converted to the amide nitrogen of glutamine, which passes to the liver, then into liver mitochondria. Glutamine or glutamate or both are present in higher concentrations than other amino acids in most tissues. In skeletal muscle, excess amino groups are generally transferred to pyruvate to form alanine, another important molecule in the transport of amino groups to the liver. We begin with a discussion of the breakdown of dietary proteins, then proceed to a general description of the metabolic fates of amino groups.

Dietary Protein Is Enzymatically Degraded to Amino Acids

In humans, the degradation of ingested proteins to their constituent amino acids occurs in the gastrointestinal tract. Entry of dietary protein into the stomach stimulates the gastric mucosa to secrete the hormone **gastrin**, which in turn stimulates the secretion of hydrochloric acid by the parietal cells and pepsinogen by the chief cells of the gastric glands (**Fig. 18-3a**). The acidic gastric juice (pH 1.0 to 2.5) is both an antiseptic, killing most bacteria and other foreign cells, and a denaturing agent, unfolding globular proteins and rendering their internal peptide bonds more accessible to enzymatic hydrolysis. **Pepsinogen** (M_r 40,554), an inactive precursor, or zymogen (p. 220), is converted to active pepsin (M_r 34,614) by an autocatalytic cleavage (a cleavage mediated by the pepsinogen itself) that occurs only at low pH. In the stomach, pepsin cleaves long polypeptide chains into a mixture of smaller peptides.

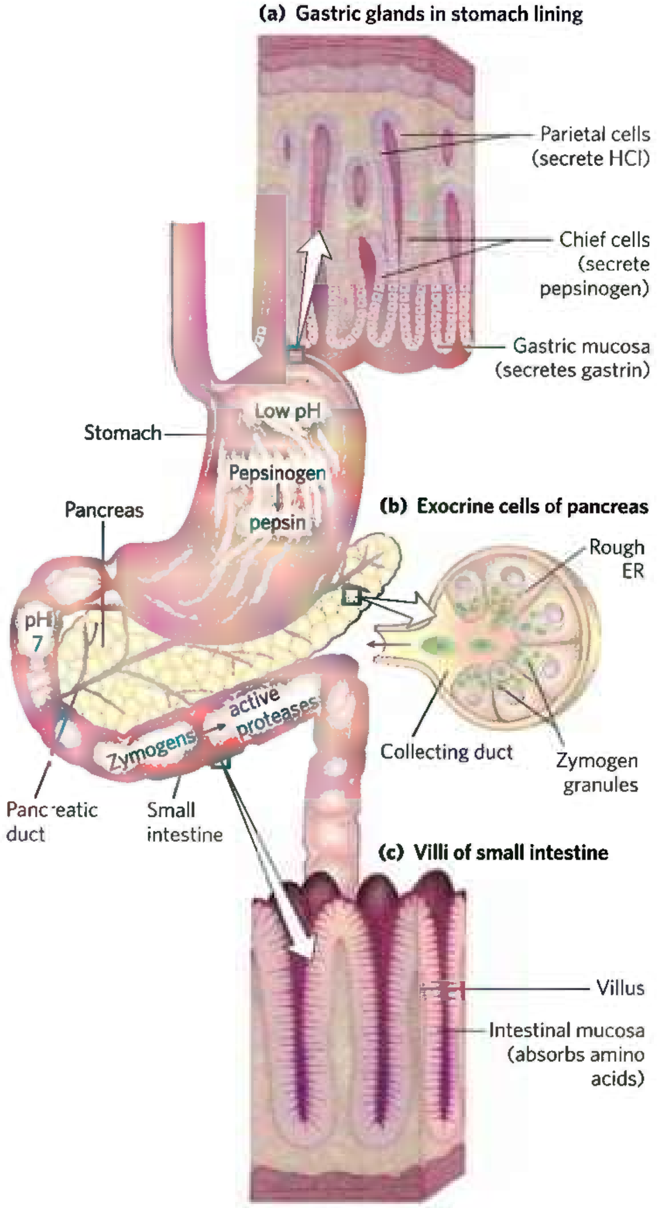

(a) Gastric glands in stomach lining
Parietal cells (secrete HCl)
Chief cells (secrete pepsinogen)
Gastric mucosa (secretes gastrin)
Stomach
Low pH
Pepsinogen
pepsin
Pancreas
pH 7
(b) Exocrine cells of pancreas
Rough ER
Zymogens
active proteases
Collecting duct
Zymogen granules
Pancreatic duct
Small intestine
(c) Villi of small intestine
Villus
Intestinal mucosa (absorbs amino acids)

FIGURE 18-3 Part of the human digestive (gastrointestinal) tract. (a) The parietal cells and chief cells of the gastric glands secrete their products in response to the hormone gastrin. Pepsin begins the process of protein degradation in the stomach. (b) The cytoplasm of exocrine cells of the pancreas is completely filled with rough endoplasmic reticulum, the site of synthesis of the zymogens of many digestive enzymes. The zymogens are concentrated in membrane-enclosed transport particles called zymogen granules. When an exocrine cell is stimulated, its plasma membrane fuses with the zymogen granule membrane and zymogens are released into the lumen of the collecting duct by exocytosis. The collecting ducts ultimately lead to the pancreatic duct and from there to the small intestine. (c) In the small intestine, amino acids are absorbed through the epithelial cell layer (intestinal mucosa) of the villi and enter the capillaries.

As the acidic stomach contents pass into the small intestine, the low pH triggers secretion of the hormone **secretin** into the blood. Secretin stimulates the pancreas to secrete bicarbonate into the small intestine to neutralize the gastric HCl, abruptly increasing the pH to about 7. Arrival of peptides in the upper part of the intestine (duodenum) causes release into the blood of the hormone **cholecystokinin**, which stimulates secretion of several pancreatic proteases with activity optima at pH 7 to 8. Trypsinogen, chymotrypsinogen, and procarboxypeptidases A and B—the zymogens of **trypsin**, **chymotrypsin**, and **carboxypeptidases A** and **B**—are synthesized and secreted by the exocrine cells of the pancreas (Fig. 18-3b). Trypsinogen is converted to its active form, trypsin, by **enteropeptidase**, a proteolytic enzyme secreted by intestinal cells. Free trypsin then catalyzes the conversion of additional trypsinogen to trypsin (see Fig. 6-42). Trypsin also activates chymotrypsinogen, the procarboxypeptidases, and proelastase.

Why this elaborate mechanism for getting active digestive enzymes into the gastrointestinal tract? Synthesis of the enzymes as inactive precursors protects the exocrine cells from destructive proteolytic attack. The pancreas further protects itself against self-digestion by making a specific inhibitor, a protein called **pancreatic trypsin inhibitor** (p. 220). Given the key role of trypsin in proteolytic activation pathways, inhibition of trypsin effectively prevents premature production of active proteolytic enzymes within pancreatic cells.

Trypsin and chymotrypsin further hydrolyze the peptides that were produced by pepsin in the stomach. This stage of protein digestion is accomplished very efficiently, because pepsin, trypsin, chymotrypsin, and the carboxypeptidases have different catalytic specificities and cleave different sets of peptide bonds (see Table 3-6). The resulting mixture of free amino acids is transported into the epithelial cells lining the small intestine (Fig. 18-3c), through which the amino acids enter the blood capillaries in the villi and travel to the liver.

Acute pancreatitis is a disease caused by obstruction of the normal pathway by which pancreatic secretions enter the intestine. The zymogens of the proteolytic enzymes are converted to their catalytically active forms prematurely, *inside* the pancreatic cells, and attack the pancreatic tissue itself. This causes excruciating pain and damage to the organ that can prove fatal. ■

Pyridoxal Phosphate Participates in the Transfer of α-Amino Groups to α-Ketoglutarate

P1 The first step in the catabolism of most L-amino acids, once they have reached the liver, is removal of the α-amino groups, promoted by enzymes called **aminotransferases** or **transaminases**. In these **transamination** reactions, the α-amino group is transferred to the α-carbon atom of α-ketoglutarate, leaving behind the corresponding α-keto acid analog of the amino acid (**Fig. 18-4**). There is no net deamination (loss of amino groups) in these reactions, because the α-ketoglutarate becomes aminated as the α-amino acid is deaminated. P2 These are the reactions that effectively collect the amino groups from many different amino acids in the form of L-glutamate.

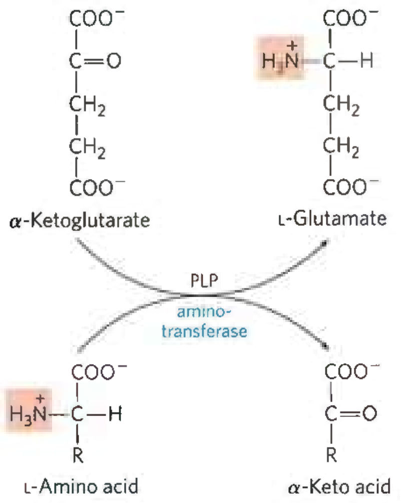


FIGURE 18-4 Enzyme-catalyzed transaminations. In many aminotransferase reactions, α-ketoglutarate is the amino group acceptor. All aminotransferases have pyridoxal phosphate (PLP) as cofactor. Although the reaction is shown here in the direction of transfer of the amino group to α-ketoglutarate, it is readily reversible.

Cells contain different types of aminotransferases. Many are specific for α-ketoglutarate as the amino group acceptor but differ in their specificity for the L-amino acid. The enzymes are named for the amino group donor (alanine aminotransferase and aspartate aminotransferase, for example). The reactions catalyzed by aminotransferases are freely reversible, having an equilibrium constant of about 1.0 ($\Delta G'^{\circ} \approx 0$ kJ/mol).

P1 All aminotransferases have **pyridoxal phosphate (PLP)**, the coenzyme form of pyridoxine, or vitamin B_6, as a prosthetic group. We encountered pyridoxal phosphate in Chapter 15, as a coenzyme in the glycogen phosphorylase reaction, but its role in that reaction is not representative of its usual coenzyme function. Its primary role in cells is in the metabolism of molecules with amino groups.

Pyridoxal phosphate functions as an intermediate carrier of amino groups at the active site of aminotransferases. It undergoes reversible transformations between its aldehyde form, pyridoxal phosphate, which can accept an amino group, and its aminated form, pyridoxamine phosphate, which can donate its amino group to an α-keto acid (**Fig. 18-5a**). Pyridoxal phosphate is generally covalently bound to the enzyme's active site through an aldimine (Schiff base) linkage to the ε-amino group of a Lys residue (Fig. 18-5b, c). This linkage is replaced by the amino group of the amino acid as the first step in most PLP-catalyzed reactions (Fig. 18-5d).

Pyridoxal phosphate participates in a variety of reactions in amino acid metabolism (Section 18.3 and Chapter 22), facilitating reactions at the α, β, and γ carbons (C-2 to C-4) of amino acids. Reactions at the α carbon (**Fig. 18-6**) include racemizations (interconverting L- and D-amino acids) and decarboxylations, as well as transaminations. Pyridoxal phosphate plays the same chemical role in each of these reactions. A bond to the α carbon of the substrate is broken, removing either a proton or a carboxyl group. Pyridoxal phosphate provides resonance stabilization of the otherwise unstable carbanion

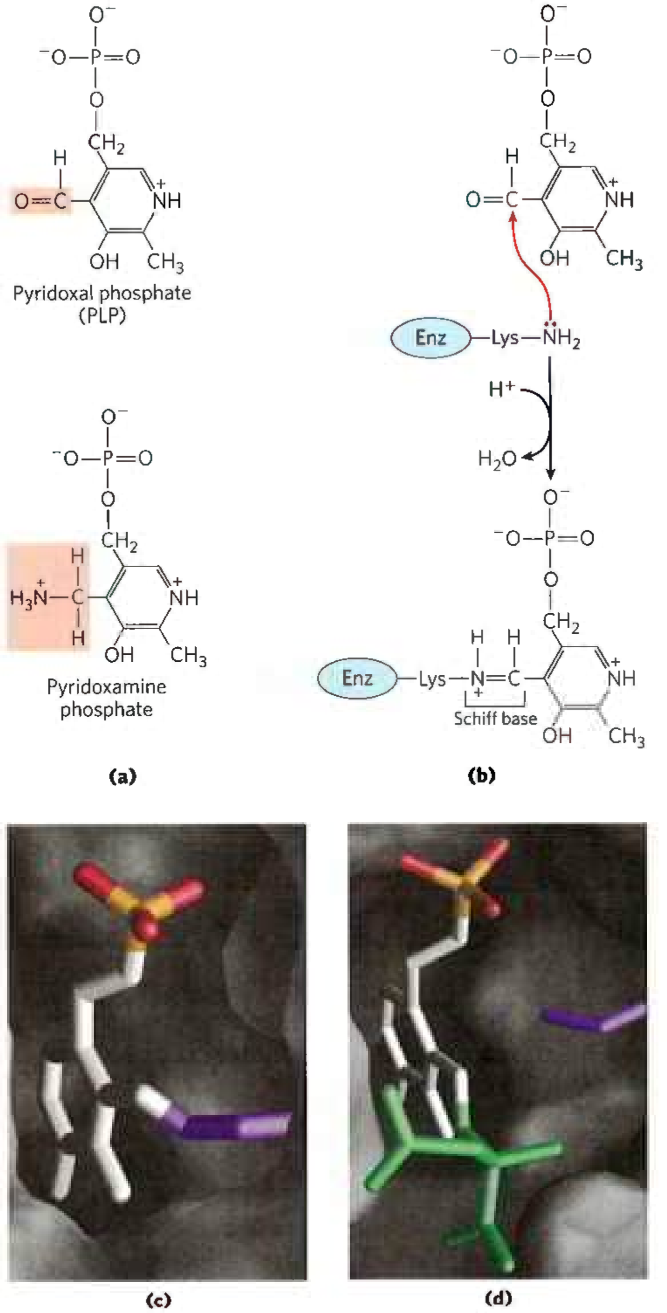
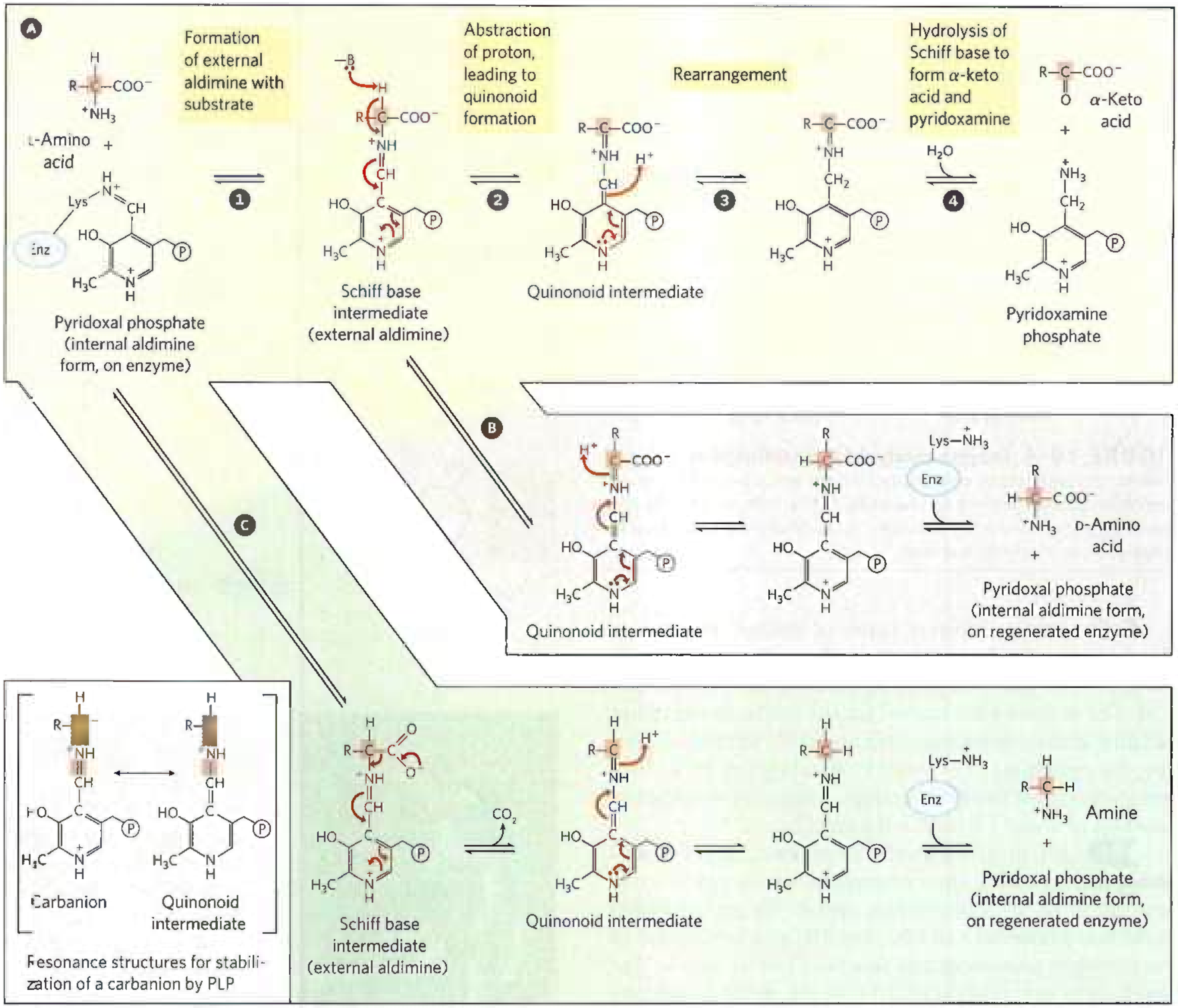


FIGURE 18-5 Pyridoxal phosphate, the prosthetic group of aminotransferases. (a) Pyridoxal phosphate (PLP) and its aminated form, pyridoxamine phosphate, are the tightly bound coenzymes of aminotransferases. The functional groups are shaded. (b) Pyridoxal phosphate is bound to the enzyme through noncovalent interactions and a Schiff-base (aldimine) linkage to a Lys residue at the active site. The steps in the formation of a Schiff base from a primary amine and a carbonyl group are detailed in Figure 14-5. (c, d) Close-up views of the active site of aspartate aminotransferase, with PLP (white, with the phosphoryl group in orange and red). In (c) PLP is in aldimine linkage with the side chain of Lys^{258} (purple). In (d) PLP is linked to the substrate analog 2-methylaspartate (green) via a Schiff base. [(c, d) Data from PDB ID 1AJS, S. Rhee et al., *J. Biol. Chem.* 272:17,293, 1997.]


A
L-Amino acid
Pyridoxal phosphate (internal aldimine form, on enzyme)
Formation of external aldimine with substrate
1
Schiff base intermediate (external aldimine)
Abstraction of proton, leading to quinonoid formation
2
Quinonoid intermediate
Rearrangement
3
Hydrolysis of Schiff base to form α-keto acid and pyridoxamine
H2O
4
α-Keto acid
Pyridoxamine phosphate
B
Quinonoid intermediate
Enz
Lys—NH3
D-Amino acid
Pyridoxal phosphate (internal aldimine form, on regenerated enzyme)
C
CO2
Schiff base intermediate (external aldimine)
Quinonoid intermediate
Amine
Pyridoxal phosphate (internal aldimine form, on regenerated enzyme)
Carbanion
Quinonoid intermediate
Resonance structures for stabilization of a carbanion by PLP

MECHANISM FIGURE 18-6 Some amino acid transformations at the α carbon that are facilitated by pyridoxal phosphate. Pyridoxal phosphate is generally bonded to the enzyme through a Schiff base, also called an internal aldimine. This activated form of PLP readily undergoes transamination to form a new Schiff base (external aldimine) with the α-amino group of the substrate amino acid (see Fig. 18-5b, d). Three alternative fates for the external aldimine are shown: Ⓐ transamination, Ⓑ racemization, and Ⓒ decarboxylation. The PLP–amino acid Schiff base is in conjugation with the pyridine ring, an electron sink that permits delocalization of an electron pair to avoid formation of an unstable carbanion on the α carbon (inset). A quinonoid intermediate is involved in all three types of reactions. The transamination route Ⓐ is especially important in the pathways described in this chapter. This pathway (shown left to right) represents only part of the overall reaction catalyzed by aminotransferases. To complete the process, a second α-keto acid replaces the one that is released, and this is converted to an amino acid in a reversal of the reaction steps (right to left). Pyridoxal phosphate is also involved in certain reactions at the β and γ carbons of some amino acids (not shown).

intermediate (Fig. 18-6, inset). The highly conjugated structure of PLP (an electron sink) permits delocalization of the negative charge.

Aminotransferases (Fig. 18-6a) are classic examples of enzymes catalyzing bimolecular Ping-Pong reactions (see Fig. 6-15b, d), in which the first substrate reacts and the product must leave the active site before the second substrate can bind. Thus, the incoming amino acid binds to the active site, donates its amino group to pyridoxal phosphate, and departs in the form of an α-keto acid. The incoming α-keto acid then binds, accepts the amino group from pyridoxamine phosphate, and departs in the form of an amino acid.

Glutamate Releases Its Amino Group as Ammonia in the Liver

As we shall see, the urea cycle begins with free ammonia in the mitochondria of hepatocytes. P1 The delivery of NH_4^+ to these mitochondria is streamlined by collecting the amino groups of many different α-amino acids in the liver in one of two forms: the amino group of L-glutamate or the amide nitrogen of glutamine (Fig. 18-2).

As the product of many aminotransferase reactions, glutamate has a central role. In hepatocytes, glutamate is transported from the cytosol into mitochondria. Here, it undergoes **oxidative deamination** catalyzed by **L-glutamate dehydrogenase** (M_r 330,000) to produce NH_4^+ and α-ketoglutarate. In mammals, this enzyme is present in the mitochondrial matrix. It is unusual in that it can use either NAD^+ or $NADP^+$ as the acceptor of reducing equivalents (**Fig. 18-7**).

The combined action of an aminotransferase and glutamate dehydrogenase is referred to as **transdeamination**. A few amino acids bypass the transdeamination pathway and undergo direct oxidative deamination. The fate of the NH_4^+ produced by any of these deamination processes is discussed in detail in Section 18.2. P3 The α-ketoglutarate formed from glutamate deamination can be used either in the citric acid cycle or for glucose synthesis.

P1 P2 P3 Glutamate dehydrogenase operates at an important intersection of carbon and nitrogen metabolism. Its α-ketoglutarate product can be oxidized as fuel or serve as a glucose precursor in gluconeogenesis. An allosteric enzyme with six identical subunits, its activity is influenced by a complicated array of allosteric modulators. The best-studied of these are the positive modulator ADP and the negative modulator GTP. Although the metabolic rationale for this regulatory pattern has not been elucidated in detail, ADP can signal low glucose levels and GTP is a product of the citric acid cycle that can signal high levels of α-ketoglutarate (see Fig. 16-7).

Mutations that alter the allosteric binding site for GTP or otherwise cause permanent activation of glutamate dehydrogenase lead to a human genetic disorder called hyperinsulinism-hyperammonemia syndrome, characterized by hypersecretion of insulin after a protein meal. This results in elevated levels of ammonia in the bloodstream and hypoglycemia. ■

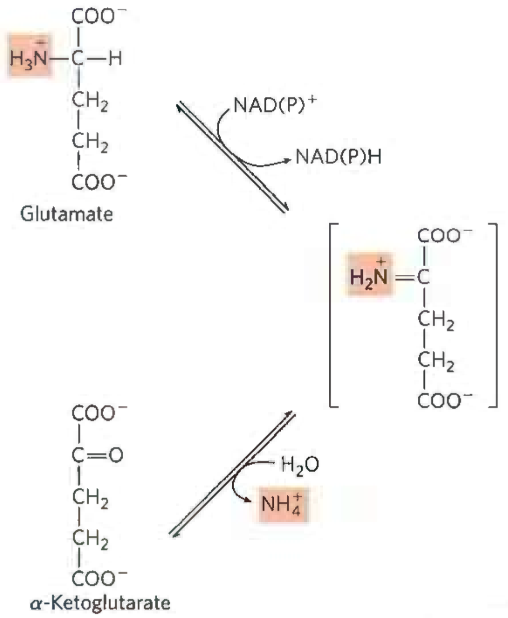


FIGURE 18-7 Reaction catalyzed by glutamate dehydrogenase. The glutamate dehydrogenase of mammalian liver has the unusual capacity to use either NAD^+ or $NADP^+$ as cofactor. The glutamate dehydrogenases of plants and microorganisms are generally specific for one or the other. The mammalian enzyme is allosterically regulated by GTP and ADP.

Glutamine Transports Ammonia in the Bloodstream

Glutamine is the second major source of ammonia in hepatocyte mitochondria and is particularly important for intercellular transport of ammonia. P4 Ammonia is quite toxic to animal tissues (we examine some possible reasons for this toxicity later). Significant amounts are present in blood, but the levels are tightly controlled. In many tissues, including the brain, some processes such as nucleotide degradation generate free ammonia. In most animals, much of the free ammonia is converted to a nontoxic compound before export from the extrahepatic tissues into the blood and transport to the liver or kidneys. For this transport function, glutamate, critical to *intracellular* amino group metabolism, is supplanted by L-glutamine. The free ammonia produced in tissues is combined with glutamate to yield glutamine by the action of **glutamine synthetase**. This reaction requires ATP and occurs in two steps (**Fig. 18-8**). First, glutamate and ATP react to form ADP and a γ-glutamyl phosphate intermediate, which then reacts with ammonia to produce glutamine and inorganic phosphate. In addition to its transport role, glutamine serves as a source of amino groups in a variety of biosynthetic reactions. Glutamine synthetase is found in all organisms, always playing a central metabolic role. In microorganisms, the enzyme serves as an essential portal for the entry of fixed nitrogen into biological systems. (The roles of glutamine and glutamine synthetase in metabolism are further discussed in Chapter 22.)

In most terrestrial animals, glutamine in excess of that required for biosynthesis is transported in the blood to the intestine, liver, and kidneys for processing. In these tissues, the amide nitrogen is released as ammonium ion in the mitochondria, where the enzyme **glutaminase** converts glutamine to glutamate and NH_4^+ (Fig. 18-8). The NH_4^+ from intestine and kidney is transported in the blood to the liver. In the liver, the ammonia from all sources is disposed of by urea synthesis. Some of the glutamate produced in the glutaminase reaction may be further processed in the liver by glutamate dehydrogenase (Fig. 18-7), releasing more ammonia and producing carbon skeletons for metabolic fuel.

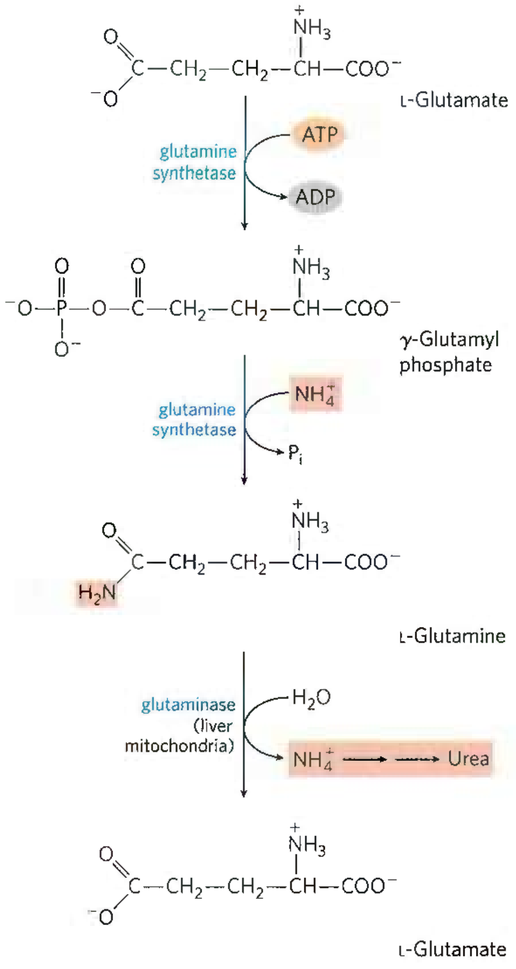

L-Glutamate
glutamine synthetase
ATP
ADP
γ-Glutamyl phosphate
glutamine synthetase
NH_4^+
P_i
L-Glutamine
glutaminase (liver mitochondria)
H_2O
NH_4^+
Urea
L-Glutamate

FIGURE 18-8 Ammonia transport in the form of glutamine. Excess ammonia in tissues is added to glutamate to form glutamine, a process catalyzed by glutamine synthetase. After transport in the bloodstream, the glutamine enters the liver, and NH_4^+ is liberated in mitochondria by the enzyme glutaminase.

In metabolic acidosis (p. 621) there is a regulated increase in glutamine processing by the kidneys. Much of the excess NH_4^+ thus produced is not destined for the bloodstream or converted to urea; instead, it is excreted directly into the urine where it forms salts with metabolic acids. The glutamine breakdown thus facilitates removal of those acids in the urine. Bicarbonate produced by the decarboxylation of α-ketoglutarate in the citric acid cycle can also serve as a buffer in blood plasma. Taken together, these effects of glutamine metabolism in the kidney tend to counteract acidosis. ■

Alanine Transports Ammonia from Skeletal Muscles to the Liver

Vigorously contracting skeletal muscles operate anaerobically, producing large amounts of pyruvate and lactate from glycolysis as well as ammonia from protein breakdown. These products must find their way to the liver, where pyruvate and lactate are incorporated into glucose, which is returned to the muscles, and ammonia is converted to urea for excretion. Pyruvate and alanine are readily interconverted via transamination with glutamate by the action of **alanine aminotransferase**). Thus, alanine largely supplants glutamine in the transport of amino groups from muscle to the liver in a nontoxic form (Fig. 18-2a), ultimately delivering the free ammonia to hepatocyte mitochondria via glutamate in a pathway called the **glucose-alanine cycle** (Fig. 18-9). In the cytosol of hepatocytes, alanine aminotransferase acts in reverse to transfer the amino group from alanine to α-ketoglutarate, forming pyruvate and glutamate. Glutamate can then enter mitochondria, where the glutamate dehydrogenase reaction releases NH_4^+ (Fig. 18-7), or it can undergo transamination with oxaloacetate to form aspartate, another nitrogen donor in urea synthesis, as we shall see.

The use of alanine to transport ammonia from skeletal muscles to the liver is another example of the intrinsic economy of living organisms. The energetic burden of

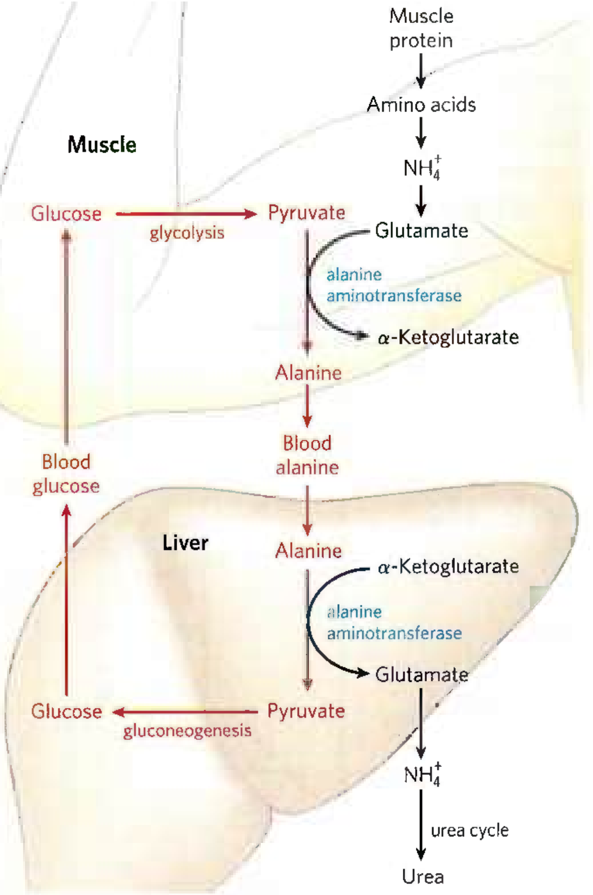

Muscle protein
Amino acids
NH_4^+
Muscle
Glucose
glycolysis
Pyruvate
Glutamate
alanine aminotransferase
α-Ketoglutarate
Alanine
Blood glucose
Blood alanine
Liver
Alanine
α-Ketoglutarate
alanine aminotransferase
Glutamate
Glucose
gluconeogenesis
Pyruvate
NH_4^+
urea cycle
Urea

FIGURE 18-9 Glucose-alanine cycle. Alanine serves as a carrier of ammonia and of the carbon skeleton of pyruvate from skeletal muscle to liver. The ammonia is excreted and the pyruvate is used to produce glucose, which is returned to the muscle.

gluconeogenesis is imposed on the liver rather than the muscle, and all available ATP in muscle is devoted to muscle contraction. The glucose-alanine cycle, in concert with the Cori cycle (see Box 14-2 and Fig. 23-17), accomplishes this transaction.

Ammonia Is Toxic to Animals

The catabolic production of ammonia poses a serious biochemical problem, because ammonia is very toxic. The brain is particularly sensitive; damage from ammonia toxicity causes cognitive impairment, ataxia, and epileptic seizures. In extreme cases there is swelling of the brain leading to death. The molecular bases for this toxicity are gradually coming into focus. In the blood, about 98% of ammonia is in the protonated form (NH_4^+), which does not cross the plasma membrane. The small amount of NH_3 present readily crosses all membranes, including the blood-brain barrier, allowing it to enter cells, where much of it becomes protonated and can accumulate inside cells as NH_4^+.

Ridding the cytosol of ammonia requires reductive amination of α-ketoglutarate to glutamate by glutamate dehydrogenase (the reverse of the reaction described earlier; Fig. 18-7) and conversion of glutamate to glutamine by glutamine synthetase. In the brain, only astrocytes — star-shaped cells of the nervous system that provide nutrients, support, and insulation for neurons — express glutamine synthetase. Glutamate and its derivative γ-aminobutyrate (GABA; see Fig. 22-31) are important neurotransmitters; some of the sensitivity of the brain to ammonia may reflect depletion of glutamate in the glutamine synthetase reaction. However, glutamine synthetase activity is insufficient to deal with excess ammonia, or to fully explain its toxicity.

Increased [NH_4^+] also alters the capacity of astrocytes to maintain potassium homeostasis across the membrane. NH_4^+ competes with K^+ for transport into the cell through the Na^+K^+ ATPase, resulting in elevated extracellular [K^+]. The excess extracellular K^+ enters neurons through a symporter, **Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1)**, bringing Na^+ and $2Cl^-$ with it. Excess Cl^- in these neurons alters their response when the neurotransmitter GABA interacts with their $GABA_A$ receptors, producing abnormal depolarization and increased neuronal activity that likely account for the neuromuscular incoordination and seizures that often result from ammonia poisoning. If extracellular [NH_4^+] remains elevated, the perturbation of ion and aquaporin channels in astrocytes causes the cells to swell, resulting in fatal brain edema. ■

As we close this discussion of amino group metabolism, note that we have described several processes that deposit excess ammonia in the mitochondria of hepatocytes (Fig. 18-2). We now look at the fate of that ammonia.

SUMMARY 18.1 *Metabolic Fates of Amino Groups*

- Humans derive a small fraction of their oxidative energy from the catabolism of amino acids. Proteases degrade ingested proteins in the stomach and small intestine. Most proteases are initially synthesized as inactive zymogens.
- An early step in the catabolism of amino acids is the separation of the amino group from the carbon skeleton. In most cases, the amino group is transferred to α-ketoglutarate to form glutamate. This transamination reaction requires pyridoxal phosphate, a coenzyme globally involved in amino acid metabolism.
- Glutamate, a central reservoir of metabolized amino groups, is transported to liver mitochondria, where glutamate dehydrogenase liberates the amino group as ammonium ion (NH_4^+).
- Ammonia formed in most other tissues is transported to the liver as the amide nitrogen of glutamine.
- To make use of the pyruvate and amino groups generated in hardworking skeletal muscle, the pyruvate is converted to alanine and transported to the liver within the glucose-alanine cycle.
- Free ammonia is toxic. Excess ammonia is often manifested in serious neurological damage.

18.2 Nitrogen Excretion and the Urea Cycle

In ureotelic organisms, the ammonia deposited in the mitochondria of hepatocytes is converted to urea in the **urea cycle** (**Fig. 18-10**). This pathway was discovered in 1932 by Hans Krebs (who later also discovered the citric acid cycle) and a medical student associate, Kurt Henseleit. Urea production occurs almost exclusively in the liver and is the fate of most of the ammonia channeled there. The urea passes into the bloodstream and thus to the kidneys and is excreted into the urine. The production of urea now becomes the focus of our discussion.

Urea Is Produced from Ammonia in Five Enzymatic Steps

The urea cycle begins inside liver mitochondria, but three of the subsequent steps take place in the cytosol; the cycle thus spans two cellular compartments (Fig. 18-10). The first amino group to enter the urea cycle is derived from ammonia in the mitochondrial matrix — most of this NH_4^+ arises by the pathways described in Section 18.1. The liver also receives some ammonia via the portal vein from the intestine, from the bacterial oxidation of amino acids. Whatever its source, the NH_4^+ generated in liver mitochondria is immediately used, together with CO_2 (as HCO_3^-) produced by mitochondrial respiration,

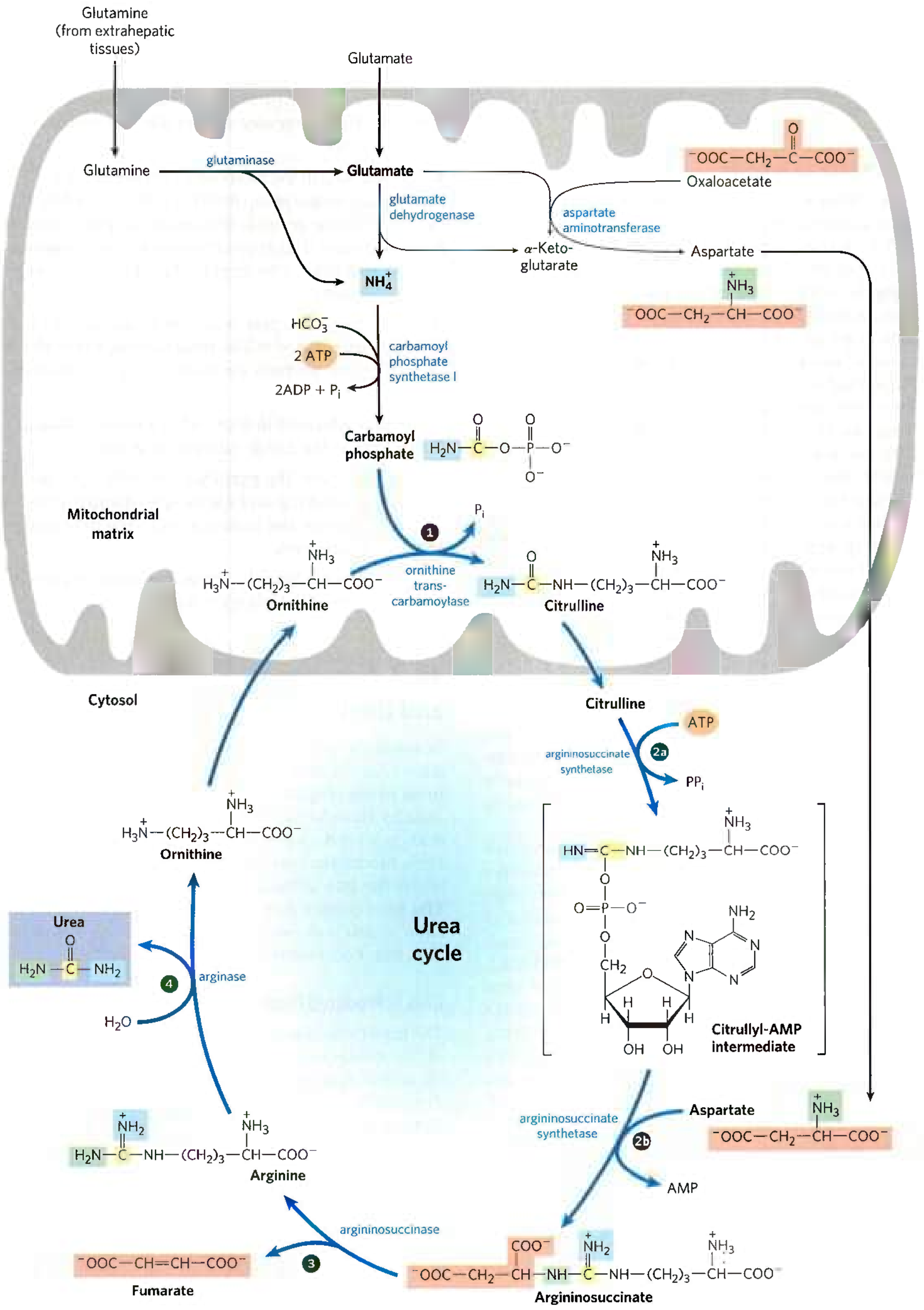
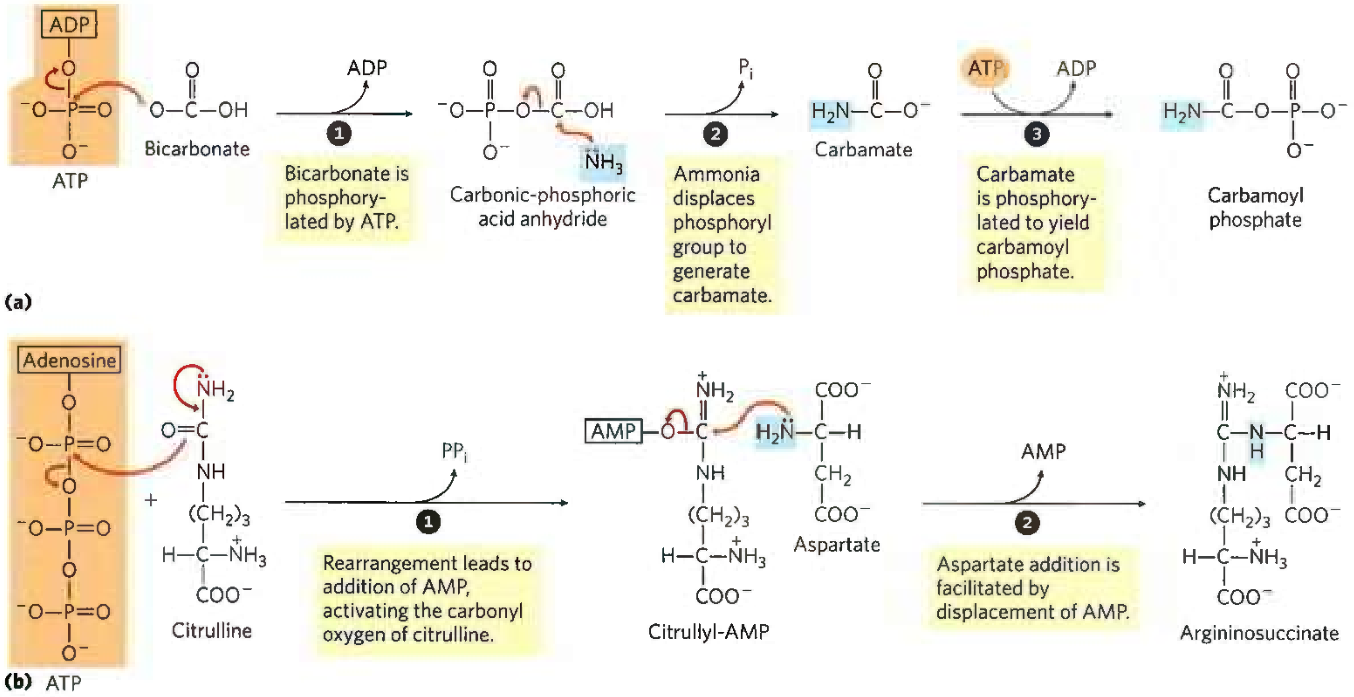

Glutamine (from extrahepatic tissues)
Glutamate
Glutamine
glutaminase
Glutamate
glutamate dehydrogenase
NH_4^+
$^-OOC-CH_2-C(=O)-COO^-$
Oxaloacetate
aspartate aminotransferase
α-Ketoglutarate
Aspartate
$^-OOC-CH_2-CH(\overset{+}{N}H_3)-COO^-$
HCO_3^-
2 ATP
2ADP + P_i
carbamoyl phosphate synthetase I
Carbamoyl phosphate
$H_2N-C(=O)-O-P(=O)(O^-)-O^-$
Mitochondrial matrix
P_i
1
$H_3\overset{+}{N}-(CH_2)_3-CH(\overset{+}{N}H_3)-COO^-$
Ornithine
ornithine transcarbamoylase
$H_2N-C(=O)-NH-(CH_2)_3-CH(\overset{+}{N}H_3)-COO^-$
Citrulline
Cytosol
Citrulline
ATP
argininosuccinate synthetase
2a
PP_i
$HN=C(-O-PO_2^--O-CH_2\text{-ribose-adenine})-NH-(CH_2)_3-CH(\overset{+}{N}H_3)-COO^-$
NH_2
N
O
H
OH
Citrullyl-AMP intermediate
Urea cycle
$H_3\overset{+}{N}-(CH_2)_3-CH(\overset{+}{N}H_3)-COO^-$
Ornithine
Urea
$H_2N-C(=O)-NH_2$
4
arginase
H_2O
argininosuccinate synthetase
Aspartate
$^-OOC-CH_2-CH(\overset{+}{N}H_3)-COO^-$
2b
AMP
$H_2N-C(=\overset{+}{N}H_2)-NH-(CH_2)_3-CH(\overset{+}{N}H_3)-COO^-$
Arginine
argininosuccinase
3
$^-OOC-CH=CH-COO^-$
Fumarate
$^-OOC-CH_2-CH(COO^-)-NH-C(=\overset{+}{N}H_2)-NH-(CH_2)_3-CH(\overset{+}{N}H_3)-COO^-$
Argininosuccinate

FIGURE 18-10 The urea cycle and reactions that feed amino groups into the cycle. The enzymes catalyzing these reactions (named in the text) are distributed between the mitochondrial matrix and the cytosol. One amino group enters the urea cycle as carbamoyl phosphate, formed in the matrix; the other enters as aspartate, formed in the matrix by transamination of oxaloacetate and glutamate, catalyzed by aspartate aminotransferase. The urea cycle consists of four steps: ① Formation of citrulline from ornithine and carbamoyl phosphate (entry of the first amino group); the citrulline passes into the cytosol. ② Formation of argininosuccinate through a citrullyl-AMP intermediate (entry of the second amino group). ③ Formation of arginine from argininosuccinate; this reaction releases fumarate, which enters the citric acid cycle. ④ Formation of urea; this reaction also regenerates ornithine.

to form carbamoyl phosphate in the matrix (as shown in Fig. 18-10 and explained in detail in **Fig. 18-11a**). This ATP-dependent reaction is catalyzed by **carbamoyl phosphate synthetase I**, a regulatory enzyme (see below). The mitochondrial form of the enzyme is distinct from the cytosolic (II) form, which has a separate function in pyrimidine biosynthesis (Chapter 22).

The carbamoyl phosphate, which functions as an activated carbamoyl group donor, now enters the urea cycle. The cycle has only four enzymatic steps. First, carbamoyl phosphate donates its carbamoyl group to ornithine to form citrulline, with the release of P_i (Fig. 18-10, step ①). The reaction is catalyzed by **ornithine transcarbamoylase**. Ornithine is not one of the 20 common amino acids found in proteins, but it is a key intermediate in arginine biosynthesis and nitrogen metabolism in general. It is synthesized from glutamate in a five-step pathway described in Chapter 22. Ornithine plays a role resembling that of oxaloacetate in the citric acid cycle, accepting material at each turn of the urea cycle. The citrulline produced in the first step of the urea cycle passes from the mitochondrion to the cytosol.

The next two steps bring in the second amino group, featuring aspartate as the amino group donor. The aspartate is generated in the mitochondria by transamination between glutamate and oxaloacetate, and then transported into the cytosol. A condensation reaction between the amino group of aspartate and the ureido (carbonyl) group of citrulline forms argininosuccinate (step ② in Fig. 18-10). This cytosolic reaction, catalyzed by **argininosuccinate synthetase**, requires ATP and proceeds through a citrullyl-AMP intermediate (Fig. 18-11b). The argininosuccinate is then cleaved by **argininosuccinase** (step ③ in Fig. 18-10) to form free arginine and fumarate, the latter being converted to malate before entering mitochondria to join the pool of citric acid cycle intermediates. This is the only reversible step in the urea cycle. In the last reaction of the urea cycle (step ④), the cytosolic enzyme **arginase** cleaves arginine to yield **urea** and ornithine. Ornithine is transported into the mitochondrion to initiate another round of the urea cycle.


ADP
ATP
Bicarbonate
ADP
①
Bicarbonate is phosphorylated by ATP.
Carbonic-phosphoric acid anhydride
NH_3
P_i
②
Ammonia displaces phosphoryl group to generate carbamate.
Carbamate
ATP
ADP
③
Carbamate is phosphorylated to yield carbamoyl phosphate.
Carbamoyl phosphate
(a)
Adenosine
Citrulline
PP_i
①
Rearrangement leads to addition of AMP, activating the carbonyl oxygen of citrulline.
AMP
Citrullyl-AMP
Aspartate
AMP
②
Aspartate addition is facilitated by displacement of AMP.
Argininosuccinate
(b) ATP

MECHANISM FIGURE 18-11 Nitrogen-acquiring reactions in the synthesis of urea. The urea nitrogens are acquired in two separate reactions, each requiring ATP. (a) In the first reaction, catalyzed by carbamoyl phosphate synthetase I, the first nitrogen enters from ammonia. The terminal phosphate groups of two molecules of ATP are used to form one molecule of carbamoyl phosphate. In other words, this reaction has two activation steps (① and ③). (b) In the second reaction, catalyzed by argininosuccinate synthetase, the second nitrogen enters from aspartate. This reaction has two steps. Activation of the ureido oxygen of citrulline in step ① sets up the addition of aspartate in step ②.

As we noted in Chapter 16, the enzymes of many metabolic pathways are clustered in metabolons (p. 595), with the product of one enzyme reaction being channeled directly to the next enzyme in the pathway. In the urea cycle, the mitochondrial and cytosolic enzymes seem to be clustered in this way. The citrulline transported out of the mitochondrion is not diluted into the general pool of metabolites in the cytosol but is passed directly to the active site of argininosuccinate synthetase. This channeling between enzymes continues for argininosuccinate, arginine, and ornithine. Only urea is released into the general cytosolic pool of metabolites.

The Citric Acid and Urea Cycles Can Be Linked

The fumarate produced in the argininosuccinase reaction is also an intermediate of the citric acid cycle. Thus, the cycles are, in principle, interconnected—in a process dubbed the "Krebs bicycle" (**Fig. 18-12**). However, each cycle can operate independently, and communication between them depends on the transport of key intermediates between the mitochondrion and cytosol. Major transporters in the inner mitochondrial membrane include the malate–α-ketoglutarate transporter, the glutamate-aspartate transporter, and the glutamate-OH^- transporter. Together, these transporters facilitate the movement of malate and glutamate into the mitochondrial matrix and the movement of aspartate and α-ketoglutarate out to the cytosol.

Several enzymes of the citric acid cycle, including fumarase (fumarate hydratase) and malate dehydrogenase (p. 586), are also present as isozymes in the cytosol. There is no transporter to directly move the fumarate generated in cytosolic arginine synthesis back into the mitochondrial matrix. However, fumarate can be converted to malate in the cytosol. Fumarate and malate can be further metabolized in the cytosol, or malate can be transported into mitochondria for use in the citric acid cycle. Aspartate formed in mitochondria by transamination between oxaloacetate and glutamate can be transported to the cytosol, where it serves as nitrogen donor in the urea cycle reaction catalyzed by argininosuccinate synthetase. These reactions, making up the **aspartate-argininosuccinate shunt**, provide metabolic links between the separate pathways by which the amino groups and carbon skeletons of amino acids are processed.

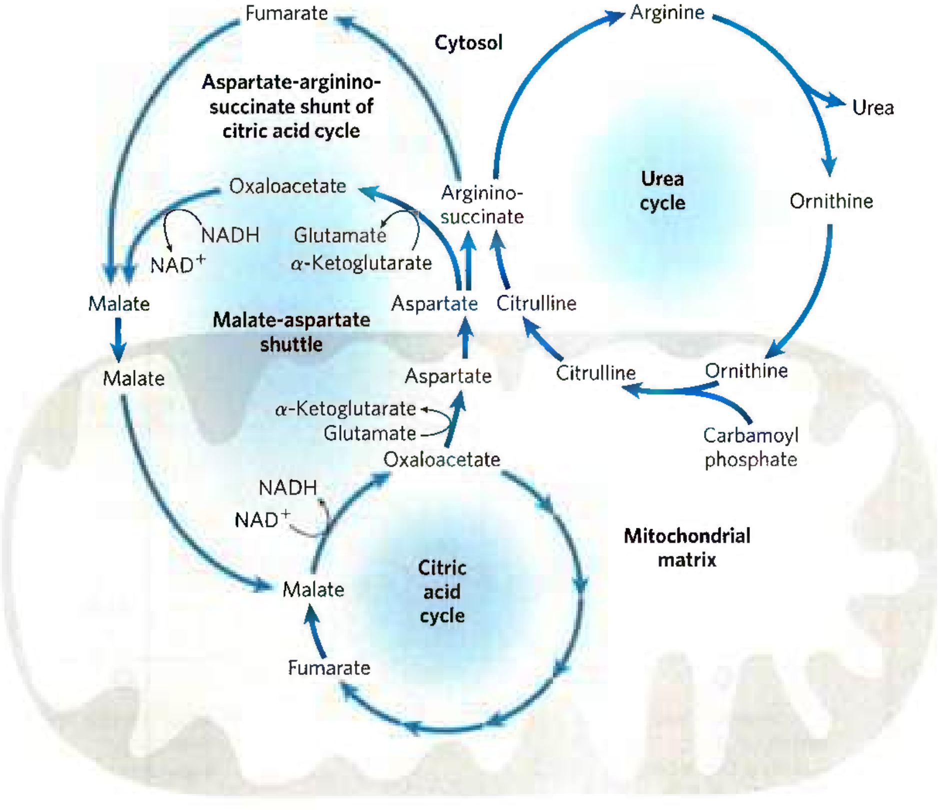

Fumarate
Cytosol
Arginine
Aspartate-arginino-succinate shunt of citric acid cycle
Urea
Urea cycle
Oxaloacetate
Arginino-succinate
Ornithine
NADH
Glutamate
NAD+
α-Ketoglutarate
Malate
Aspartate
Citrulline
Malate-aspartate shuttle
Malate
Aspartate
Citrulline
Ornithine
α-Ketoglutarate
Glutamate
Carbamoyl phosphate
Oxaloacetate
NADH
NAD+
Mitochondrial matrix
Citric acid cycle
Malate
Fumarate

FIGURE 18-12 Links between the urea cycle and citric acid cycle. The interconnected cycles have been called the "Krebs bicycle." The pathways linking the citric acid and urea cycles are known as the aspartate-argininosuccinate shunt; these effectively link the fates of the amino groups and the carbon skeletons of amino acids. The interconnections are quite elaborate. For example, some citric acid cycle enzymes, such as fumarase and malate dehydrogenase, have both cytosolic and mitochondrial isozymes. Fumarate produced in the cytosol—whether by the urea cycle, purine biosynthesis, or other processes—can be converted to cytosolic malate, which is used in the cytosol or transported into mitochondria to enter the citric acid cycle. These processes are further intertwined with the malate-aspartate shuttle, a set of reactions that brings reducing equivalents into the mitochondrion. These different cycles and processes rely on a limited number of transporters in the inner mitochondrial membrane.

The use of aspartate as a nitrogen donor in the urea cycle may appear to be a relatively complicated way to introduce the second amino group into urea. However, we shall see in Chapter 22 that this pathway for nitrogen incorporation is one of the two common ways to introduce amino groups into biomolecules. In the urea cycle, additional pathway interconnections can help explain why aspartate is used as a nitrogen donor. P3 The urea and citric acid cycles are closely tied to an additional process that brings NADH, in the form of reducing equivalents, into the mitochondrion. As detailed in the next chapter, the NADH produced by glycolysis, fatty acid oxidation, and other processes cannot be transported across the mitochondrial inner membrane. Reducing equivalents are instead brought into the mitochondrion by converting aspartate to oxaloacetate in the cytosol, reducing the oxaloacetate to malate with NADH, and transporting the malate into the mitochondrial matrix via the malate–α-ketoglutarate transporter. Once inside the mitochondrion, the malate can be reconverted to oxaloacetate while generating NADH. The oxaloacetate is converted to aspartate in the matrix and transported out of the mitochondrion by the aspartate-glutamate transporter. This malate-aspartate shuttle completes yet another cycle that functions to keep the mitochondrion supplied with NADH (Fig. 18-12; see also Fig. 19-31).

These processes require that a balance be maintained in the cytosol between the concentrations of glutamate and aspartate. The enzyme that transfers amino groups between these key amino acids is aspartate aminotransferase, AAT (also called glutamate-oxaloacetate transaminase, GOT). This is one of the most active enzymes in hepatocytes and other tissues. When tissue damage occurs, this easily assayed enzyme and others leak into the blood. Thus, measuring blood levels of liver enzymes is important in diagnosing a variety of medical conditions (**Box 18-1**).

The Activity of the Urea Cycle Is Regulated at Two Levels

The flux of nitrogen through the urea cycle in an individual animal varies with diet. When the dietary intake is primarily protein, the carbon skeletons of amino acids are used for fuel, producing much urea from the excess amino groups. During prolonged starvation, when breakdown of muscle protein begins to supply much of the organism's metabolic energy, urea production also increases substantially.

These changes in demand for urea cycle activity are met over the long term by regulation of the rates of synthesis of the four urea cycle enzymes and carbamoyl phosphate synthetase I in the liver. All five enzymes are synthesized at higher rates in starving animals and in animals on very-high-protein diets than in well-fed animals eating primarily carbohydrates and fats. Animals on protein-free diets produce lower levels of urea cycle enzymes.

On a shorter time scale, allosteric regulation of at least one key enzyme adjusts the flux through the urea cycle. The first enzyme in the pathway, carbamoyl phosphate synthetase I, is allosterically activated by ***N*-acetylglutamate**, which is synthesized from acetyl-CoA and glutamate by ***N*-acetylglutamate synthase** (**Fig. 18-13**). In plants and microorganisms, this enzyme catalyzes the first step in the de novo synthesis of arginine from glutamate (see Fig. 22-12), but in mammals,

BOX 18-1 MEDICINE

Assays for Tissue Damage

Analyses of certain enzyme activities in blood serum give valuable diagnostic information for several disease conditions.

Blood levels of alanine aminotransferase (ALT; also called glutamate-pyruvate transaminase, GPT) and aspartate aminotransferase (AAT; also called glutamate-oxaloacetate transaminase, GOT) are important in the diagnosis of liver damage, toxicity associated with long-term drug use, or infection. When tissue is damaged, a variety of enzymes, including these aminotransferases, leak from injured cells into the bloodstream. Measurements of the blood serum concentrations of the two aminotransferases by the SGPT and SGOT tests (*S* for serum) — and of another enzyme, **creatine kinase**, by the SCK test — can provide information about the severity of the damage.

The SGOT and SGPT tests are used in occupational medicine to determine whether people exposed to carbon tetrachloride, chloroform, or other industrial solvents have suffered liver damage. Liver degeneration caused by these solvents is accompanied by leakage of various enzymes from injured hepatocytes into the blood. Aminotransferases are most useful in the monitoring of people exposed to these chemicals, because these enzyme activities are high in liver and thus are likely to be among the proteins leaked from damaged hepatocytes; also, they can be detected in the bloodstream in very small amounts.

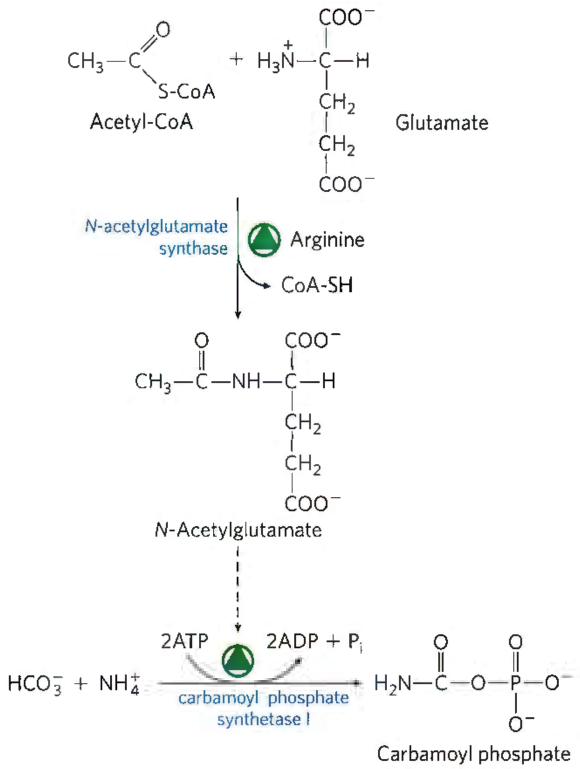


FIGURE 18-13 **Synthesis of *N*-acetylglutamate and its activation of carbamoyl phosphate synthetase I.**

N-acetylglutamate synthase activity in the liver has a purely regulatory function (mammals lack the other enzymes needed to convert glutamate to arginine). The steady-state levels of *N*-acetylglutamate are determined by the concentrations of glutamate and acetyl-CoA (the substrates for *N*-acetylglutamate synthase) and arginine (an activator of *N*-acetylglutamate synthase, and thus an activator of the urea cycle).

Pathway Interconnections Reduce the Energetic Cost of Urea Synthesis

If we consider the urea cycle in isolation, we see that the synthesis of one molecule of urea requires four high-energy phosphate groups (Fig. 18-10). Two ATP molecules are required to make carbamoyl phosphate, and one ATP to make argininosuccinate—the latter ATP undergoing a pyrophosphate cleavage to AMP and PP_i, which is hydrolyzed to two P_i. The overall equation of the urea cycle is

$$2NH_4^+ + HCO_3^- + 3ATP^{4-} + H_2O \longrightarrow \text{urea} + 2ADP^{3-} + 4P_i^{2-} + AMP^{2-} + 2H^+$$

However, this apparent cost is compensated for by the pathway interconnections detailed above. The fumarate generated by the urea cycle is converted to malate, and the malate is transported into the mitochondrion (Fig. 18-12). Inside the mitochondrial matrix, NADH is generated in the malate dehydrogenase reaction. Each NADH molecule can generate up to 2.5 ATP during mitochondrial respiration, greatly reducing the overall energetic cost of urea synthesis (mitochondrial respiration is discussed further in Chapter 19).

Genetic Defects in the Urea Cycle Can Be Life-Threatening

Infants with severe genetic defects in any enzyme involved in urea formation often appear normal at birth. However, they soon develop symptoms of hyperammonemia, including cerebral edema, lethargy, and hyperventilation. Without treatment, early death usually results. Symptoms may be less severe in patients retaining partial enzyme activity. These patients cannot tolerate protein-rich diets. Amino acids ingested in excess of the minimum daily requirements for protein synthesis are deaminated in the liver, producing free ammonia that cannot be converted to urea and exported into the bloodstream, and, as we have seen, ammonia is highly toxic. Given that most urea cycle steps are irreversible, the absent enzyme activity can often be identified by determining which cycle intermediate is present in elevated concentration in the blood and/or urine. Although the breakdown of amino acids can have serious health consequences in individuals with urea cycle deficiencies, a protein-free diet is not a treatment option. Humans are incapable of synthesizing half of the 20 common amino acids; they must obtain these **essential amino acids** in the diet (**Table 18-1**).

A variety of treatments are available for individuals with urea cycle defects. Careful administration of the aromatic acids benzoate or phenylbutyrate in the diet can help lower the level of ammonia in the blood. Benzoate is converted to benzoyl-CoA, which combines with glycine to form hippurate (**Fig. 18-14**, left). The glycine used up in this reaction must be regenerated, and ammonia is thus taken up in the glycine synthase reaction. Phenylbutyrate is converted to phenylacetate by β oxidation. The phenylacetate is then converted to phenylacetyl-CoA,

TABLE 18-1 **Nonessential and Essential Amino Acids for Humans and the Albino Rat**

Nonessential	Conditionally essential[a]	Essential
Alanine	Arginine	Histidine
Asparagine	Cysteine	Isoleucine
Aspartate	Glutamine	Leucine
Glutamate	Glycine	Lysine
Serine	Proline	Methionine
	Tyrosine	Phenylalanine
		Threonine
		Tryptophan
		Valine

[a]Required to some degree in young, growing animals and/or sometimes during illness.

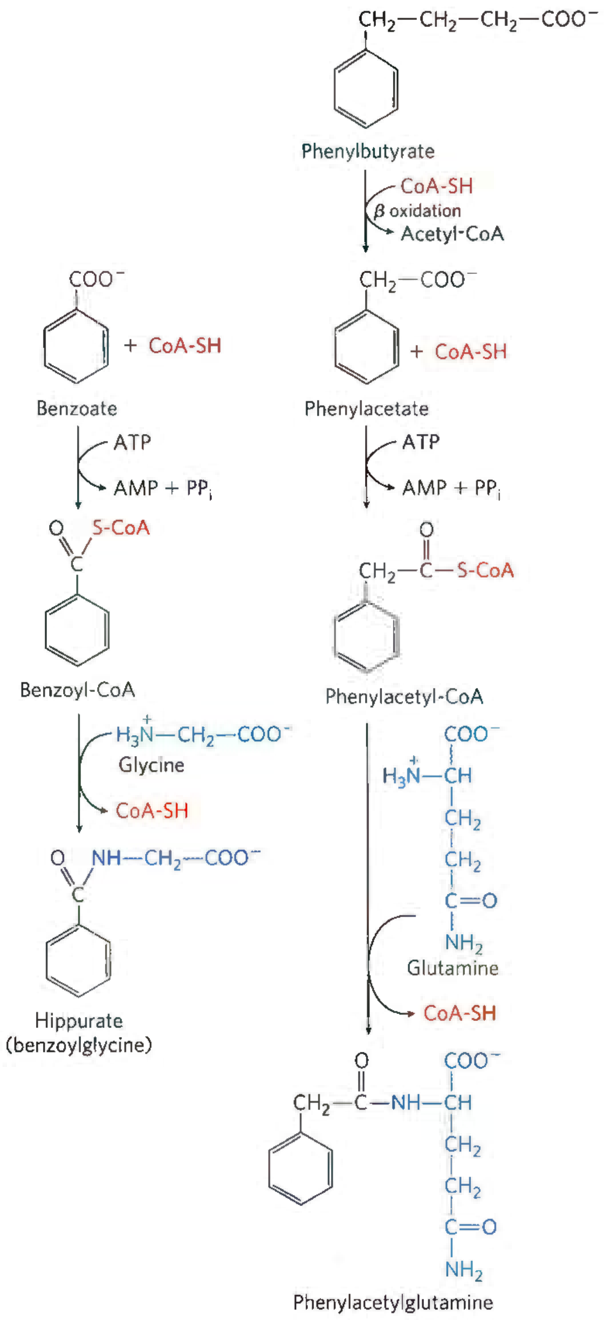
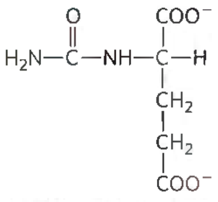


FIGURE 18-14 Treatment for deficiencies in urea cycle enzymes. The aromatic acids benzoate and phenylbutyrate, administered in the diet, are metabolized and combine with glycine and glutamine, respectively. The products are excreted in the urine. Subsequent synthesis of glycine and glutamine to replenish the pool of these intermediates removes ammonia from the bloodstream.

which combines with glutamine to form phenylacetylglutamine (Fig. 18-14, right). The resulting removal of glutamine triggers its further synthesis by glutamine synthetase (see Eqn 22-1) in a reaction that takes up ammonia. Both hippurate and phenylacetylglutamine are nontoxic compounds that are excreted in the urine. The pathways shown in Figure 18-14 make only minor contributions to normal metabolism, but they become prominent when aromatic acids are ingested.

Other therapies are more specific to a particular enzyme deficiency. Deficiency of *N*-acetylglutamate synthase results in the absence of the normal activator of carbamoyl phosphate synthetase I (Fig. 18-13). This condition can be treated by administering carbamoyl glutamate, an analog of *N*-acetylglutamate that is effective in activating carbamoyl phosphate synthetase I.



$$H_2N-\overset{\overset{\large O}{\|}}{C}-NH-\underset{\underset{\underset{\large COO^-}{|}}{\underset{CH_2}{|}}}{\underset{\underset{CH_2}{|}}{C}}\overset{COO^-}{\overset{|}{}}-H$$

Carbamoyl glutamate

Supplementing the diet with arginine is useful in treating deficiencies of ornithine transcarbamoylase, argininosuccinate synthetase, and argininosuccinase. Many of these treatments must be accompanied by strict dietary control and supplements of essential amino acids. In the rare cases of arginase deficiency, arginine, the substrate of the defective enzyme, must be excluded from the diet. ■

SUMMARY 18.2 *Nitrogen Excretion and the Urea Cycle*

- In the urea cycle, ornithine combines with ammonia, in the form of carbamoyl phosphate, to form citrulline. A second amino group is transferred to citrulline from aspartate to form arginine—the immediate precursor of urea. Arginase catalyzes hydrolysis of arginine to urea and ornithine; ornithine is regenerated in each turn of the cycle.
- The urea cycle results in a net conversion of oxaloacetate to fumarate, both of which are intermediates in the citric acid cycle. The two cycles are thus interconnected.
- The activity of the urea cycle is regulated at the level of enzyme synthesis and by allosteric regulation of the enzyme that catalyzes the formation of carbamoyl phosphate.
- The energetic cost of the urea cycle is reduced by cycle interconnections.
- Genetic diseases involving urea cycle enzyme deficiencies have serious consequences but sometimes can be managed by dietary intervention.

18.3 Pathways of Amino Acid Degradation

Amino acid catabolism normally accounts for only 10% to 15% of the human body's energy production; these pathways are not nearly as active as glycolysis and fatty acid oxidation. Flux through these catabolic routes also varies greatly, depending on the balance between requirements for biosynthetic processes and the availability of a particular amino acid. The 20 catabolic pathways converge to form only six major products: pyruvate, acetyl-CoA, α-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate. All of these enter the citric acid cycle (**Fig. 18-15**). From here the carbon skeletons are diverted to gluconeogenesis or ketogenesis; alternatively, they are completely oxidized as fuel to CO_2 and H_2O.

We summarize the individual pathways for the 20 amino acids in flow diagrams, each leading to a specific point of entry into the citric acid cycle. In these diagrams, the carbon atoms that enter the citric acid cycle are shown in color. Note that some amino acids appear more than once, reflecting different fates for different parts of their carbon skeletons. Rather than examining every step of every pathway in amino acid catabolism, we single out for special discussion some enzymatic reactions that are particularly noteworthy for their mechanisms or their medical significance.

Some Amino Acids Can Contribute to Gluconeogenesis, Others to Ketone Body Formation

P5 The seven amino acids that are degraded entirely or in part to acetoacetyl-CoA and/or acetyl-CoA—phenylalanine, tyrosine, isoleucine, leucine, tryptophan, threonine, and lysine—can yield ketone bodies in the liver, where acetoacetyl-CoA is converted to acetoacetate and then to acetone and β-hydroxybutyrate (see Fig. 17-16). These are the **ketogenic** amino acids (Fig. 18-15). Their ability to form ketone bodies is particularly evident in uncontrolled diabetes mellitus, in which the liver produces large amounts of ketone bodies from both fatty acids and the ketogenic amino acids. Ketone bodies may also be metabolized in the brain as fuel in place of glucose in cases during starvation.

The amino acids that are degraded to pyruvate, α-ketoglutarate, succinyl-CoA, fumarate, and/or oxaloacetate can be converted to glucose and glycogen by pathways described in Chapter 14. They are the **glucogenic** amino acids. The division between ketogenic and glucogenic amino acids is not sharp; five amino acids—tryptophan, phenylalanine, tyrosine, threonine, and isoleucine—are both ketogenic and glucogenic. All amino acids except for lysine and leucine can make some contribution to gluconeogenesis. Catabolism of amino acids is particularly critical to the survival of animals with high-protein diets or during starvation. Leucine is an exclusively ketogenic amino acid that is very common

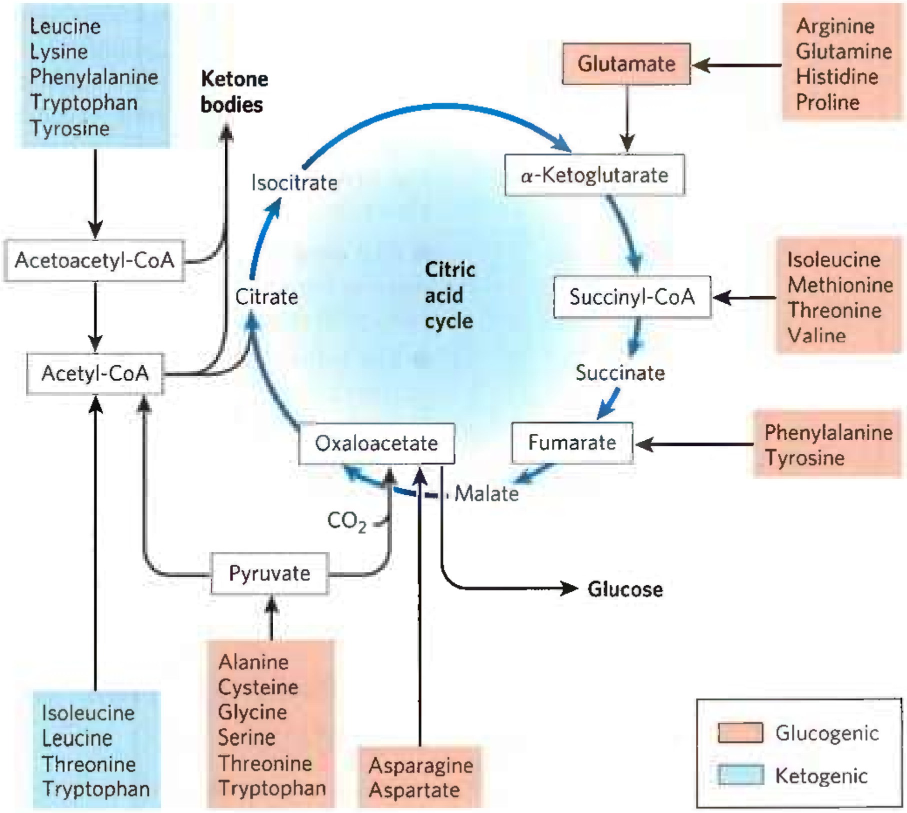

Leucine
Lysine
Phenylalanine
Tryptophan
Tyrosine
Ketone bodies
Acetoacetyl-CoA
Acetyl-CoA
Isocitrate
Citrate
Citric acid cycle
Glutamate
Arginine
Glutamine
Histidine
Proline
α-Ketoglutarate
Succinyl-CoA
Isoleucine
Methionine
Threonine
Valine
Succinate
Fumarate
Phenylalanine
Tyrosine
Oxaloacetate
Malate
CO₂
Pyruvate
Glucose
Isoleucine
Leucine
Threonine
Tryptophan
Alanine
Cysteine
Glycine
Serine
Threonine
Tryptophan
Asparagine
Aspartate
Glucogenic
Ketogenic

FIGURE 18-15 Summary of amino acid catabolism. Amino acids are grouped according to their major degradative end product. Some amino acids are listed more than once because different parts of their carbon skeletons are degraded to different end products. The figure shows the most important catabolic pathways in vertebrates, but there are minor variations among vertebrate species. Threonine, for instance, is degraded by at least two different pathways (see Figs 18-19, 18-27), and the importance of a given pathway can vary with the organism and its metabolic conditions. The glucogenic and ketogenic amino acids are also delineated in the figure, by color shading. Notice that five of the amino acids are both glucogenic and ketogenic. The amino acids degraded to pyruvate are also potentially ketogenic. Only two amino acids, leucine and lysine, are exclusively ketogenic.

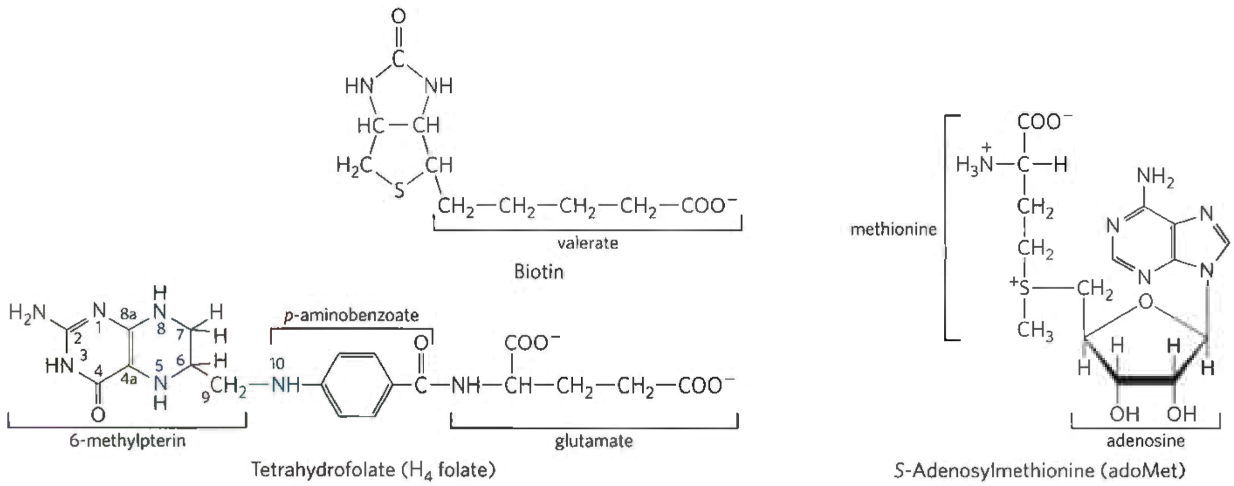


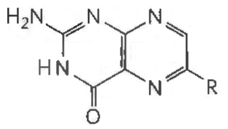
FIGURE 18-16 Some enzyme cofactors important in one-carbon transfer reactions. The nitrogen atoms to which one-carbon groups are attached in tetrahydrofolate are shown in blue.

in proteins. Its degradation makes a substantial contribution to ketosis under starvation conditions.

Several Enzyme Cofactors Play Important Roles in Amino Acid Catabolism

The structural diversity of amino acids is reflected in the varied reaction types encountered in their breakdown pathways. We begin our study of these pathways by noting important classes of reactions that recur and introducing their enzyme cofactors. We have already considered one important class: transamination reactions requiring pyridoxal phosphate. One-carbon transfers are another common type of reaction in amino acid catabolism. Such transfers usually involve one of three cofactors: biotin, tetrahydrofolate, or *S*-adenosylmethionine (**Fig. 18-16**). These cofactors transfer one-carbon groups in different oxidation states: biotin transfers carbon in its most oxidized state, CO_2 (see Fig. 14-17); tetrahydrofolate transfers one-carbon groups in intermediate oxidation states and sometimes as methyl groups; and *S*-adenosylmethionine transfers methyl groups, the most reduced state of carbon. The latter two cofactors are especially important in amino acid and nucleotide metabolism.

Tetrahydrofolate (H_4 folate), synthesized in bacteria, consists of substituted pterin (6-methylpterin), *p*-aminobenzoate, and glutamate moieties (Fig. 18-16).



Pterin

The oxidized form, folate, is a vitamin for mammals; it is converted in two steps to tetrahydrofolate by the enzyme dihydrofolate reductase. The one-carbon group undergoing transfer, in any of three oxidation states, is bonded to N-5 or N-10 or both. The most reduced form of the cofactor carries a methyl group, a more oxidized form carries a methylene group, and the most oxidized forms carry a methenyl, formyl, or formimino group (**Fig. 18-17**). Most forms of tetrahydrofolate are interconvertible and serve as donors of one-carbon units in a variety of metabolic reactions. The primary source of one-carbon units for tetrahydrofolate is the carbon removed in the conversion of serine to glycine, producing N^5,N^{10}-methylenetetrahydrofolate.

Although tetrahydrofolate can carry a methyl group at N-5, the transfer potential of this methyl group is insufficient for most biosynthetic reactions. ***S*-Adenosylmethionine (adoMet)** is the preferred cofactor for biological methyl group transfers. It is synthesized from ATP and methionine by the action of **methionine adenosyl transferase** (**Fig. 18-18**, step ❶). This reaction is unusual in that the nucleophilic sulfur atom of methionine attacks the 5′ carbon of the ribose moiety of ATP rather than one of the phosphorus atoms. Triphosphate is released and is cleaved to P_i and PP_i on the enzyme, and the PP_i is cleaved by inorganic pyrophosphatase; thus three bonds, including two bonds of high-energy phosphate groups, are broken in this reaction. The only other known reaction in which triphosphate is displaced from ATP occurs in the synthesis of coenzyme B_{12} (see Box 17-2, Fig. 3).

S-Adenosylmethionine is a potent alkylating agent by virtue of its destabilizing sulfonium ion. The methyl group is subject to attack by nucleophiles and is about 1,000 times more reactive than the methyl group of N^5-methyltetrahydrofolate.

Transfer of the methyl group from *S*-adenosylmethionine to an acceptor yields ***S*-adenosylhomocysteine** (Fig. 18-18, step ❷), which is subsequently broken down to homocysteine and adenosine (step ❸). Methionine is

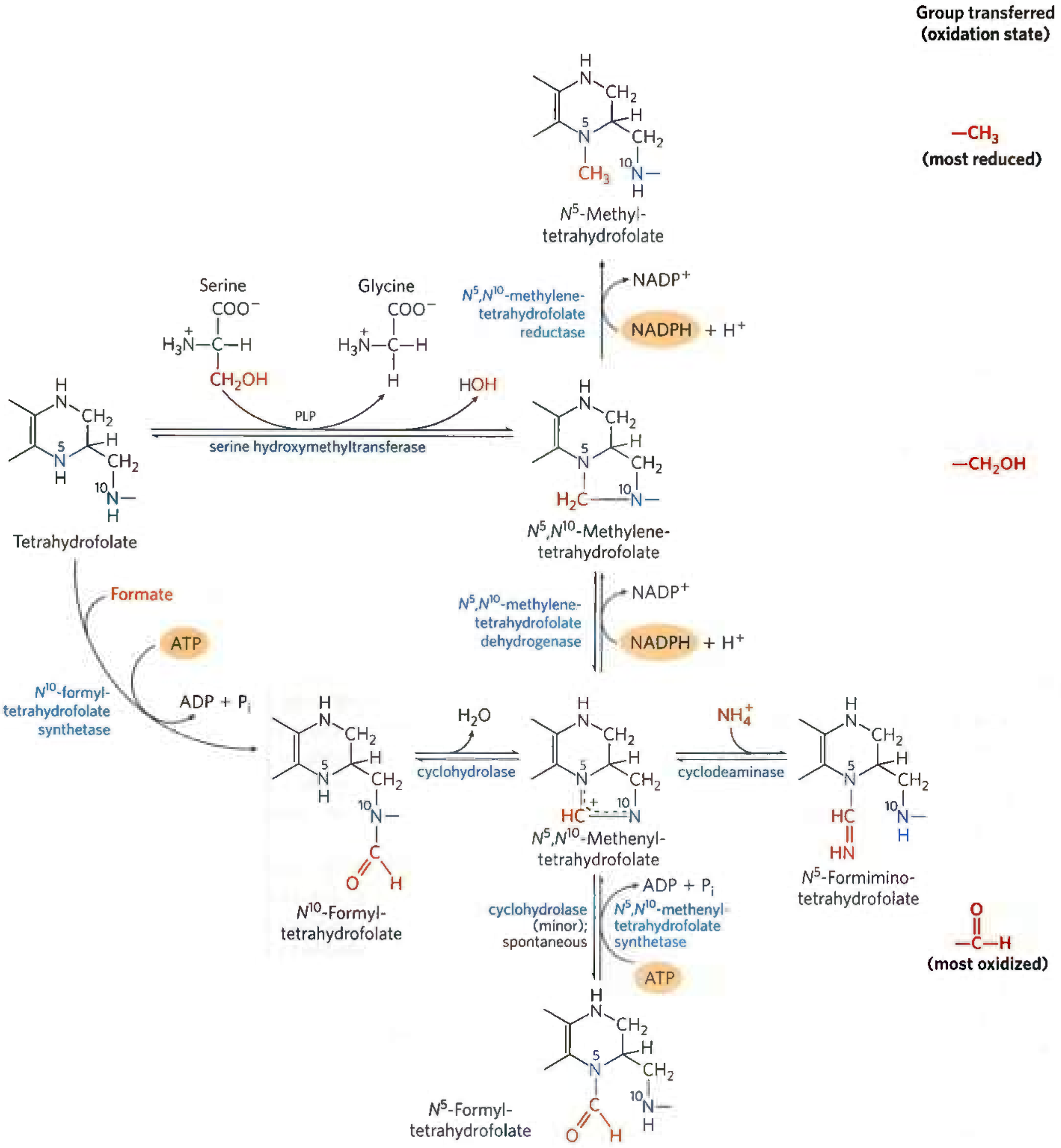

Group transferred (oxidation state)
N^5-Methyl-tetrahydrofolate
—CH₃ (most reduced)
N^5,N^{10}-methylene-tetrahydrofolate reductase
NADP⁺
NADPH + H⁺
Serine
Glycine
Tetrahydrofolate
PLP
HOH
serine hydroxymethyltransferase
N^5,N^{10}-Methylene-tetrahydrofolate
—CH₂OH
N^5,N^{10}-methylene-tetrahydrofolate dehydrogenase
NADP⁺
NADPH + H⁺
Formate
ATP
ADP + Pᵢ
N^{10}-formyl-tetrahydrofolate synthetase
H₂O
cyclohydrolase
NH₄⁺
cyclodeaminase
N^{10}-Formyl-tetrahydrofolate
N^5,N^{10}-Methenyl-tetrahydrofolate
N^5-Formimino-tetrahydrofolate
ADP + Pᵢ
cyclohydrolase (minor); spontaneous
N^5,N^{10}-methenyl-tetrahydrofolate synthetase
ATP
—C(=O)—H (most oxidized)
N^5-Formyl-tetrahydrofolate

FIGURE 18-17 Conversions of one-carbon units on tetrahydrofolate. The different molecular species are grouped according to oxidation state, with the most reduced at the top and most oxidized at the bottom. All species within a single shaded box are at the same oxidation state. The conversion of N^5,N^{10}-methylenetetrahydrofolate to N^5-methyltetrahydrofolate is effectively irreversible. The enzymatic transfer of formyl groups, as in purine synthesis (see Fig. 22-35) and in the formation of formylmethionine in bacteria (Chapter 27), generally uses N^{10}-formyltetrahydrofolate rather than N^5-formyltetrahydrofolate. The latter species is significantly more stable and therefore a weaker donor of formyl groups. N^5-Formyltetrahydrofolate is a minor byproduct of the cyclohydrolase reaction, and can also form spontaneously. Conversion of N^5-formyltetrahydrofolate to N^5,N^{10}-methenyltetrahydrofolate requires ATP, because of an otherwise unfavorable equilibrium. Note that N^5-formiminotetrahydrofolate is derived from histidine in a pathway shown in Figure 18-26.

regenerated by transfer of a methyl group to homocysteine in a reaction catalyzed by methionine synthase (step ④), and methionine is reconverted to S-adenosylmethionine to complete an activated-methyl cycle.

One form of methionine synthase common in bacteria uses N^5-methyltetrahydrofolate as a methyl donor. Another form of the enzyme present in some bacteria and mammals uses N^5-methyltetrahydrofolate, but the methyl group is first transferred to cobalamin, derived from coenzyme B_{12}, to form methylcobalamin as the methyl donor in methionine formation. This reaction and the rearrangement of L-methylmalonyl-CoA to

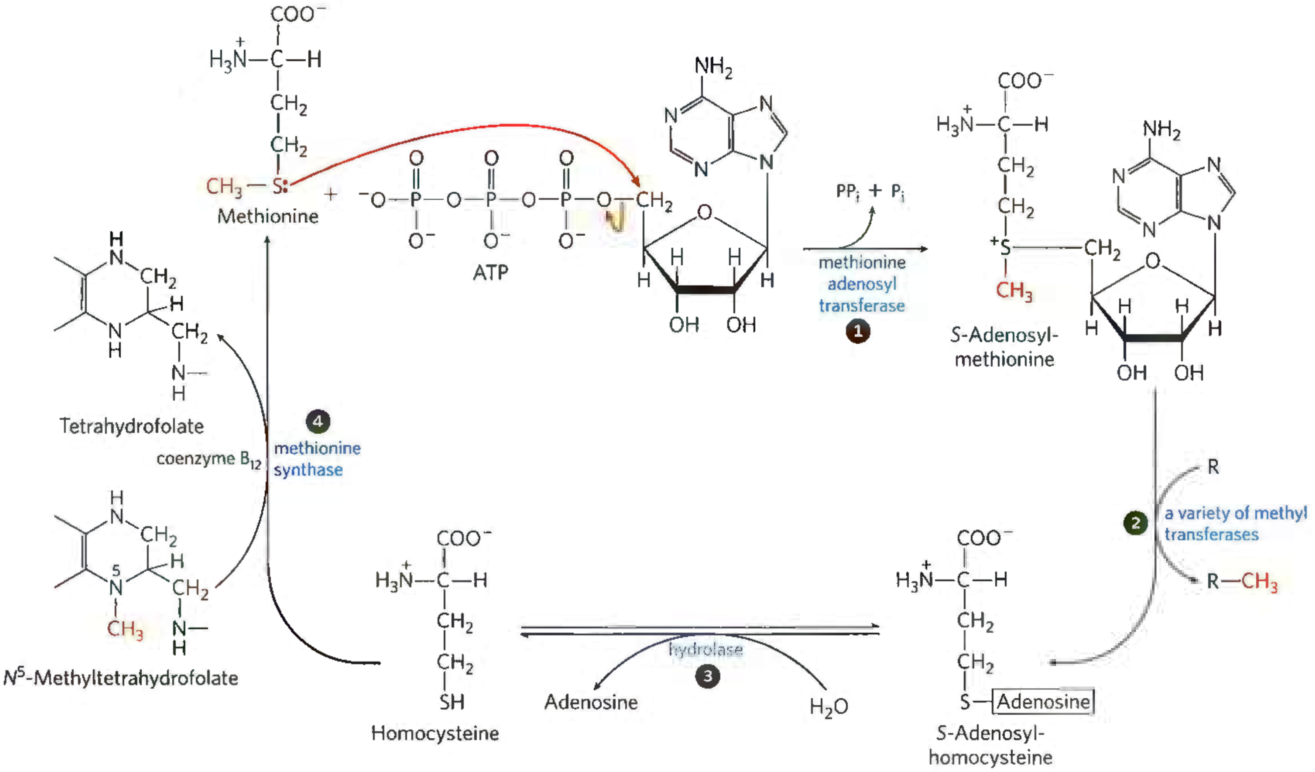


FIGURE 18-18 Synthesis of methionine and *S*-adenosylmethionine in an activated-methyl cycle. The steps are described in the text. In the methionine synthase reaction (step ④), the methyl group is transferred to cobalamin to form methylcobalamin, which is the methyl donor in the formation of methionine. *S*-Adenosylmethionine, which has a positively charged sulfur (and is thus a sulfonium ion), is a powerful methylating agent in several biosynthetic reactions. The methyl group acceptor (step ②) is designated R.

succinyl-CoA (see Box 17-2, Fig. 1a) are the only known coenzyme B_{12}–dependent reactions in mammals.

The vitamins B_{12} and folate are closely linked in these pathways. The anemia observed in the rare B_{12} deficiency disease **pernicious anemia** (Box 17-2) can be traced to the methionine synthase reaction. As noted above, the methyl group of methylcobalamin is derived from N^5-methyltetrahydrofolate, and this is the only reaction in mammals that uses N^5-methyltetrahydrofolate. The reaction converting the N^5,N^{10}-methylene form to the N^5-methyl form of tetrahydrofolate is irreversible (Fig. 18-17). Thus, if coenzyme B_{12} is not available for the synthesis of methylcobalamin, metabolic folates become trapped in the N^5-methyl form.

The anemia associated with vitamin B_{12} deficiency is called **megaloblastic anemia**. It manifests as a decline in the production of mature erythrocytes (red blood cells) and the appearance in the bone marrow of immature precursor cells, or **megaloblasts**. Erythrocytes are gradually replaced in the blood by smaller numbers of abnormally large erythrocytes called **macrocytes**. The defect in erythrocyte development is a direct consequence of the depletion of the N^5,N^{10}-methylenetetrahydrofolate, which is required for synthesis of the thymidine nucleotides needed for DNA synthesis (see Chapter 22). Folate deficiency, in which all forms of tetrahydrofolate are depleted, also produces anemia, for much the same reasons. The anemia symptoms of B_{12} deficiency can be alleviated by administering either vitamin B_{12} or folate.

However, it is dangerous to treat pernicious anemia by folate supplementation alone, because the neurological symptoms of B_{12} deficiency will progress. These symptoms do not arise from the defect in the methionine synthase reaction. Instead, the impaired methylmalonyl-CoA mutase (see Box 17-2 and Fig. 17-12) causes accumulation of unusual, odd-number fatty acids in neuronal membranes. The anemia associated with folate deficiency is thus often treated by administering both folate and vitamin B_{12}, at least until the metabolic source of the anemia is unambiguously defined. Early diagnosis of B_{12} deficiency is important because some of its associated neurological conditions may be irreversible.

Folate deficiency also reduces the availability of the N^5-methyltetrahydrofolate required for methionine synthase function. This leads to a rise in homocysteine levels in blood, a condition linked to heart disease, hypertension, and stroke. High levels of homocysteine may be responsible for 10% of all cases of heart disease. The condition is treated with folate supplements. ■

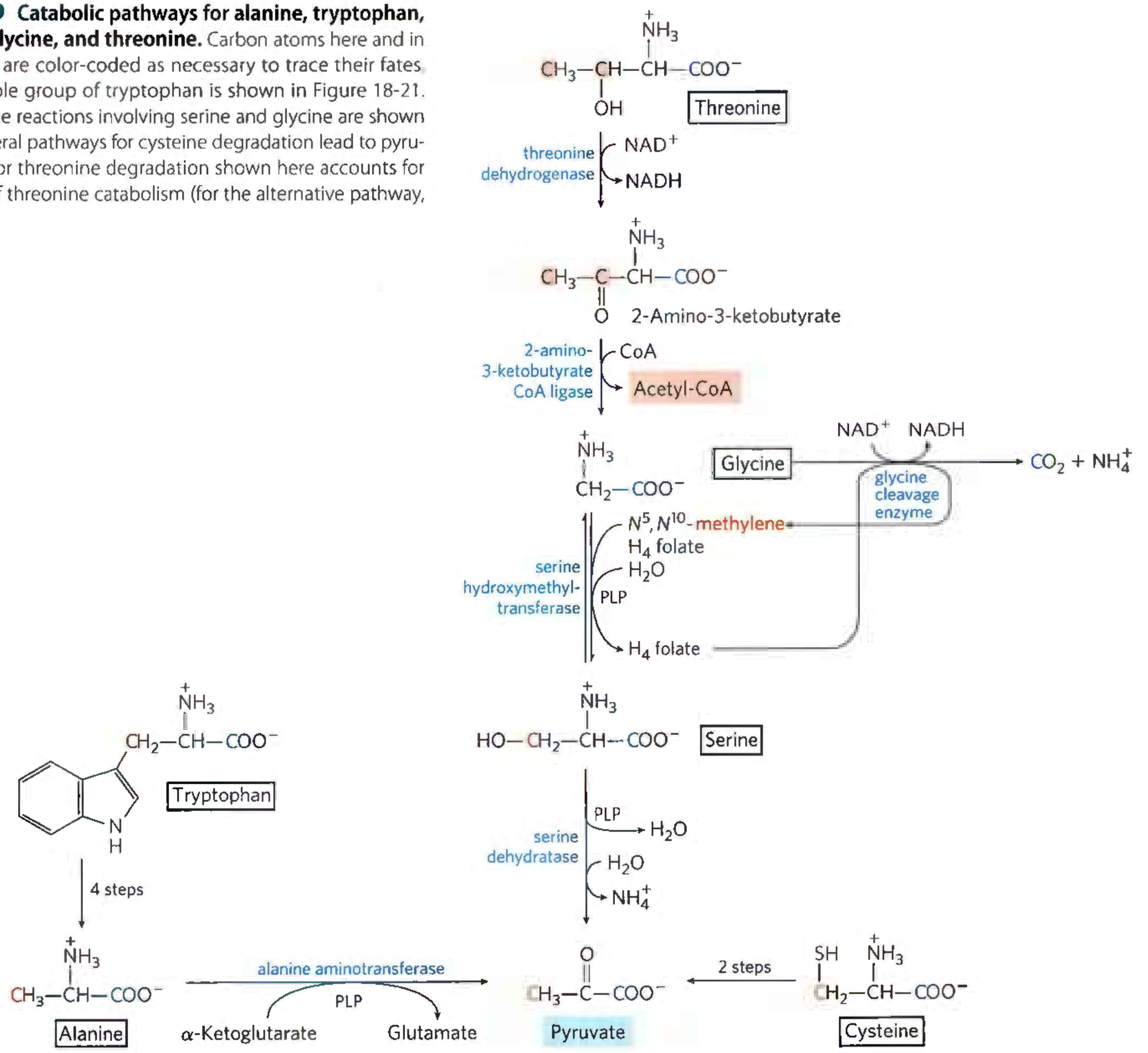
Tetrahydrobiopterin, another cofactor of amino acid catabolism, is similar to the pterin moiety of tetrahydrofolate, but it is not involved in one-carbon transfers; instead it participates in oxidation reactions. We consider its mode of action when we discuss phenylalanine degradation (see Fig. 18-24).

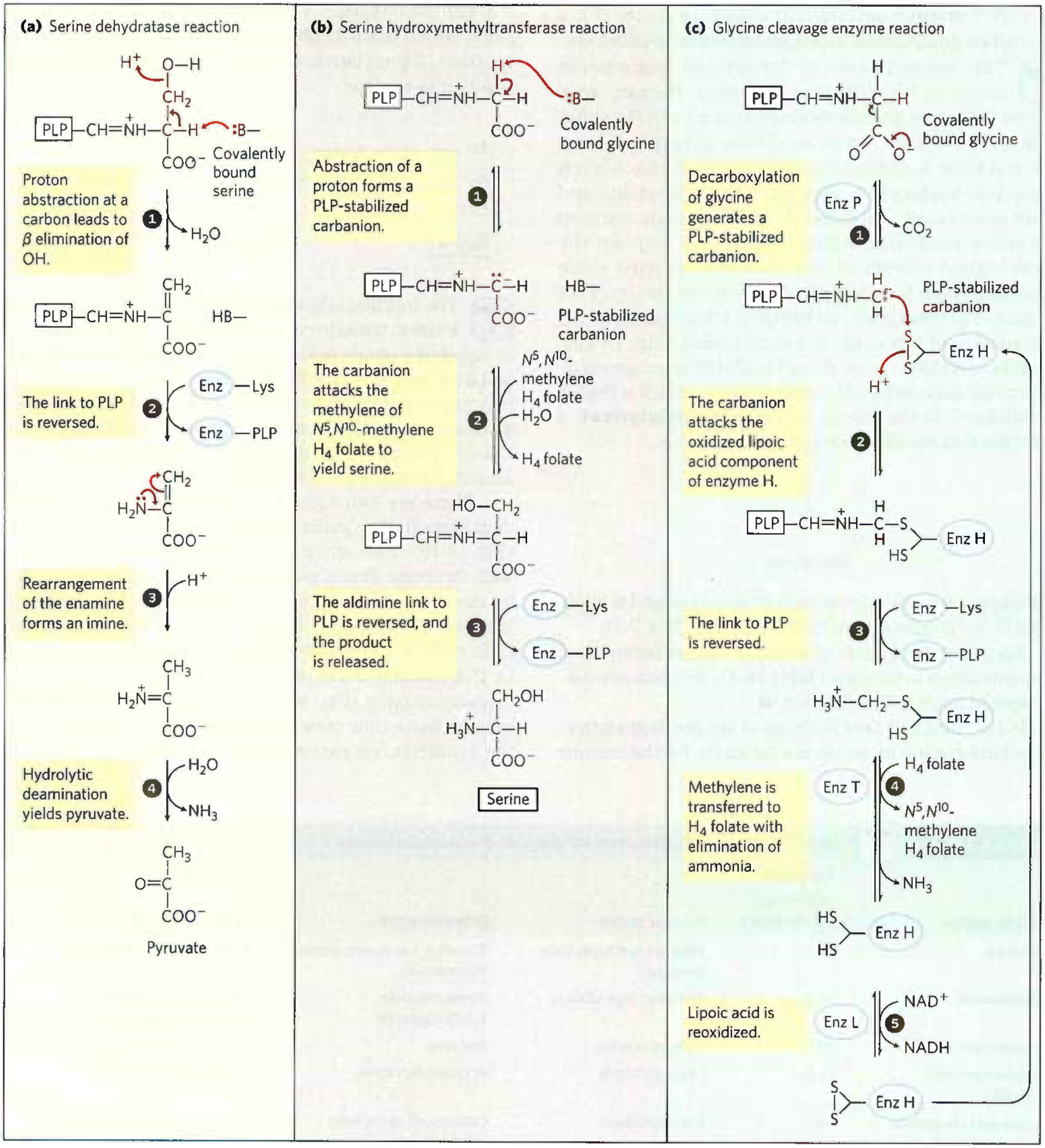
Six Amino Acids Are Degraded to Pyruvate

P1 P3 P5 The carbon skeletons of six amino acids — alanine, tryptophan, cysteine, serine, glycine, and threonine — are converted in whole or in part to pyruvate. The pyruvate can then be converted to acetyl-CoA and eventually oxidized via the citric acid cycle, or to oxaloacetate and shunted into gluconeogenesis (**Fig. 18-19**). **Alanine** yields pyruvate directly on transamination with α-ketoglutarate, and the side chain of **tryptophan** is cleaved to yield alanine and thus pyruvate. **Cysteine** is converted to pyruvate in two steps; one removes the sulfur atom, the other is a transamination. **Serine** is converted to pyruvate by serine dehydratase. Both the β-hydroxyl and the α-amino groups of serine are removed in this single pyridoxal phosphate–dependent reaction (**Fig. 18-20a**).

Glycine is degraded via three pathways, only one of which leads to pyruvate. Glycine is converted to serine by enzymatic addition of a hydroxymethyl group (Figs 18-19, 18-20b). This reaction, catalyzed by **serine hydroxymethyltransferase**, requires the coenzymes tetrahydrofolate and pyridoxal phosphate. The serine is converted to pyruvate as described above. In the second pathway, which predominates in animals, glycine undergoes oxidative cleavage to CO_2, NH_4^+, and a methylene group ($—CH_2—$) (Figs. 18-19, 18-20c). This readily reversible reaction, catalyzed by **glycine cleavage enzyme** (also called glycine synthase), also requires tetrahydrofolate, which accepts the methylene group. In this oxidative cleavage pathway, the two carbon atoms of glycine do not enter the citric acid cycle. One carbon is lost as CO_2 and the other becomes the methylene group

FIGURE 18-19 Catabolic pathways for alanine, tryptophan, cysteine, serine, glycine, and threonine. Carbon atoms here and in subsequent figures are color-coded as necessary to trace their fates. The fate of the indole group of tryptophan is shown in Figure 18-21. Details of most of the reactions involving serine and glycine are shown in Figure 18-20. Several pathways for cysteine degradation lead to pyruvate. The pathway for threonine degradation shown here accounts for only about a third of threonine catabolism (for the alternative pathway, see Fig 18-27).

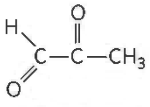

Threonine
threonine dehydrogenase
NAD⁺
NADH
2-Amino-3-ketobutyrate
2-amino-3-ketobutyrate CoA ligase
CoA
Acetyl-CoA
Glycine
NAD⁺ NADH
glycine cleavage enzyme
CO₂ + NH₄⁺
N⁵,N¹⁰-methylene H₄ folate
H₂O
serine hydroxymethyltransferase
PLP
H₄ folate
Serine
serine dehydratase
PLP
H₂O
H₂O
NH₄⁺
Tryptophan
4 steps
Alanine
alanine aminotransferase
PLP
α-Ketoglutarate
Glutamate
Pyruvate
2 steps
Cysteine


(a) Serine dehydratase reaction
PLP
Covalently bound serine
Proton abstraction at α carbon leads to β elimination of OH.
①
H₂O
HB—
:B—
The link to PLP is reversed.
②
Enz —Lys
Enz —PLP
Rearrangement of the enamine forms an imine.
③
H⁺
Hydrolytic deamination yields pyruvate.
④
H₂O
NH₃
Pyruvate
(b) Serine hydroxymethyltransferase reaction
Covalently bound glycine
Abstraction of a proton forms a PLP-stabilized carbanion.
①
PLP-stabilized carbanion
The carbanion attacks the methylene of N⁵,N¹⁰-methylene H₄ folate to yield serine.
②
N⁵,N¹⁰-methylene H₄ folate
H₂O
H₄ folate
The aldimine link to PLP is reversed, and the product is released.
③
Enz —Lys
Enz —PLP
Serine
(c) Glycine cleavage enzyme reaction
Covalently bound glycine
Decarboxylation of glycine generates a PLP-stabilized carbanion.
Enz P
①
CO₂
PLP-stabilized carbanion
Enz H
H⁺
The carbanion attacks the oxidized lipoic acid component of enzyme H.
②
The link to PLP is reversed.
③
Enz —Lys
Enz —PLP
Methylene is transferred to H₄ folate with elimination of ammonia.
Enz T
④
H₄ folate
N⁵,N¹⁰-methylene H₄ folate
NH₃
Lipoic acid is reoxidized.
Enz L
⑤
NAD⁺
NADH

MECHANISM FIGURE 18-20 Interplay of the pyridoxal phosphate and tetrahydrofolate cofactors in serine and glycine metabolism. The first step in each of these reactions (not shown) involves the formation of a covalent imine linkage between enzyme-bound PLP and the substrate amino acid—serine in (a), glycine in (b) and (c). (a) A PLP-catalyzed elimination of water in the serine dehydratase reaction (step ①) begins the pathway to pyruvate. (b) In the serine hydroxymethyltransferase reaction, a PLP-stabilized carbanion (product of step ①) is a key intermediate in the reversible transfer of the methylene group (as—CH_2—OH) from N^5,N^{10}-methylenetetrahydrofolate to form serine. (c) The glycine cleavage enzyme is a multienzyme complex, with components P, H, T, and L. The overall reaction, which is reversible, converts glycine to CO_2 and NH_4^+, with the second glycine carbon taken up by tetrahydrofolate to form N^5,N^{10}-methylenetetrahydrofolate. Pyridoxal phosphate activates the α carbon of amino acids at critical stages in all these reactions, and tetrahydrofolate carries a one-carbon unit in two of them (see Figs 18-6, 18-17).

of N^5,N^{10}-methylenetetrahydrofolate (Fig. 18-17), a one-carbon group donor in certain biosynthetic pathways.

This second pathway for glycine degradation seems to be critical in mammals. Humans with serious defects in glycine cleavage enzyme activity suffer from a condition known as nonketotic hyperglycinemia. The condition is characterized by elevated serum levels of glycine, leading to severe intellectual disability and death in very early childhood. At high levels, glycine is an inhibitory neurotransmitter, which may explain the neurological effects of the disease. Perhaps more important, high levels of glycine increase the levels of 2-amino-3-ketobutyrate, an unstable intermediate in the degradation of threonine in mitochondria (Fig. 18-19). 2-Amino-3-ketobutyrate decarboxylates spontaneously to form the toxic metabolite aminoacetone, which is readily metabolized to the highly reactive **methylglyoxal**, a molecule that modifies both protein and DNA.

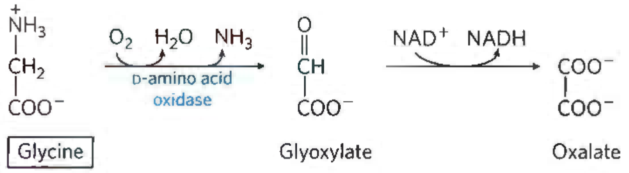


Methylglyoxal

Methylglyoxal is also a byproduct of glycolysis and is implicated in the progression of type 2 diabetes (Box 7-2).

Many genetic defects of amino acid metabolism have been identified in humans (**Table 18-2**). We shall encounter several more in this chapter. ■

In the third and final pathway of glycine degradation, the achiral glycine molecule is a substrate for the enzyme D-amino acid oxidase. The glycine is converted to glyoxylate, an alternative substrate for lactate dehydrogenase (p. 526). Glyoxylate is oxidized in an NAD^+-dependent reaction to oxalate:



The function of D-amino acid oxidase, present at high levels in the kidney, is thought to be the detoxification of ingested D-amino acids derived from bacterial cell walls and from grilled foodstuffs (high heat causes some spontaneous racemization of the L-amino acids in proteins). Oxalate, whether obtained in foods or produced enzymatically in the kidneys, has medical significance. Crystals of calcium oxalate account for up to 75% of all kidney stones. ■

There are two significant pathways for **threonine** degradation. One pathway leads to pyruvate via glycine (Fig. 18-19). The conversion to glycine occurs in two steps, with threonine first converted to 2-amino-3-ketobutyrate by the action of threonine dehydrogenase. This pathway is important in a few classes of rapidly dividing human cells, such as embryonic stem cells. The glycine generated by this pathway is broken down primarily by the glycine cleavage enzyme (Fig. 18-19). The N^5,N^{10}-methylenetetrahydrofolate thus generated (Fig. 18-20c) is needed for the synthesis, via pathways described in Chapter 22, of

TABLE 18-2 **Some Human Genetic Disorders Affecting Amino Acid Catabolism**

Medical condition	Approximate incidence (per 100,000 births)	Defective process	Defective enzyme	Symptoms and effects
Albinism	<3	Melanin synthesis from tyrosine	Tyrosine 3-monooxygenase (tyrosinase)	Lack of pigmentation; white hair, pink skin
Alkaptonuria	<0.4	Tyrosine degradation	Homogentisate 1,2-dioxygenase	Dark pigment in urine; late-developing arthritis
Argininemia	<0.5	Urea synthesis	Arginase	Intellectual disability
Argininosuccinic acidemia	<1.5	Urea synthesis	Argininosuccinase	Vomiting; convulsions
Carbamoyl phosphate synthetase I deficiency	<0.5	Urea synthesis	Carbamoyl phosphate synthetase I	Lethargy; convulsions; early death
Homocystinuria	<0.5	Methionine degradation	Cystathionine β-synthase	Faulty bone development; intellectual disability
Maple syrup urine disease (branched-chain ketoaciduria)	<0.4	Isoleucine, leucine, and valine degradation	Branched-chain α-keto acid dehydrogenase complex	Vomiting; convulsions; intellectual disability; early death
Methylmalonic acidemia	<0.5	Conversion of propionyl-CoA to succinyl-CoA	Methylmalonyl-CoA mutase	Vomiting; convulsions; intellectual disability; early death
Phenylketonuria	<8	Conversion of phenylalanine to tyrosine	Phenylalanine hydroxylase	Neonatal vomiting; intellectual disability

nucleotides used in DNA replication. However, in most human tissues, the degradation of threonine via glycine is a relatively minor pathway, accounting for 10% to 30% of threonine catabolism. It is more important in some other mammals. The major pathway in most human tissues leads to succinyl-CoA, as described later in this chapter.

Seven Amino Acids Are Degraded to Acetyl-CoA

P1 P5 Portions of the carbon skeletons of seven amino acids—**tryptophan**, **lysine**, **phenylalanine**, **tyrosine**, **leucine**, **isoleucine**, and **threonine**—yield acetyl-CoA and/or acetoacetyl-CoA, the latter being converted to acetyl-CoA (**Fig. 18-21**). Some of the final steps in the degradative pathways for leucine, lysine, and tryptophan resemble steps in the oxidation of fatty acids (see Fig. 17-9). Threonine (not shown in Fig. 18-21) yields some acetyl-CoA via the minor pathway illustrated in Figure 18-19.

The degradative pathways of two of these seven amino acids deserve special mention. Tryptophan breakdown is the most complex of all the pathways of amino acid catabolism in animal tissues; portions of tryptophan (four of its carbons) yield acetyl-CoA via acetoacetyl-CoA. Some of the intermediates in tryptophan catabolism are precursors for the synthesis of other

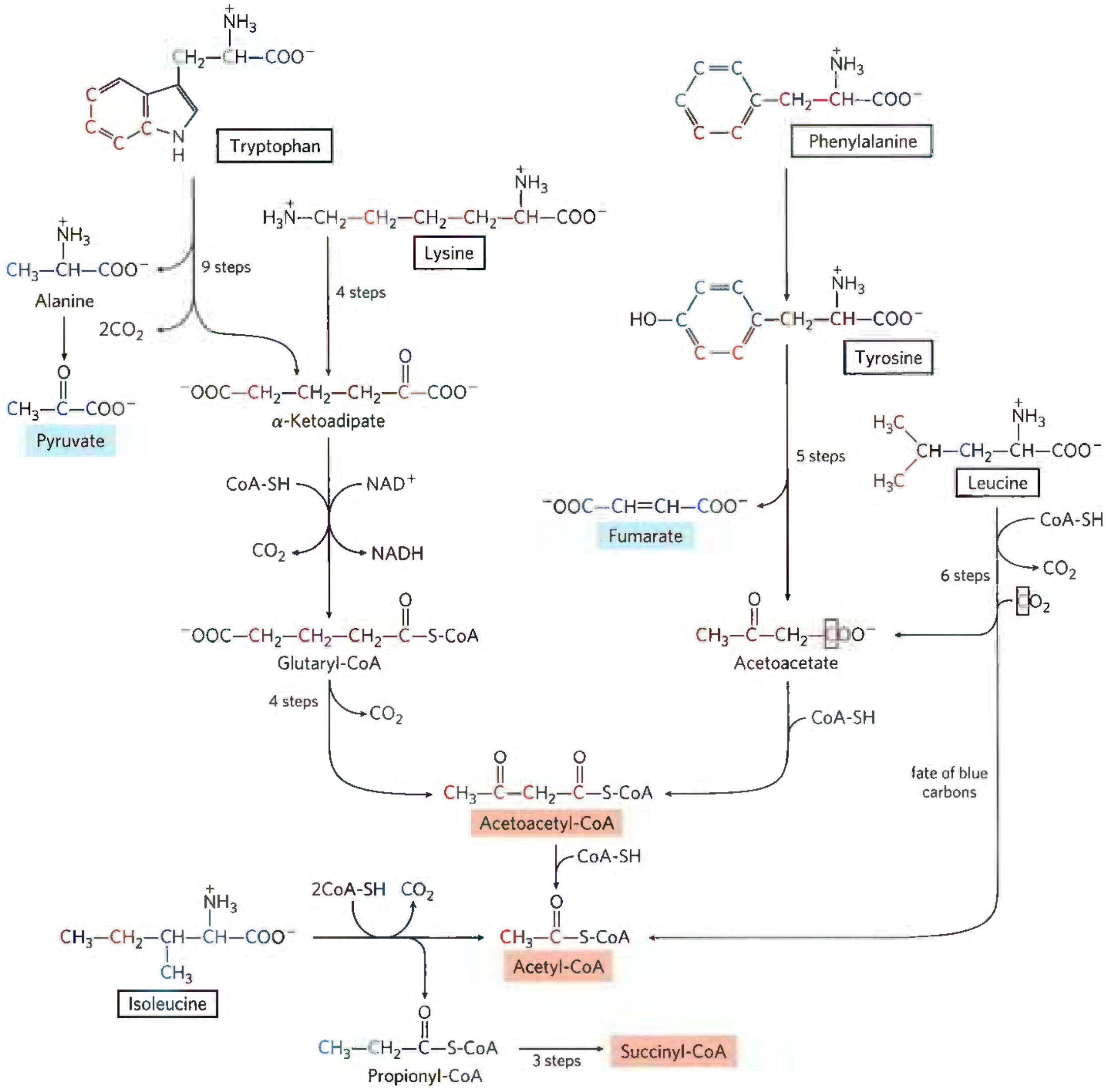

Tryptophan
9 steps
Alanine
2CO₂
Pyruvate
Lysine
4 steps
α-Ketoadipate
CoA-SH
NAD⁺
CO₂
NADH
Glutaryl-CoA
4 steps
CO₂
Phenylalanine
Tyrosine
5 steps
Fumarate
Leucine
CoA-SH
6 steps
CO₂
CO₂
Acetoacetate
CoA-SH
fate of blue carbons
Acetoacetyl-CoA
CoA-SH
Acetyl-CoA
2CoA-SH
CO₂
Isoleucine
Propionyl-CoA
3 steps
Succinyl-CoA

FIGURE 18-21 Catabolic pathways for tryptophan, lysine, phenylalanine, tyrosine, leucine, and isoleucine. These amino acids donate some of their carbons (red) to acetoacetate and acetyl-CoA. In the pathway for leucine catabolism, the carbon in acetoacetate that is donated by CO_2 is surrounded by a box to distinguish it from the three carbons that come from leucine itself. Tryptophan, phenylalanine, tyrosine, and isoleucine also contribute carbons (blue) to pyruvate or citric acid cycle intermediates. The phenylalanine pathway is described in more detail in Figure 18-23. The fate of nitrogen atoms is not traced in this scheme; in most cases they are transferred to α-ketoglutarate to form glutamate.

biomolecules (**Fig. 18-22**), including nicotinate, a precursor of NAD and NADP in animals; serotonin, a neurotransmitter in vertebrates; and indoleacetate, a growth factor in plants. Some of these biosynthetic pathways are described in more detail in Chapter 22 (see Figs. 22-30, 22-31).

The breakdown of phenylalanine is noteworthy because genetic defects in the enzymes of this pathway lead to several inheritable human diseases (**Fig. 18-23**), as discussed below. Phenylalanine and its oxidation product tyrosine (both with nine carbons) are degraded into two fragments, both of which can enter the citric acid cycle: four of the nine carbon atoms yield free acetoacetate, which is converted to acetoacetyl-CoA and thus acetyl-CoA, and a second four-carbon fragment is recovered as fumarate. Eight of the nine carbons of these two amino acids thus enter the citric acid cycle; the remaining carbon is lost as CO_2. Phenylalanine, after its hydroxylation to tyrosine, is also the precursor of dopamine, a neurotransmitter, and of norepinephrine and epinephrine, hormones secreted by the adrenal medulla (see Fig. 22-31). Melanin, the black pigment of skin and hair, is also derived from tyrosine.

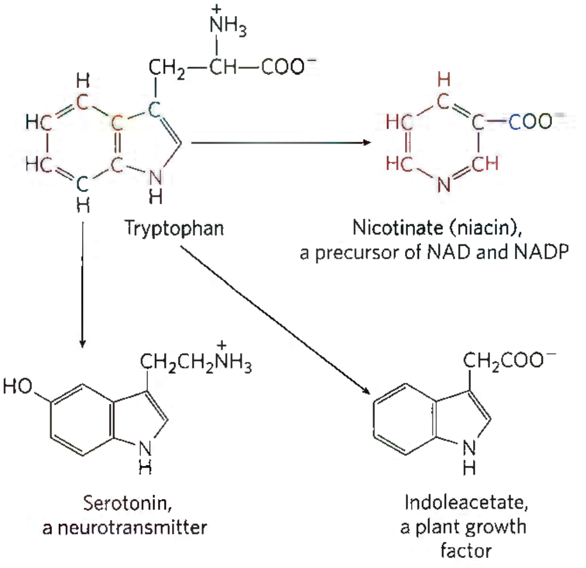

Tryptophan
Nicotinate (niacin), a precursor of NAD and NADP
Serotonin, a neurotransmitter
Indoleacetate, a plant growth factor

FIGURE 18-22 Tryptophan as precursor. The aromatic rings of tryptophan give rise to nicotinate (niacin), indoleacetate, and serotonin. Colored atoms trace the source of the ring atoms in nicotinate.

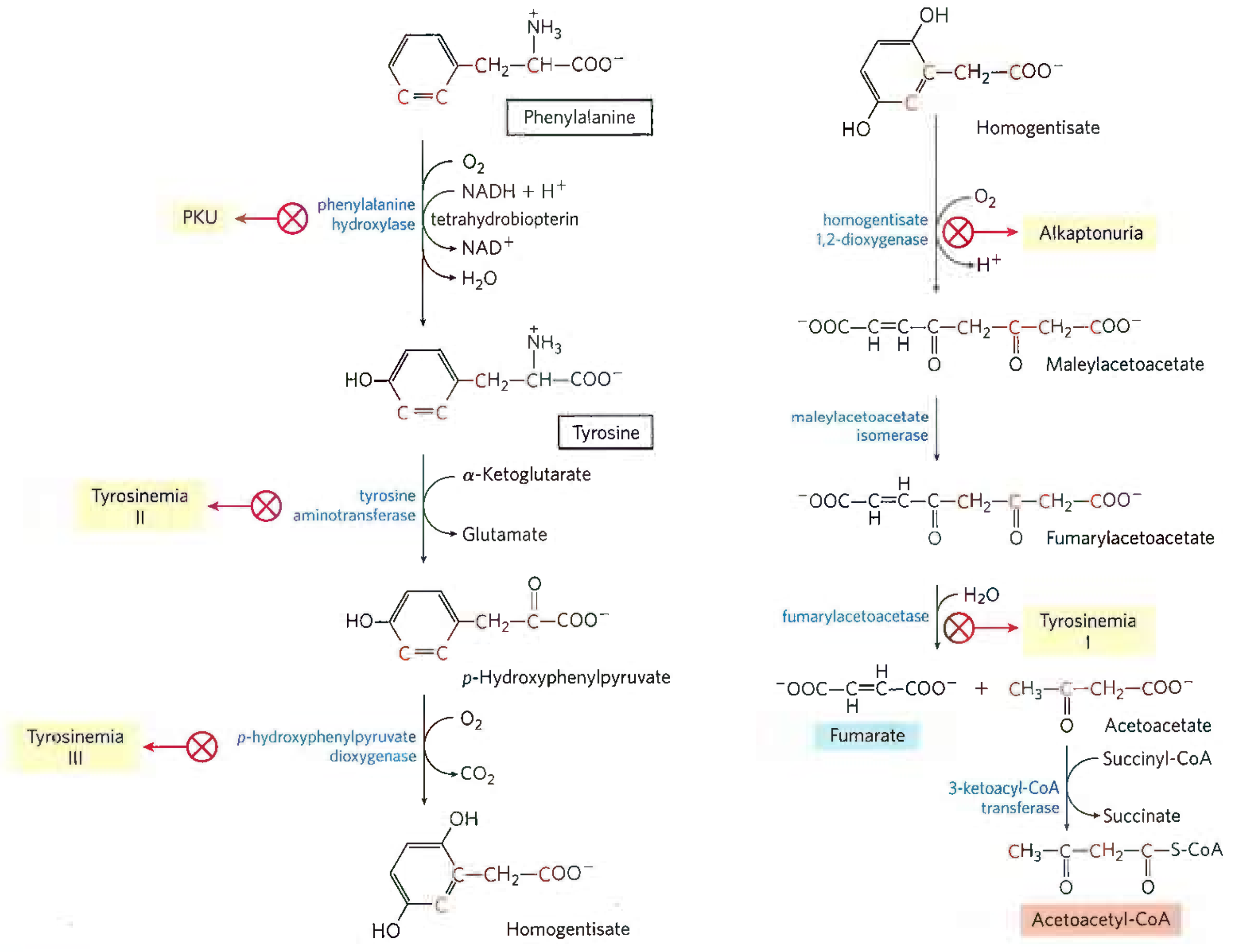

Phenylalanine
PKU
phenylalanine hydroxylase
O2
NADH + H+
tetrahydrobiopterin
NAD+
H2O
Tyrosine
Tyrosinemia II
tyrosine aminotransferase
α-Ketoglutarate
Glutamate
p-Hydroxyphenylpyruvate
Tyrosinemia III
p-hydroxyphenylpyruvate dioxygenase
O2
CO2
Homogentisate
Homogentisate
homogentisate 1,2-dioxygenase
O2
H+
Alkaptonuria
Maleylacetoacetate
maleylacetoacetate isomerase
Fumarylacetoacetate
fumarylacetoacetase
H2O
Tyrosinemia I
Fumarate
Acetoacetate
3-ketoacyl-CoA transferase
Succinyl-CoA
Succinate
Acetoacetyl-CoA

FIGURE 18-23 Catabolic pathways for phenylalanine and tyrosine. In humans these amino acids are normally converted to acetoacetyl-CoA and fumarate. Genetic defects in many of these enzymes cause inheritable human diseases (shaded yellow).

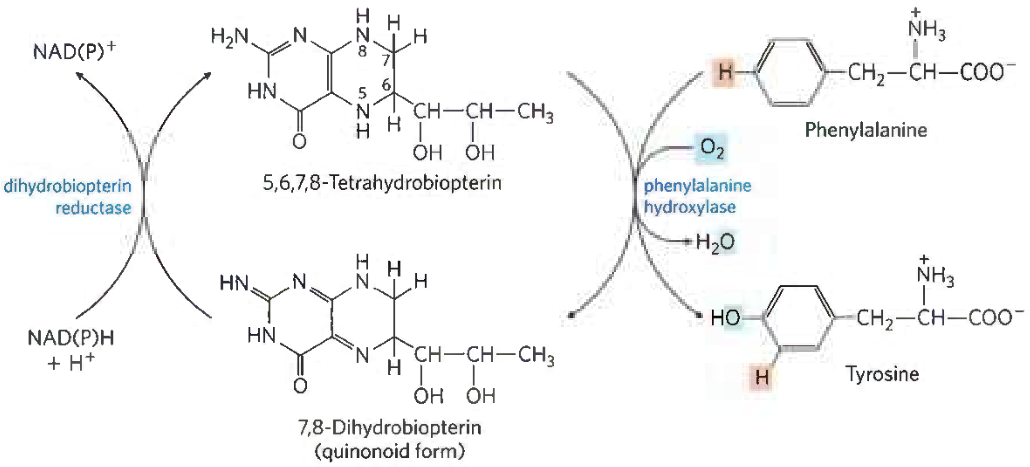

NAD(P)$^+$
dihydrobiopterin reductase
NAD(P)H + H$^+$
5,6,7,8-Tetrahydrobiopterin
7,8-Dihydrobiopterin (quinonoid form)
O_2
phenylalanine hydroxylase
H_2O
Phenylalanine
Tyrosine

FIGURE 18-24 Role of tetrahydrobiopterin in the phenylalanine hydroxylase reaction. The H atom shaded pink is transferred directly from C-4 to C-3 in the reaction. This feature, discovered at the National Institutes of Health, is called the NIH shift.

Phenylalanine Catabolism Is Genetically Defective in Some People

Given that many amino acids are either neurotransmitters or precursors or antagonists of neurotransmitters, it is not surprising that genetic defects of amino acid metabolism can cause defective neural development and intellectual deficits. In most such diseases, specific intermediates accumulate. For example, a genetic defect in **phenylalanine hydroxylase**, the first enzyme in the catabolic pathway for phenylalanine (Fig. 18-23), is responsible for the disease **phenylketonuria (PKU)**, the most common cause of elevated levels of phenylalanine in the blood (hyperphenylalaninemia).

Phenylalanine hydroxylase (also called phenylalanine-4-monooxygenase) is one of a general class of enzymes called **mixed-function oxygenases** (see Box 21-1), all of which catalyze simultaneous hydroxylation of a substrate by an oxygen atom of O_2 and reduction of the other oxygen atom to H_2O. Phenylalanine hydroxylase requires the cofactor tetrahydrobiopterin, which carries electrons from NADPH to O_2 and becomes oxidized to dihydrobiopterin in the process (**Fig. 18-24**). It is subsequently reduced by the enzyme **dihydrobiopterin reductase** in a reaction that requires NADPH.

In individuals with PKU, a secondary, normally little-used pathway of phenylalanine metabolism comes into play. In this pathway phenylalanine undergoes transamination with pyruvate to yield **phenylpyruvate** (**Fig. 18-25**). Phenylalanine and phenylpyruvate accumulate in the blood and tissues and are excreted in the urine—hence the name "phenylketonuria." Much of the phenylpyruvate, rather than being excreted as such, is either decarboxylated to phenylacetate or reduced to phenyllactate. Phenylacetate imparts a characteristic honeylike odor to the urine, which nurses have traditionally used to detect PKU in infants. The accumulation of phenylalanine or its metabolites in early life impairs normal development of the brain, causing severe intellectual deficits. This may be caused by excess phenylalanine competing with other amino acids for transport across the blood-brain barrier, resulting in a deficit of required metabolites.

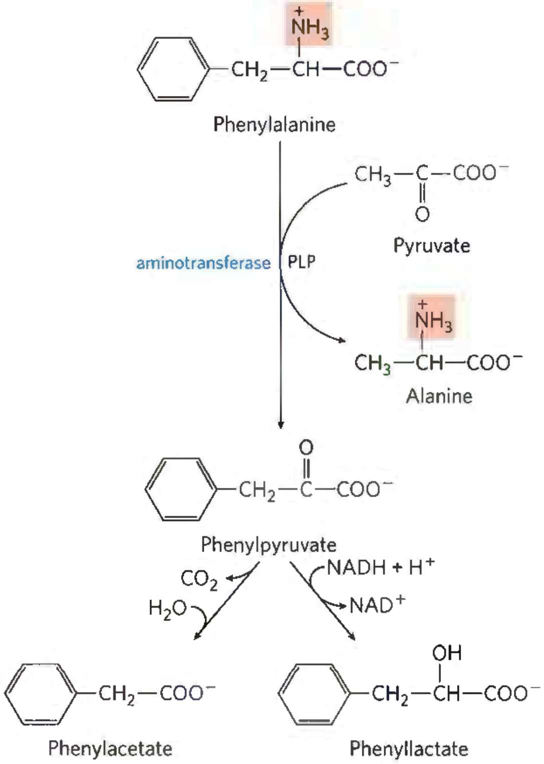


FIGURE 18-25 Alternative pathways for catabolism of phenylalanine in phenylketonuria. In PKU, phenylpyruvate accumulates in the tissues, blood, and urine. The urine may also contain phenylacetate and phenyllactate.

Phenylketonuria was among the first inheritable metabolic defects discovered in humans. When this condition is recognized early in infancy, intellectual disability can be prevented by rigid dietary control. The diet must supply only enough phenylalanine and tyrosine to meet the needs for protein synthesis. Consumption of protein-rich foods must be curtailed. Natural proteins, such as casein of milk, must first be hydrolyzed and much of the phenylalanine removed to provide an appropriate diet, at least through childhood. Because the artificial sweetener aspartame is a dipeptide of aspartate and the methyl ester of phenylalanine (see Fig. 1-23a), foods sweetened with aspartame bear warnings addressed to individuals on phenylalanine-controlled diets.

The dietary restrictions are difficult to follow perfectly for a lifetime, and thus often do not completely eliminate neurological symptoms. An enzyme substitution treatment has been developed in which the enzyme phenylalanine ammonia lyase is modified with polyethylene glycol (PEGylated) and injected subcutaneously to degrade phenylalanine in proteins ingested as part of a somewhat less restricted diet. Derived from plants, bacteria, and many yeast and fungi, phenylalanine ammonia lyase normally contributes to the biosynthesis of polyphenol compounds such as flavonoids. It degrades phenylalanine to the harmless metabolite *trans*-cinnamic acid and ammonia; the small amounts of ammonia generated are not toxic. The treatment was approved for patients in 2018. The long-term effects of the treatment continue to be studied.

Phenylketonuria can also be caused by a defect in the enzyme that catalyzes the regeneration of tetrahydrobiopterin (Fig. 18-24). The treatment in this case is more complex than restricting the intake of phenylalanine and tyrosine. Tetrahydrobiopterin is also required for the formation of L-3,4-dihydroxyphenylalanine (L-dopa) and 5-hydroxytryptophan—precursors of the neurotransmitters norepinephrine and serotonin, respectively. In phenylketonuria of this type, these precursors must be supplied in the diet, along with tetrahydrobiopterin.

Screening newborns for genetic diseases can be highly cost-effective, especially in the case of PKU. The tests (no longer relying on urine odor) are relatively inexpensive, and the detection and early treatment of PKU in infants (eight to ten cases per 100,000 newborns) saves millions of dollars in later health care costs each year. More importantly, the emotional trauma avoided by early detection with these simple tests is inestimable.

Another inheritable disease of phenylalanine catabolism is **alkaptonuria**, in which the defective enzyme is **homogentisate dioxygenase** (Fig. 18-23). Less serious than PKU, this condition produces few ill effects, although large amounts of homogentisate are excreted and its oxidation turns the urine black. Individuals with alkaptonuria are also prone to develop a form of arthritis. Alkaptonuria is of considerable historical interest. Archibald Garrod discovered in the early 1900s that this condition is inherited, and he traced the cause to the absence of a single enzyme. Garrod was the first to make a connection between an inheritable trait and an enzyme—a great advance on the path that ultimately led to our current understanding of genes and the information pathways described in Part III. ■

Five Amino Acids Are Converted to α-Ketoglutarate

P5 The carbon skeletons of five amino acids (proline, glutamate, glutamine, arginine, and histidine) enter the citric acid cycle as α-ketoglutarate (**Fig. 18-26**). **Proline**, **glutamate**, and **glutamine** have five-carbon skeletons. The cyclic structure of proline is opened by oxidation of the carbon most distant from the carboxyl group to create a Schiff base, then hydrolysis of the Schiff base to form a linear semialdehyde, glutamate γ-semialdehyde. This intermediate is further oxidized at the same carbon to produce glutamate. The action of glutaminase, or any of several enzyme reactions in which glutamine donates its amide nitrogen to an acceptor, converts glutamine to glutamate. Transamination or deamination of glutamate produces α-ketoglutarate.

Arginine and **histidine** contain five adjacent carbons and a sixth carbon attached through a nitrogen atom. The catabolic conversion of these amino acids to glutamate is therefore slightly more complex than the path from proline or glutamine (Fig. 18-26). Arginine is converted to the five-carbon skeleton of ornithine in the urea cycle (Fig. 18-10), and the ornithine is transaminated to glutamate γ-semialdehyde. Conversion of histidine to the five-carbon glutamate occurs in a multistep pathway; the extra carbon is removed in a step that uses tetrahydrofolate as cofactor.

Four Amino Acids Are Converted to Succinyl-CoA

P5 The carbon skeletons of methionine, isoleucine, threonine, and valine are degraded by pathways that yield succinyl-CoA (**Fig. 18-27**), an intermediate of the citric acid cycle. **Methionine** donates its methyl group to one of several possible acceptors through *S*-adenosyl methionine, and three of its four remaining carbon atoms are converted to the propionate of propionyl-CoA, a precursor of succinyl-CoA. **Isoleucine** undergoes transamination, followed by oxidative decarboxylation of the resulting α-keto acid. The remaining five-carbon skeleton is further oxidized to acetyl-CoA and propionyl-CoA. **Valine** undergoes transamination and decarboxylation, then a series of oxidation reactions that convert the remaining four carbons to propionyl-CoA. Some parts of the valine and isoleucine degradative pathways closely parallel steps in fatty acid degradation (see Fig. 17-9). In human tissues, **threonine** is also converted in two

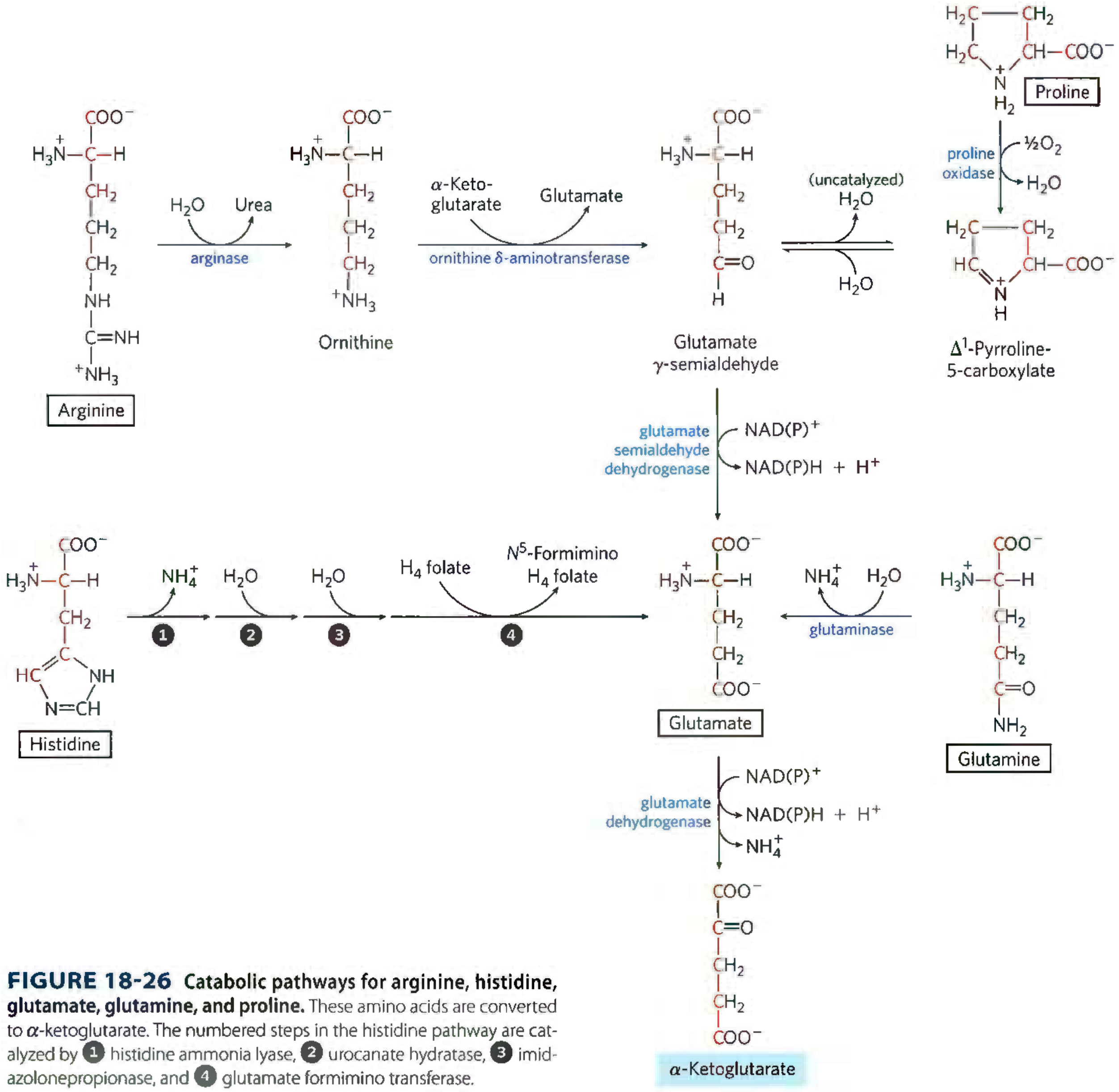

Arginine
H_2O
Urea
arginase
Ornithine
α-Keto-glutarate
Glutamate
ornithine δ-aminotransferase
Glutamate γ-semialdehyde
(uncatalyzed)
H_2O
H_2O
Proline
proline oxidase
½O_2
H_2O
Δ^1-Pyrroline-5-carboxylate
glutamate semialdehyde dehydrogenase
$NAD(P)^+$
$NAD(P)H + H^+$
Histidine
NH_4^+
H_2O
H_2O
H_4 folate
N^5-Formimino H_4 folate
❶
❷
❸
❹
Glutamate
NH_4^+
H_2O
glutaminase
Glutamine
glutamate dehydrogenase
$NAD(P)^+$
$NAD(P)H + H^+$
NH_4^+
α-Ketoglutarate

FIGURE 18-26 Catabolic pathways for arginine, histidine, glutamate, glutamine, and proline. These amino acids are converted to α-ketoglutarate. The numbered steps in the histidine pathway are catalyzed by ❶ histidine ammonia lyase, ❷ urocanate hydratase, ❸ imidazolonepropionase, and ❹ glutamate formimino transferase.

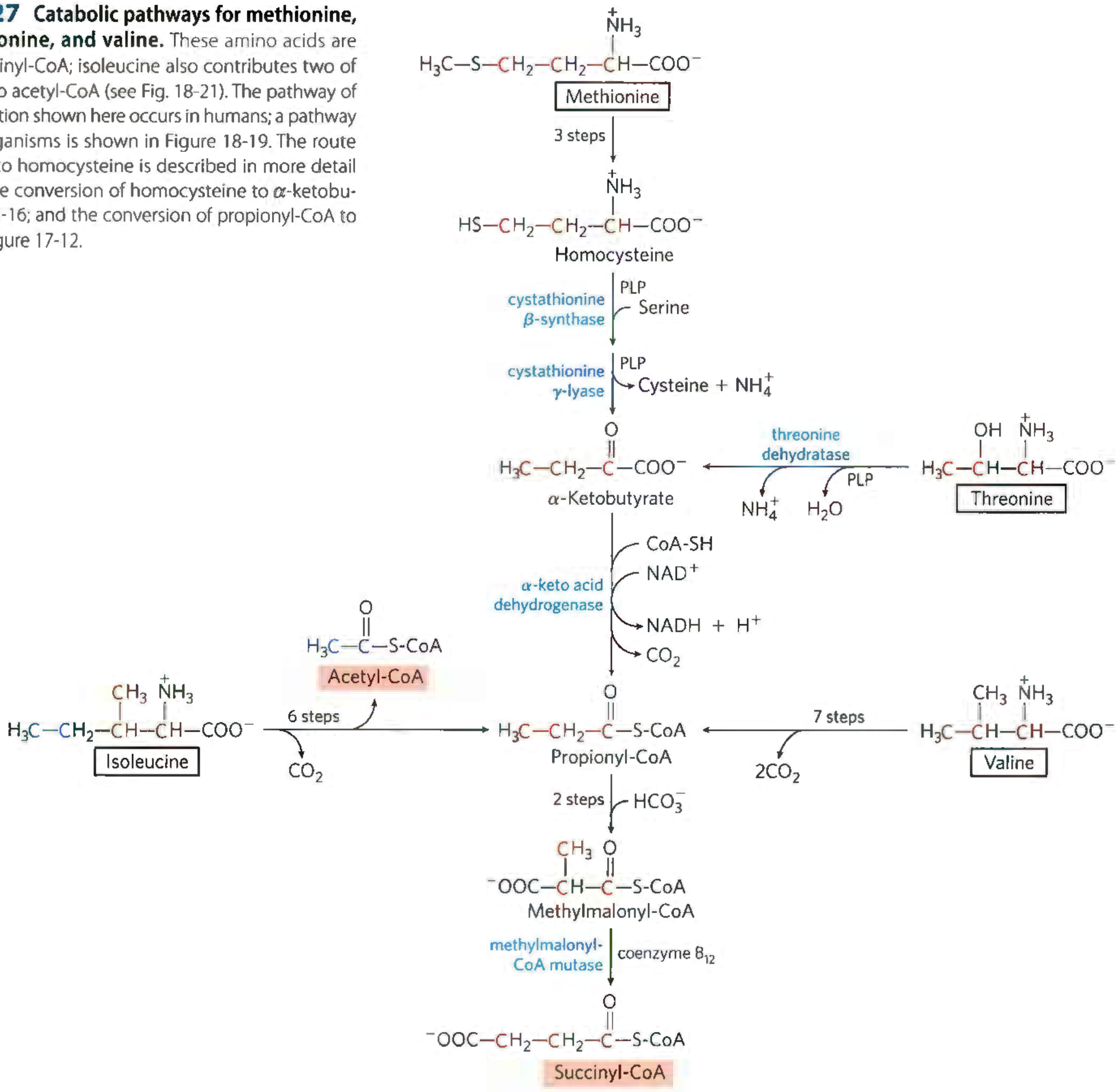
steps to propionyl-CoA. This is the primary pathway for threonine degradation in humans (see Fig. 18-19 for the alternative pathway). The mechanism of the first step is analogous to that catalyzed by serine dehydratase, and the serine and threonine dehydratases may actually be the same enzyme.

The propionyl-CoA derived from these three amino acids is converted to succinyl-CoA by a pathway described in Chapter 17: carboxylation to methylmalonyl-CoA, epimerization of the methylmalonyl-CoA, and conversion to succinyl-CoA by the coenzyme B_{12}–dependent methylmalonyl-CoA mutase (see Fig. 17-12). In the rare genetic disease known as methylmalonic acidemia, methylmalonyl-CoA mutase is lacking—with serious metabolic consequences (Table 18-2; **Box 18-2**).

Branched-Chain Amino Acids Are Not Degraded in the Liver

Although much of the catabolism of amino acids takes place in the liver, the three amino acids with branched side chains (leucine, isoleucine, and valine) are oxidized as fuels primarily in muscle, adipose, kidney, and brain tissue. These extrahepatic tissues contain an aminotransferase, absent in liver, that acts on all three branched-chain amino acids to produce the corresponding α-keto acids (**Fig. 18-28**). The **branched-chain α-keto acid dehydrogenase complex** then catalyzes oxidative decarboxylation of all three α-keto acids, in each case releasing the carboxyl group as CO_2 and producing the acyl-CoA derivative. This reaction is formally analogous

FIGURE 18-27 Catabolic pathways for methionine, isoleucine, threonine, and valine. These amino acids are converted to succinyl-CoA; isoleucine also contributes two of its carbon atoms to acetyl-CoA (see Fig. 18-21). The pathway of threonine degradation shown here occurs in humans; a pathway found in other organisms is shown in Figure 18-19. The route from methionine to homocysteine is described in more detail in Figure 18-18; the conversion of homocysteine to α-ketobutyrate, in Figure 22-16; and the conversion of propionyl-CoA to succinyl-CoA, in Figure 17-12.


Methionine
3 steps
Homocysteine
cystathionine β-synthase
PLP
Serine
cystathionine γ-lyase
PLP
Cysteine + NH4+
α-Ketobutyrate
threonine dehydratase
PLP
NH4+
H2O
Threonine
CoA-SH
NAD+
α-keto acid dehydrogenase
NADH + H+
CO2
Acetyl-CoA
Isoleucine
6 steps
CO2
Propionyl-CoA
7 steps
2CO2
Valine
2 steps
HCO3−
Methylmalonyl-CoA
methylmalonyl-CoA mutase
coenzyme B12
Succinyl-CoA

to two other oxidative decarboxylations encountered in Chapter 16: oxidation of pyruvate to acetyl-CoA by the pyruvate dehydrogenase complex and oxidation of α-ketoglutarate to succinyl-CoA by the α-ketoglutarate dehydrogenase complex (see Fig. 16-12). In fact, all three enzyme complexes are similar in structure and share essentially the same reaction mechanism. Five cofactors (thiamine pyrophosphate, FAD, NAD, lipoate, and coenzyme A) participate, and the three proteins in each complex catalyze homologous reactions. This is clearly a case in which enzymatic machinery that evolved to catalyze one reaction was "borrowed" by gene duplication and further evolved to catalyze similar reactions in other pathways.

Experiments with rats have shown that the branched-chain α-keto acid dehydrogenase complex is regulated by covalent modification in response to the content of branched-chain amino acids in the diet. With little or no excess dietary intake of branched-chain amino acids, the enzyme complex is phosphorylated by a protein kinase and thereby inactivated. Addition of excess branched-chain amino acids to the diet results in dephosphorylation and consequent activation of the enzyme. Recall that the pyruvate dehydrogenase complex is subject to similar regulation by phosphorylation and dephosphorylation (see Fig. 16-19).

There is a relatively rare genetic disease in which the three branched-chain α-keto acids (as well as their precursor amino acids, especially leucine) accumulate in the blood and "spill over" into the urine. This condition, called **maple syrup urine disease**

BOX 18-2 MEDICINE

MMA: Sometimes More than a Genetic Disease

Without treatment, an infant born with a genetic deficiency involving the enzyme methylmalonyl-CoA mutase is subject to severe symptoms ranging from seizures and vomiting to lethargy, developmental disease, progressive encephalopathy (brain disease), and early death. A rare and recessive genetic disorder, the condition, methylmalonic acidemia or MMA, affects about 1 child in 48,000 (Fig. 1). For most parents of a child with MMA, the condition brings trials and some heartbreak. For Patricia Stallings, the condition of her undiagnosed child led to much worse. Biochemistry eventually came to the rescue.

In the summer of 1989, Stallings took her baby son Ryan to the emergency room at the Cardinal Glennon Children's Hospital in St. Louis. To the attending physician, a toxicologist, Ryan's fit of vomiting and labored breathing suggested ingestion of the antifreeze ingredient ethylene glycol. The toxicologist suspected foul play and alerted authorities. Analysis of Ryan's milk bottles by two laboratories seemed to confirm the physician's fears. Ryan was placed in foster care as soon as he recovered. Unfortunately for both Ryan and his mother, Ryan subsequently died immediately after a visit from Patricia. Patricia was arrested and charged with murder.

While awaiting trial, Patricia learned that she was again pregnant. She gave birth to a second son, David, who was placed in foster care. David developed symptoms similar to Ryan's and was diagnosed with MMA. Although the symptoms of MMA can mimic those observed in cases of ethylene glycol poisoning, the revelation did not help Patricia. The information about David's diagnosis was excluded at her trial for the murder of Ryan. In 1991, Patricia Stallings was sentenced to prison for life.

The situation was ultimately resolved when William Sly, chair of the Department of Biochemistry and Molecular Biology at St. Louis University, became interested in the case. Working with metabolic disease experts James Shoemaker and Piero Rinaldo, he had new analyses performed on Ryan's blood and the original milk bottles. The blood showed high levels of methylmalonic acid and ketones, diagnostic of MMA. Surprisingly, unlike the earlier lab reports, they found no ethylene glycol in any of the samples. Could both a hospital lab and a commercial lab have been mistaken? What the biochemists saw provided the final breakthrough. The biochemists presented all of their materials to the Missouri state prosecutor who had handled the case, George McElroy. Both of the original lab analyses had been so unusually shoddy that they were quickly dismissed. Patricia Stallings was cleared of charges and released on September 20, 1991.

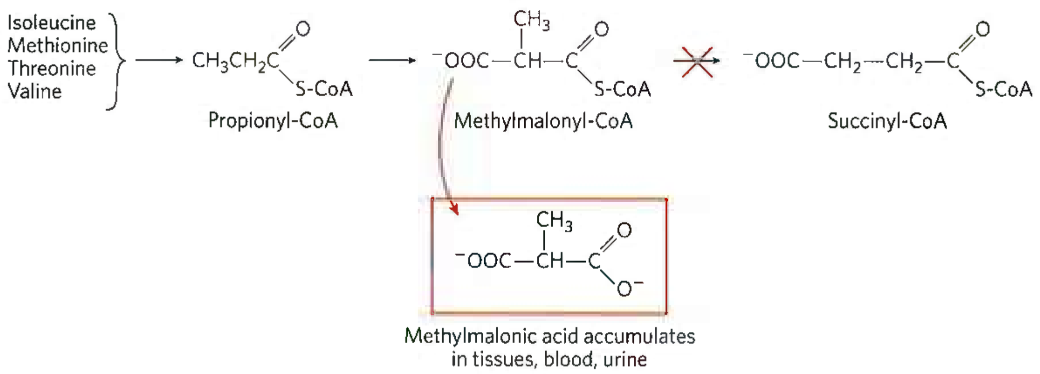


FIGURE 1 Children with a mutation (red X) that inactivates the enzyme methylmalonyl-CoA mutase cannot degrade isoleucine, methionine, threonine, and valine normally. Instead, a potentially fatal accumulation of methylmalonic acid occurs, with symptoms similar to those of ethylene glycol poisoning.

because of the characteristic odor imparted to the urine by the α-keto acids, results from a defective branched-chain α-keto acid dehydrogenase complex. Untreated, the disease results in abnormal development of the brain and death in early infancy. Treatment entails rigid control of the diet, limiting the intake of valine, isoleucine, and leucine to the minimum required to permit normal growth. ■

Asparagine and Aspartate Are Degraded to Oxaloacetate

The carbon skeletons of **asparagine** and **aspartate** ultimately enter the citric acid cycle as malate in mammals or oxaloacetate in bacteria. The enzyme **asparaginase** catalyzes the hydrolysis of asparagine to aspartate, which undergoes transamination with α-ketoglutarate to yield glutamate and oxaloacetate (**Fig. 18-29**).

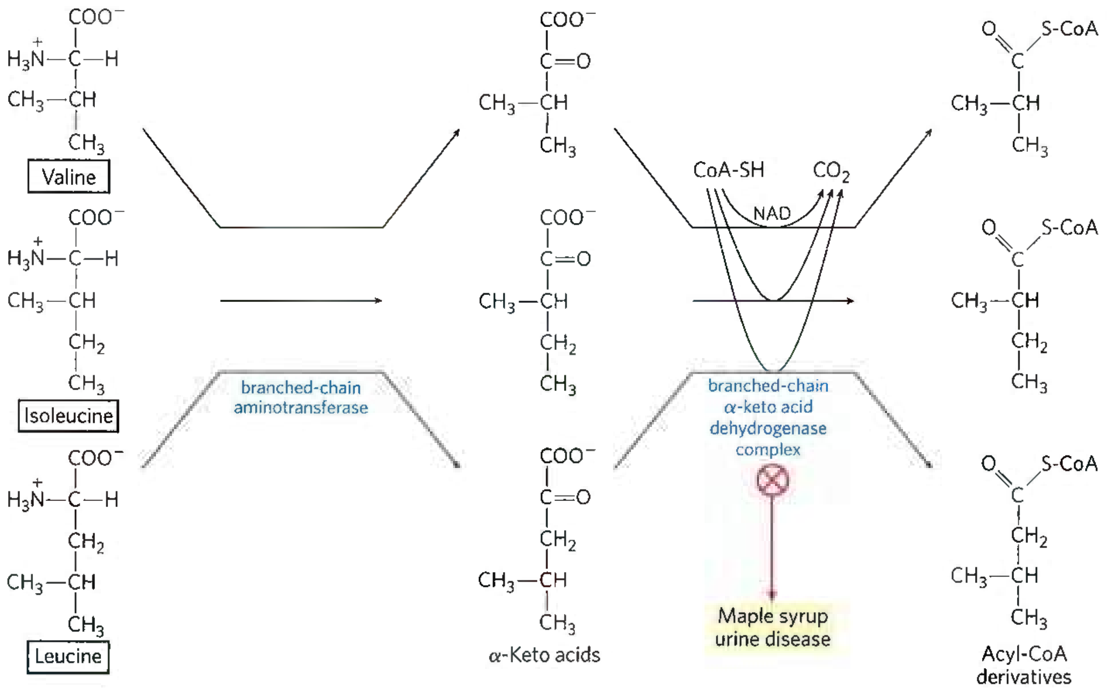

Valine
Isoleucine
Leucine
branched-chain aminotransferase
α-Keto acids
CoA-SH
CO₂
NAD
branched-chain α-keto acid dehydrogenase complex
Maple syrup urine disease
Acyl-CoA derivatives

FIGURE 18-28 Catabolic pathways for the three branched-chain amino acids: valine, isoleucine, and leucine. All three pathways occur in extrahepatic tissues and share the first two enzymes, as shown here. The branched-chain α-keto acid dehydrogenase complex is analogous to the pyruvate and α-ketoglutarate dehydrogenase complexes and requires the same five cofactors (some not shown here). This enzyme is defective in people with maple syrup urine disease.

The oxaloacetate is converted to malate in the cytosol and then transported into the mitochondrial matrix through the malate–α-ketoglutarate transporter. In bacteria, the oxaloacetate produced in the transamination reaction can be used directly in the citric acid cycle.

We have now seen how the 20 common amino acids, after losing their nitrogen atoms, are degraded by dehydrogenation, decarboxylation, and other reactions to yield portions of their carbon backbones in the form of six central metabolites that can enter the citric acid cycle. Those portions degraded to acetyl-CoA are completely oxidized to carbon dioxide and water, with generation of ATP by oxidative phosphorylation. Those portions degraded to other citric acid cycle intermediates can either be oxidized or contribute to gluconeogenesis, depending on the metabolic state.

As was the case for carbohydrates and lipids, the degradation of amino acids results ultimately in the generation of reducing equivalents (NADH and $FADH_2$) through the action of the citric acid cycle. Our survey of catabolic processes concludes in the next chapter with a discussion of respiration, in which these reducing equivalents fuel the ultimate oxidative and energy-generating process in aerobic organisms.

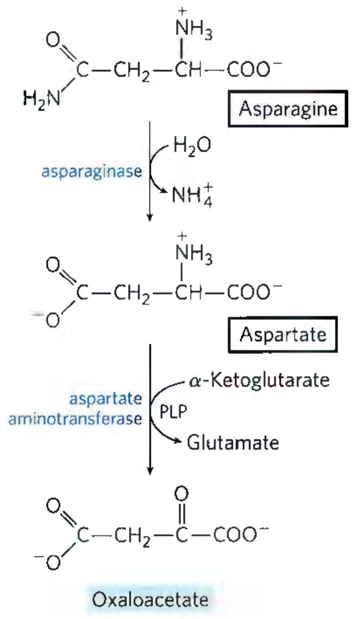

Asparagine
H₂O
asparaginase
NH₄⁺
Aspartate
α-Ketoglutarate
aspartate aminotransferase
PLP
Glutamate
Oxaloacetate

FIGURE 18-29 Catabolic pathway for asparagine and aspartate. Both amino acids are converted to oxaloacetate.

SUMMARY 18.3 ***Pathways of Amino Acid Degradation***

- After the removal of amino groups, the carbon skeletons of amino acids undergo oxidation to compounds that can enter the citric acid cycle. The citric acid cycle products can be oxidized as fuel. Alternatively, depending on their degradative end product, some amino acids can be converted to ketone bodies, some to glucose, and some to both. Thus, amino acid degradation is integrated into intermediary metabolism and can be critical to survival under conditions in which amino acids are a significant source of metabolic energy.

- The reactions of these pathways require several cofactors, including tetrahydrofolate and *S*-adenosylmethionine in one-carbon transfer reactions and tetrahydrobiopterin in the oxidation of phenylalanine by phenylalanine hydroxylase.
- Alanine, cysteine, glycine, serine, threonine, and tryptophan are converted in whole or in part to pyruvate.
- Leucine, lysine, phenylalanine, tyrosine, and tryptophan yield acetyl-CoA via acetoacetyl-CoA. Isoleucine, leucine, threonine, and tryptophan also form acetyl-CoA directly. Leucine and lysine are the only amino acids that cannot contribute to gluconeogenesis. Four carbon atoms of phenylalanine and tyrosine give rise to fumarate.
- Many human genetic diseases are traced to deficiencies in enzymes catalyzing steps in amino acid degradation. Two well-studied examples, phenylketonuria and alkaptonuria, feature defects in phenylalanine degradation. Most amino acid degradation deficiencies are treated with dietary intervention.
- Arginine, glutamate, glutamine, histidine, and proline are degraded to α-ketoglutarate.
- Isoleucine, methionine, threonine, and valine produce succinyl-CoA.
- The branched-chain amino acids (isoleucine, leucine, and valine), unlike the other amino acids, are degraded only in extrahepatic tissues.
- Asparagine and aspartate produce oxaloacetate.

KEY TERMS

Terms in bold are defined in the glossary.

ammonotelic	626
ureotelic	626
uricotelic	626
aminotransferases	628
transaminases	628
transamination	628
pyridoxal phosphate (PLP)	629
oxidative deamination	631
L-glutamate dehydrogenase	631
glutamine synthetase	631
glutaminase	631
glucose-alanine cycle	632
urea cycle	633
urea	635
creatine kinase	637
essential amino acids	638
ketogenic	640
glucogenic	640
tetrahydrofolate	641
***S*-adenosylmethionine (adoMet)**	641
tetrahydrobiopterin	644
phenylketonuria (PKU)	649
mixed-function oxygenases	649
alkaptonuria	650
maple syrup urine disease	652

PROBLEMS

1. Products of Amino Acid Transamination Name and draw the structure of the α-keto acid resulting when each of the four amino acids listed undergoes transamination with α-ketoglutarate: **(a)** aspartate, **(b)** glutamate, **(c)** alanine, **(d)** phenylalanine.

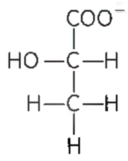
2. Measurement of Alanine Aminotransferase Activity The measurement of alanine aminotransferase activity (reaction rate) usually includes an excess of pure lactate dehydrogenase and NADH in the reaction system. The rate of alanine disappearance is equal to the rate of NADH disappearance measured spectrophotometrically. Explain how this assay works.

3. Alanine and Glutamine in the Blood Normal human blood plasma contains all the amino acids required for the synthesis of body proteins, but not in equal concentrations. Alanine and glutamine are present in much higher concentrations than any other amino acids. Suggest why.

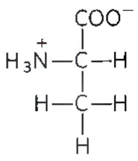
4. Glutamate Dehydrogenase Function Increases in ATP levels in blood trigger insulin release by the pancreas, which in turn stimulates the uptake of blood glucose (see Chapter 23, p. 860). Knowing this, suggest why a mutation that prevents inhibition of glutamate dehydrogenase by GTP results in insulin release and hypoglycemia.

5. Distribution of Amino Nitrogen If your diet is rich in alanine but deficient in aspartate, will you show signs of aspartate deficiency? Explain.

6. Lactate versus Alanine as Metabolic Fuel: The Cost of Nitrogen Removal The three carbons in lactate and alanine have identical oxidation states, and animals can use either carbon source as a metabolic fuel. Compare the net ATP yield (moles of ATP per mole of substrate) for the complete oxidation (to CO_2 and H_2O) of lactate versus alanine when the cost of nitrogen excretion as urea is included.



Lactate



Alanine

7. Ammonia Toxicity Resulting from an Arginine-Deficient Diet In a study, cats were fasted overnight then given a single meal complete in all amino acids except arginine. Within 2 hours, blood ammonia levels increased from a normal level of 18 μg/L to 140 μg/L, and the cats showed the clinical symptoms of ammonia toxicity. A control group fed a complete amino acid diet or an amino acid diet in which arginine was replaced by ornithine showed no unusual clinical symptoms.
(a) What was the role of fasting in the experiment?
(b) What caused the ammonia levels to rise in the experimental group? Why did the absence of arginine lead to ammonia toxicity? Is arginine an essential amino acid in cats? Why or why not?
(c) Why can ornithine be substituted for arginine?

8. Oxidation of Glutamate Write a series of balanced equations and an overall equation for the net reaction describing the oxidation of 2 mol of glutamate to 2 mol of α-ketoglutarate and 1 mol of urea.

9. Transamination and the Urea Cycle Aspartate aminotransferase has the highest activity of all the mammalian liver aminotransferases. Why?

10. The Case against the Liquid Protein Diet A weight-reducing diet heavily promoted some years ago

required the daily intake of a "liquid protein" soup made of hydrolyzed gelatin (derived from collagen), water, and an assortment of vitamins. All other food and drink were to be avoided. People on this diet typically lost 10 to 14 lb in the first week.
(a) Opponents argued that the weight loss was almost entirely due to water loss and would be regained very soon after a normal diet was resumed. What is the biochemical basis for this argument?
(b) A few people on this diet died. What are some of the dangers inherent in the diet, and how can they lead to death?

11. Ketogenic Amino Acids Which amino acids are exclusively ketogenic?

12. A Genetic Defect in Amino Acid Metabolism: A Case History A two-year-old child was taken to the hospital. His mother said that he vomited frequently, especially after feedings. The child's weight and physical development were below normal. His hair, although dark, contained patches of white. A urine sample treated with ferric chloride ($FeCl_3$) gave a green color characteristic of the presence of phenylpyruvate. Quantitative analysis of urine samples gave the results shown in the table.

	Concentration (mM)	
Substance	**Patient's urine**	**Normal urine**
Phenylalanine	7.0	0.01
Phenylpyruvate	4.8	0
Phenyllactate	10.3	0

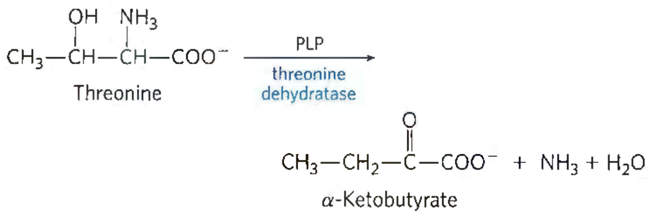
(a) Suggest which enzyme might be deficient in this child. Propose a treatment.
(b) Why does phenylalanine appear in the urine in large amounts?
(c) What is the source of phenylpyruvate and phenyllactate? Why does this pathway (normally not functional) come into play when the concentration of phenylalanine rises?
(d) Why does the boy's hair contain patches of white?

13. Role of Cobalamin in Amino Acid Catabolism Pernicious anemia is caused by impaired absorption of vitamin B_{12}. What is the effect of this impairment on the catabolism of amino acids? Are all amino acids equally affected? (Hint: See Box 17-2.)

14. Vegetarian Diets Vegetarian diets can provide high levels of antioxidants and a lipid profile that can help prevent coronary disease. However, there can be some associated problems. Blood samples were taken from a large group of volunteer subjects who were vegans (strict vegetarians: no animal products), lactovegetarians (vegetarians who eat dairy products), or omnivores (individuals with a varied diet, including meat). In each case, the volunteers had followed the diet for several years. The blood levels of both homocysteine and methylmalonate were elevated in the vegan group, somewhat lower in the lactovegetarian group, and much lower in the omnivore group. Explain.

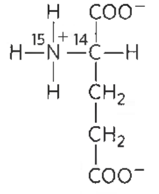
15. Pernicious Anemia Vitamin B_{12} deficiency can arise from a few rare genetic diseases that lead to low B_{12} levels despite a normal diet that includes B_{12}-rich meat and dairy sources. These conditions cannot be treated with dietary B_{12} supplements. Explain.

16. Pyridoxal Phosphate Reaction Mechanisms Threonine can be broken down by the enzyme threonine dehydratase, which catalyzes the conversion of threonine to α-ketobutyrate and ammonia. The enzyme uses PLP as a cofactor. Suggest a mechanism for this reaction, based on the mechanisms in Figure 18-6. Note that this reaction includes an elimination at the β carbon of threonine.



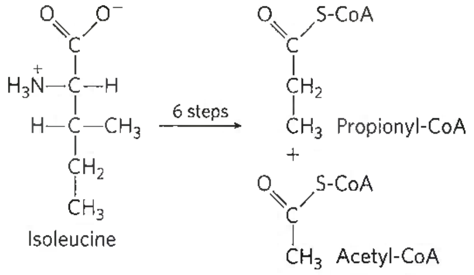
$$\underset{\text{Threonine}}{CH_3-\overset{OH}{CH}-\overset{NH_3}{CH}-COO^-} \xrightarrow[\text{threonine dehydratase}]{PLP} \underset{\alpha\text{-Ketobutyrate}}{CH_3-CH_2-\overset{O}{\overset{\|}{C}}-COO^-} + NH_3 + H_2O$$

17. Pathway of Carbon and Nitrogen in Glutamate Metabolism When [2-^{14}C,^{15}N]glutamate undergoes oxidative degradation in the liver of a rat, in which atoms of the following metabolites will each isotope be found? **(a)** urea **(b)** succinate **(c)** arginine **(d)** citrulline **(e)** ornithine **(f)** aspartate

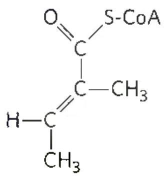


Labeled glutamate

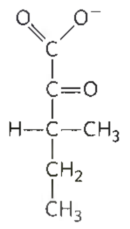
18. Chemical Strategy of Isoleucine Catabolism Isoleucine is degraded in six steps to propionyl-CoA and acetyl-CoA.



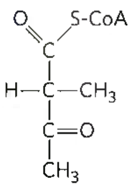
(a) The chemical process of isoleucine degradation includes strategies analogous to those used in the citric acid cycle and the β oxidation of fatty acids. The intermediates of isoleucine degradation (I to V) shown below are not in the proper order. Use your knowledge and understanding of the citric acid cycle and β-oxidation pathway to arrange the intermediates in the proper metabolic sequence for isoleucine degradation.



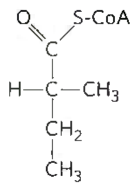
I



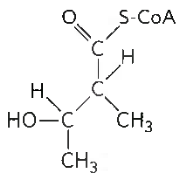
II



III



IV

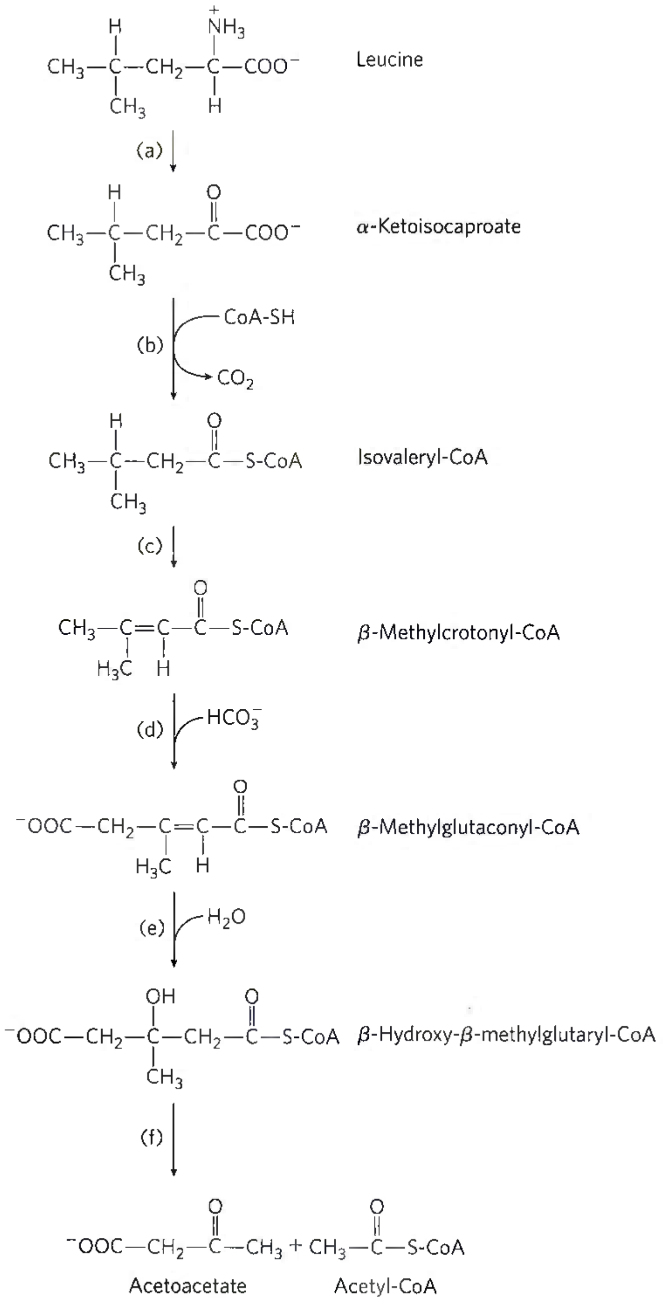


V

(b) For each step you propose, describe the chemical process, provide an analogous example from the citric acid cycle or β-oxidation pathway (where possible), and indicate any necessary cofactors.

19. Role of Pyridoxal Phosphate in Glycine Metabolism The enzyme serine hydroxymethyltransferase requires pyridoxal phosphate as a cofactor. Propose a mechanism for the reaction catalyzed by this enzyme, in the direction of serine degradation (glycine production). (Hint: See Figs 18-19 and 18-20b.)

20. Parallel Pathways for Amino Acid and Fatty Acid Degradation The carbon skeleton of leucine is degraded by a series of reactions closely analogous to those of the citric acid cycle and β oxidation. For each reaction, (a) through (f), shown below, indicate its type, provide an analogous example from the citric acid cycle or β-oxidation pathway (where possible), and note any necessary cofactors.



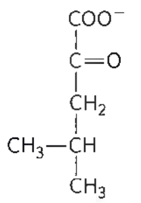
21. Treatments for a Genetic Disease The strict dietary controls required to stem the progress of maple syrup urine disease are difficult to follow for a lifetime, and patients may experience poor metabolic control that leads to neurological symptoms. In these cases, treatment can involve an organ transplant from a suitable donor. Organ transplantation involves considerable risk, but success can greatly alleviate this metabolic disorder and reduce the need for dietary restrictions. Which organ could be transplanted to gain this effect, and why?

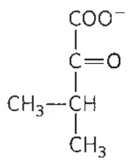
DATA ANALYSIS PROBLEM

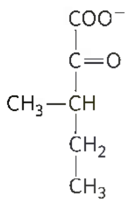
22. Maple Syrup Urine Disease Figure 18-28 shows the pathway for the degradation of branched-chain amino acids and the site of the biochemical defect that causes maple syrup urine disease. The initial findings that eventually led to the discovery of the defect in this disease were presented in three papers published in the late 1950s and early 1960s. This problem traces the history of the findings from initial clinical observations to proposal of a biochemical mechanism.

Menkes, Hurst, and Craig (1954) presented the cases of four siblings, all of whom died following a similar course of symptoms. In all four cases, the mother's pregnancy and the birth had been normal. The first 3 to 5 days of each child's life were also normal. But soon thereafter each child began having convulsions, and the children died between the ages of 11 days and 3 months. Autopsy showed considerable swelling of the brain in all cases. The children's urine had a strong, unusual "maple syrup" odor, starting from about the third day of life.

Menkes (1959) reported data collected from six more children. All showed symptoms similar to those described above and died within 15 days to 20 months of birth. In one case, Menkes was able to obtain urine samples during the last months of the infant's life. When he treated the urine with 2,4-dinitrophenylhydrazone, which forms colored precipitates with keto compounds, he found three α-keto acids in unusually large amounts:


α-Ketoisocaproate


α-Ketoisovalerate

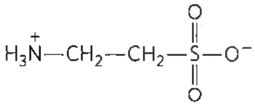

α-Keto-β-methyl-*n*-valerate

(a) These α-keto acids are produced by the deamination of amino acids. For each of the α-keto acids above, draw and name the amino acid from which it was derived.

Dancis, Levitz, and Westall (1960) collected further data that led them to propose the biochemical defect shown in Figure 18-28. In one case, they examined a patient whose urine first showed the maple syrup odor when he was 4 months old. At the age of 10 months (March 1956), the child was admitted to the hospital because he had a fever, and he showed grossly delayed motor development. At the age of 20 months (January 1957), he was readmitted and was found to have the degenerative neurological symptoms seen in previous cases of maple syrup urine disease; he died soon after. Results of his blood and urine analyses are shown in the table, along with normal values for each component.

	Urine concentration (mg/24 h)			Plasma concentration (mg/mL)	
	Normal	**Patient**		**Normal**	**Patient**
Amino acid(s)		**Mar. 1956**	**Jan. 1957**		**Jan. 1957**
Alanine	5–15	0.2	0.4	3.0–4.8	0.6
Asparagine and glutamine	5–15	0.4	0	3.0–5.0	2.0
Aspartic acid	1–2	0.2	1.5	0.1–0.2	0.04
Arginine	1.5–3	0.3	0.7	0.8–1.4	0.8
Cystine	2–4	0.5	0.3	1.0–1.5	0
Glutamic acid	1.5–3	0.7	1.6	1.0–1.5	0.9
Glycine	20–40	4.6	20.7	1.0–2.0	1.5
Histidine	8–15	0.3	4.7	1.0–1.7	0.7
Isoleucine	2–5	2.0	13.5	0.8–1.5	2.2
Leucine	3–8	2.7	39.4	1.7–2.4	14.5
Lysine	2–12	1.6	4.3	1.5–2.7	1.1
Methionine	2–5	1.4	1.4	0.3–0.6	2.7
Ornithine	1–2	0	1.3	0.6–0.8	0.5
Phenylalanine	2–4	0.4	2.6	1.0–1.7	0.8
Proline	2–4	0.5	0.3	1.5–3.0	0.9
Serine	5–15	1.2	0	1.3–2.2	0.9
Taurine	1–10	0.2	18.7	0.9–1.8	0.4
Threonine	5–10	0.6	0	1.2–1.6	0.3
Tryptophan	3–8	0.9	2.3	Not measured	0
Tyrosine	4–8	0.3	3.7	1.5–2.3	0.7
Valine	2–4	1.6	15.4	2.0–3.0	13.1

(b) The table includes taurine, an amino acid not normally found in proteins. Taurine is often produced as a byproduct of cell damage. Its structure is



$$H_3\overset{+}{N}-CH_2-CH_2-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O}{\|}}{S}}-O^-$$

Based on its structure and the information in this chapter, what is the most likely amino acid precursor of taurine? Explain your reasoning.

(c) Compared with the normal values given in the table, which amino acids showed significantly elevated levels in the patient's blood in January 1957? Which ones in the patient's urine?

Based on their results and their knowledge of the pathway shown in Figure 18-28, Dancis and coauthors concluded that "although it appears most likely to the authors that the primary block is in the metabolic degradative pathway of the branched-chain amino acids, this cannot be considered established beyond question."

(d) How do the data presented here support this conclusion?

(e) Which data presented here do *not* fit this model of maple syrup urine disease? How do you explain these seemingly contradictory data?

(f) What data would you need to collect to be more secure in your conclusion?

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Dancis, J., M. Levitz, and R. Westall. 1960. Maple syrup urine disease: branched-chain ketoaciduria. *Pediatrics* 25:72–79.

Menkes, J.H. 1959. Maple syrup disease: isolation and identification of organic acids in the urine. *Pediatrics* 23:348–353.

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Chapter 19

OXIDATIVE PHOSPHORYLATION

19.1 The Mitochondrial Respiratory Chain *660*

19.2 ATP Synthesis *674*

19.3 Regulation of Oxidative Phosphorylation *686*

19.4 Mitochondria in Thermogenesis, Steroid Synthesis, and Apoptosis *689*

19.5 Mitochondrial Genes: Their Origin and the Effects of Mutations *692*

Oxidative phosphorylation is the culmination of energy-yielding metabolism (catabolism) in aerobic organisms. All oxidative steps in the degradation of carbohydrates, fats, and amino acids converge at this final stage of cellular respiration, in which the energy of oxidation drives the synthesis of ATP. Oxidative phosphorylation accounts for most of the ATP synthesized by nonphotosynthetic organisms under most circumstances. In eukaryotes, oxidative phosphorylation occurs in mitochondria and involves huge protein complexes embedded in the mitochondrial membranes. The pathway to ATP synthesis in mitochondria challenged and fascinated biochemists for much of the twentieth century. Our current exploration of the topic is guided by five principles:

P1 **Mitochondria play a central role in eukaryotic aerobic metabolism.** ATP production is not the only important mitochondrial function. Mitochondria play host to the citric acid cycle, the fatty acid β-oxidation pathway, and the pathways of amino acid oxidation. The mitochondria also act in thermogenesis, steroid synthesis, and apoptosis (programmed cell death). The discovery of these diverse and important roles of mitochondria has stimulated much current research on the biochemistry of this organelle.

P2 **Mitochondria trace their evolutionary origin to bacteria.** Over 1.45 billion years ago, an endosymbiotic relationship arose between bacteria and a primitive eukaryote or eukaryotic progenitor. Mitochondria are ubiquitous in modern eukaryotes, and their bacterial origin is evident in almost every aspect of their structure and function.

P3 **Electrons flow from electron donors (oxidizable substrates) through a chain of membrane-bound carriers to a final electron acceptor with a large reduction potential.** The final acceptor is molecular oxygen, O_2. The appearance of oxygen in the atmosphere some 2.3 billion years ago, and its harnessing in living systems via the evolution of oxidative phosphorylation, made more complex life forms possible.

P4 **The free energy made available by "downhill" (exergonic) electron flow is coupled to the "uphill" transport of protons across a proton-impermeable membrane.** The free energy of fuel oxidation is thus conserved as a transmembrane electrochemical potential.

P5 **The transmembrane flow of protons back down their electrochemical gradient through specific protein channels provides the free energy for synthesis of ATP.** This process is catalyzed by a membrane protein complex (ATP synthase) that couples proton flow to phosphorylation of ADP.

Principles 3 through 5, illustrated in **Figure 19-1**, encapsulate the theory, introduced by Peter Mitchell in 1961, that transmembrane differences in proton concentration are the reservoir for the energy extracted from biological oxidation reactions. This **chemiosmotic theory** has been accepted as one of the great unifying ideas of twentieth-century biology. It provides insight into the processes of oxidative phosphorylation and photophosphorylation in plants, and into such apparently disparate energy transductions as active transport across membranes and the motion of bacterial flagella.

This chapter begins with a description of the components of the mitochondrial electron-transfer chain—the respiratory chain—and their organization into large functional complexes in the inner mitochondrial membrane, the path of electron flow through these complexes, and the proton movements that accompany this flow. We then

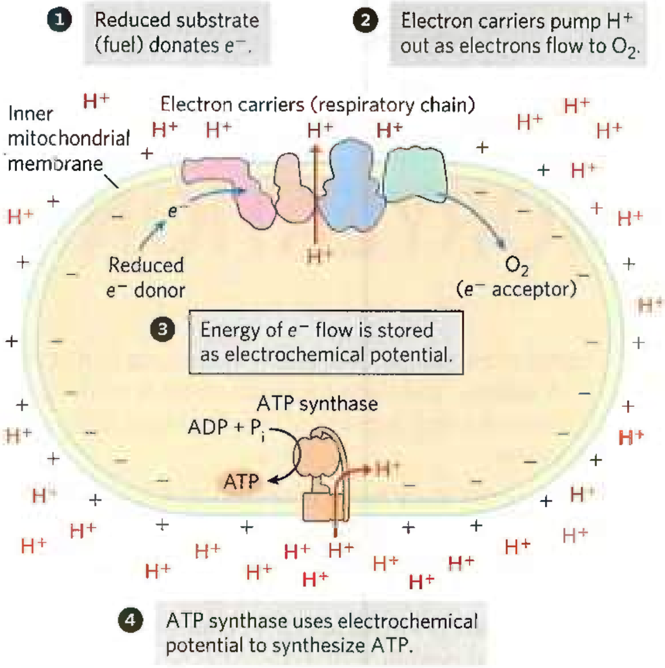

1 Reduced substrate (fuel) donates e⁻.
2 Electron carriers pump H⁺ out as electrons flow to O₂.
Inner mitochondrial membrane
Electron carriers (respiratory chain)
H⁺
e⁻
Reduced e⁻ donor
O₂ (e⁻ acceptor)
3 Energy of e⁻ flow is stored as electrochemical potential.
ATP synthase
ADP + Pᵢ
ATP
4 ATP synthase uses electrochemical potential to synthesize ATP.

FIGURE 19-1 The chemiosmotic mechanism for ATP synthesis in mitochondria. Electrons move spontaneously through a chain of membrane-bound carriers, the respiratory chain, driven by the high reduction potential of oxygen and the relatively low reduction potentials of the various reduced substrates (fuels) that undergo oxidation in the mitochondrion. Electron flow creates an electrochemical potential by the transmembrane movement of protons and positive charge. This electrochemical potential drives ATP synthesis by a membrane-bound enzyme (ATP synthase) that is fundamentally similar in mitochondria and chloroplasts, and in bacteria and archaea as well.

consider the remarkable enzyme complex that captures, by "rotational catalysis," the energy of proton flow in ATP, and the regulatory mechanisms that coordinate oxidative phosphorylation with the many catabolic pathways by which fuels are oxidized.

The metabolic role of mitochondria is so critical to cellular and organismal function that defects in mitochondrial function have serious medical consequences. Mitochondria are central to neuronal and muscular function, and to the regulation of whole-body energy metabolism and body weight. Human neurodegenerative diseases, as well as cancer, diabetes, and obesity, are recognized as possible results of compromised mitochondrial function, and one theory of aging is based on gradual loss of mitochondrial integrity. We consider the diverse functions of mitochondria, and the consequences of defective mitochondrial function in humans. ■

19.1 The Mitochondrial Respiratory Chain

Albert L. Lehninger, 1917–1986
[Alan Mason Chesney Medical Archives of the Johns Hopkins Medical Institutions.]

The discovery in 1948, by Eugene Kennedy and Albert Lehninger, that mitochondria are the site of oxidative phosphorylation in eukaryotes marked the beginning of the enzymological studies of biological energy transductions. Mitochondria, like gram-negative bacteria, have two membranes (**Fig. 19-2a**). The outer mitochondrial membrane is readily permeable to small molecules ($M_r < 5{,}000$) and ions, which move freely through transmembrane channels formed by a family of integral membrane proteins called porins. The inner membrane is impermeable to most small molecules and ions, including protons (H^+); the only species that cross this membrane do so through specific transporters. The inner membrane bears the components of the respiratory chain and ATP synthase.

P1 The mitochondrial matrix, enclosed by the inner membrane, contains the pyruvate dehydrogenase complex and the enzymes of the citric acid cycle, the fatty acid β-oxidation pathway, and the pathways of amino acid oxidation—all the pathways of fuel oxidation except glycolysis, which takes place in the cytosol. The selectively permeable inner mitochondrial membrane segregates the intermediates and enzymes of cytosolic metabolic pathways from those of metabolic processes occurring in the matrix. However, specific transporters carry pyruvate, fatty acids, and amino acids or their α-keto derivatives into the matrix for access to the machinery of the citric acid cycle. ADP and P_i are specifically transported into the matrix as newly synthesized ATP is transported out. Mammalian mitochondria have about 1,200 proteins, according to current best estimates. The functions of up to 25% of these remain partly or entirely enigmatic.

The bean-shaped representation of a mitochondrion in Figure 19-2a is an oversimplification, derived in part from early studies in which thin sections of cells were observed in the electron microscope. Three-dimensional images obtained either by reconstruction from serial sections or by confocal microscopy reveal great variation in mitochondrial size and shape. In living cells stained with mitochondrion-specific fluorescent dyes, large numbers of variously shaped mitochondria are seen, clustered about the nucleus (Fig. 19-2b).

Tissues with a high demand for aerobic metabolism (brain, skeletal and heart muscle, liver, and eye, for example) contain many hundreds or thousands of mitochondria per cell, and in general, mitochondria of cells with high metabolic activity have more, and more densely packed, convolutions, or **cristae** (Fig. 19-2c, d). **P2** During cell growth and division, mitochondria, like bacteria, divide by fission, and under some circumstances individual mitochondria fuse to form larger, more-extended structures. Stressful conditions, such as the presence of electron-transfer inhibitors or mutations in an electron carrier, trigger mitochondrial fission and sometimes **mitophagy**—the breakdown of mitochondria and recycling of the amino acids, nucleotides, and lipids

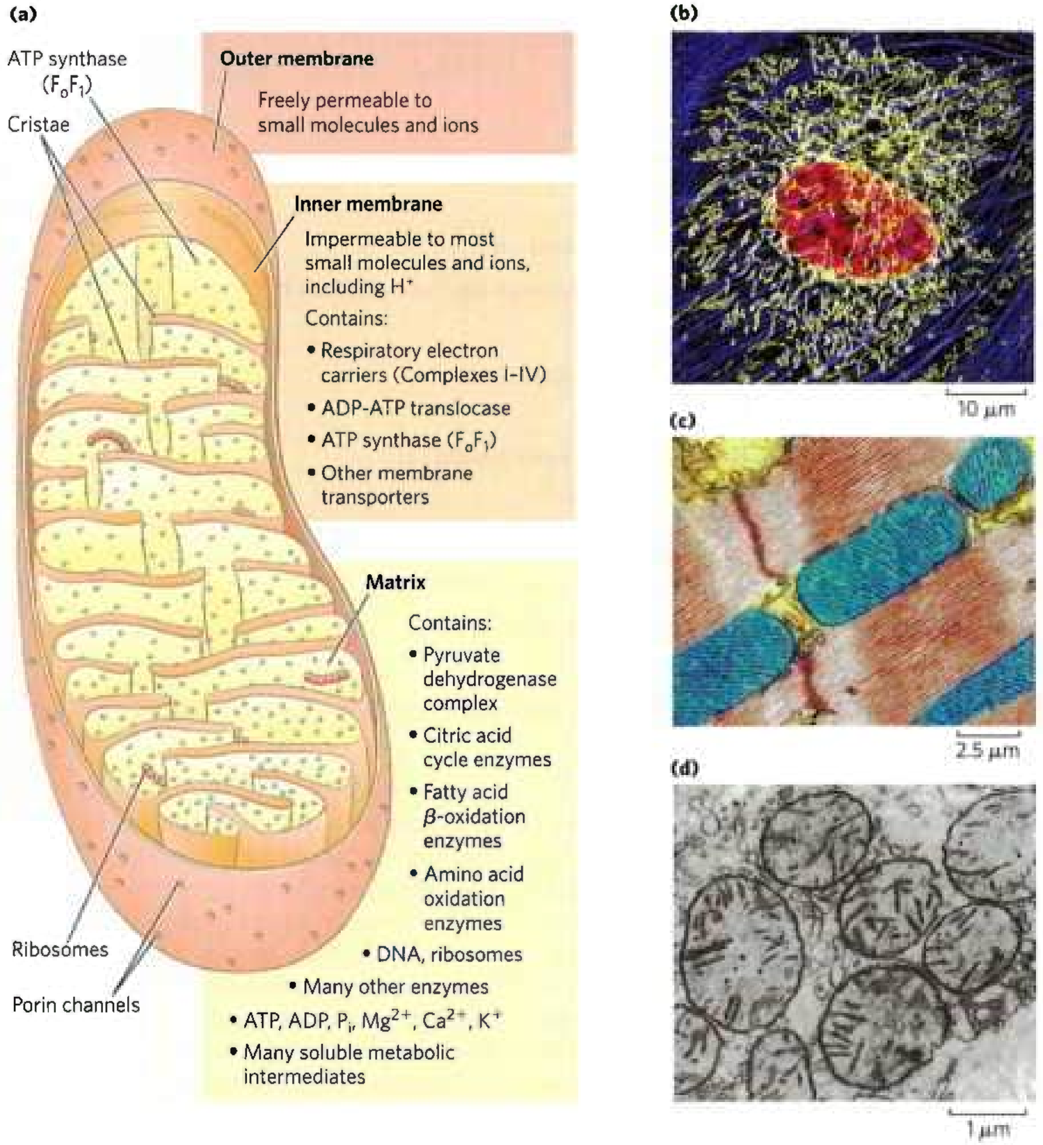

(a)
ATP synthase
(F_oF_1)
Cristae
Outer membrane
Freely permeable to small molecules and ions
Inner membrane
Impermeable to most small molecules and ions, including H^+
Contains:
• Respiratory electron carriers (Complexes I-IV)
• ADP-ATP translocase
• ATP synthase (F_oF_1)
• Other membrane transporters
Matrix
Contains:
• Pyruvate dehydrogenase complex
• Citric acid cycle enzymes
• Fatty acid β-oxidation enzymes
• Amino acid oxidation enzymes
• DNA, ribosomes
• Many other enzymes
• ATP, ADP, P_i, Mg^{2+}, Ca^{2+}, K^+
• Many soluble metabolic intermediates
Ribosomes
Porin channels
(b)
10 μm
(c)
2.5 μm
(d)
1 μm

FIGURE 19-2 Biochemical anatomy of a mitochondrion. (a) The outer membrane has pores that make it permeable to small molecules and ions, but not to proteins. The cristae provide a very large surface area. The inner membrane of a single liver mitochondrion may have more than 10,000 sets of electron-transfer systems (respiratory chains) and ATP synthase molecules, distributed over the membrane surface. (b) A typical animal cell has hundreds or thousands of mitochondria. This endothelial cell from bovine pulmonary artery was stained with fluorescent probes for actin (blue), for DNA (red), and for mitochondria (yellow). Notice the variability in length of the mitochondria. (c) The mitochondria of heart muscle (blue in this colorized electron micrograph) have more profuse cristae and thus a much larger area of inner membrane, with more than three times as many sets of respiratory chains as (d) liver mitochondria. Muscle and liver mitochondria are about the size of a bacterium — 1 to 10 μm long. The mitochondria of invertebrates, plants, and microbial eukaryotes are similar to those shown here, but with much variation in size, shape, and degree of convolution of the inner membrane. [(b) Talley Lambert/Science Source. (c) Thomas Deerinck, NCMIR/Science Source. (d) Don W. Fawcett/Science Source.]

released. As stress is relieved, small mitochondria fuse to form long, thin, tubular organelles.

Electrons Are Funneled to Universal Electron Acceptors

Oxidative phosphorylation begins with the entry of electrons into the series of electron carriers called the **respiratory chain**. Most of these electrons arise from the action of dehydrogenases that collect electrons from catabolic pathways and funnel them into universal electron acceptors — nicotinamide nucleotides (NAD^+ or $NADP^+$) or flavin nucleotides (FMN or FAD).

Nicotinamide nucleotide–linked dehydrogenases catalyze reversible reactions of the following general types:

$$\text{Reduced substrate} + NAD^+ \rightleftharpoons \text{oxidized substrate} + NADH + H^+$$

$$\text{Reduced substrate} + NADP^+ \rightleftharpoons \text{oxidized substrate} + NADPH + H^+$$

Most dehydrogenases that act in catabolism are specific for NAD^+ as electron acceptor (**Table 19-1**). Some are in the cytosol, many are in mitochondria, and still others have mitochondrial and cytosolic isozymes.

TABLE 19-1 Some Important Reactions Catalyzed by NAD(P)$^+$-Linked Dehydrogenases

Reaction[a]	Location[b]
NAD$^+$-linked	
α-Ketoglutarate + CoA + NAD$^+$ $\rightleftharpoons$ succinyl-CoA + CO_2 + NADH + H$^+$	M
L-Malate + NAD$^+$ $\rightleftharpoons$ oxaloacetate + NADH + H$^+$	M and C
Pyruvate + CoA + NAD$^+$ $\rightleftharpoons$ acetyl-CoA + CO_2 + NADH + H$^+$	M
Glyceraldehyde 3-phosphate + P_i + NAD$^+$ $\rightleftharpoons$ 1,3-bisphosphoglycerate + NADH + H$^+$	C
Lactate + NAD$^+$ $\rightleftharpoons$ pyruvate + NADH + H$^+$	C
β-Hydroxyacyl-CoA + NAD$^+$ $\rightleftharpoons$ β-ketoacyl-CoA + NADH + H$^+$	M
NADP$^+$-linked	
Glucose 6-phosphate + NADP$^+$ $\rightleftharpoons$ 6-phosphogluconate + NADPH + H$^+$	C
L-Malate + NADP$^+$ $\rightleftharpoons$ pyruvate + CO_2 + NADPH + H$^+$	C
NAD$^+$- or NADP$^+$-linked	
L-Glutamate + H_2O + NAD(P)$^+$ $\rightleftharpoons$ α-ketoglutarate + NH_4^+ + NAD(P)H	M
Isocitrate + NAD(P)$^+$ $\rightleftharpoons$ α-ketoglutarate + CO_2 + NAD(P)H + H$^+$	M and C

[a] These reactions and their enzymes are discussed in Chapters 14, 16, 17, and 18.

[b] M designates mitochondria; C designates cytosol.

NAD$^+$-linked dehydrogenases remove two hydrogen atoms from their substrates. One of these is transferred as a hydride ion (:H$^-$) to NAD$^+$, and the other is released as H$^+$ in the medium (see Fig. 13-24). NADH and NADPH are water-soluble electron carriers that associate *reversibly* with dehydrogenases. About 70% of the cellular NAD pool is in the mitochondria. NADH carries electrons from catabolic reactions to their point of entry into the respiratory chain, the NADH dehydrogenase complex described below. NADPH is primarily involved in biosynthetic (anabolic) reactions, and much of it is concentrated in the cytosol. Cells maintain separate pools of NADPH and NADH, with different redox potentials. This is accomplished by holding the ratio [reduced form]/[oxidized form] relatively high for NADPH and relatively low for NADH. Neither NADH nor NADPH can cross the inner mitochondrial membrane, but the electrons they carry can be shuttled across indirectly, as we shall see.

Flavoproteins contain a very tightly, sometimes covalently, bound flavin nucleotide, either FMN or FAD (see Fig. 13-27). P3 The oxidized flavin nucleotide can accept either one electron (yielding the semiquinone form) or two (yielding $FADH_2$ or $FMNH_2$). Electron transfer occurs because the flavoprotein has a higher reduction potential than the compound oxidized. Recall that reduction potential is a quantitative measure of the relative tendency of a given chemical species to accept electrons in an oxidation-reduction reaction (p. 490). The standard reduction potential of a flavin nucleotide, unlike that of NAD or NADP, depends on the protein with which it is associated. Local interactions with functional groups in the protein distort the electron orbitals in the flavin ring, changing the relative stabilities of oxidized and reduced forms. The relevant standard reduction potential is therefore that of the particular flavoprotein, not that of isolated FAD or FMN. The flavin nucleotide should be considered part of the flavoprotein's active site rather than a reactant or product in the electron-transfer reaction. Because flavoproteins can participate in either one- or two-electron transfers, they can serve as intermediates between reactions in which two electrons are donated (as in dehydrogenations) and those in which only one electron is accepted (as in the reduction of a quinone to a hydroquinone, described below).

Electrons Pass through a Series of Membrane-Bound Carriers

The mitochondrial respiratory chain consists of a series of sequentially acting electron carriers, most of which are integral proteins with prosthetic groups capable of accepting and donating either one or two electrons. Three types of electron transfers occur in oxidative phosphorylation: (1) direct transfer of electrons, as in the reduction of Fe^{3+} to Fe^{2+}; (2) transfer as a hydrogen atom ($H^+ + e^-$); and (3) transfer as a hydride ion (:H$^-$), which bears two electrons. The term **reducing equivalent** is used to designate a single electron equivalent transferred in an oxidation-reduction reaction.

P3 In addition to NAD and flavoproteins, three other types of electron-carrying molecules function in the respiratory chain: a hydrophobic quinone called ubiquinone, and two different types of iron-containing proteins, cytochromes and iron-sulfur proteins. **Ubiquinone** (also called **coenzyme Q**, or simply **Q**) is a lipid-soluble benzoquinone with a long isoprenoid side chain (**Fig. 19-3**). Ubiquinone can accept one electron to become the semiquinone radical ($^\bullet$QH or ubisemiquinone)

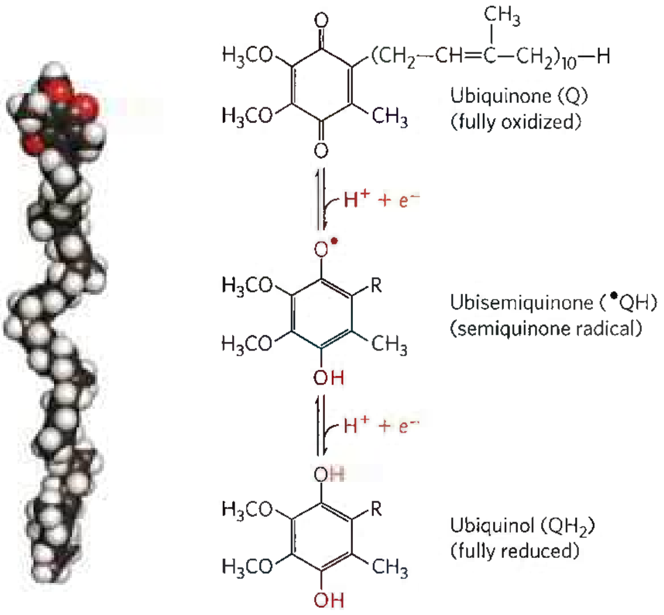
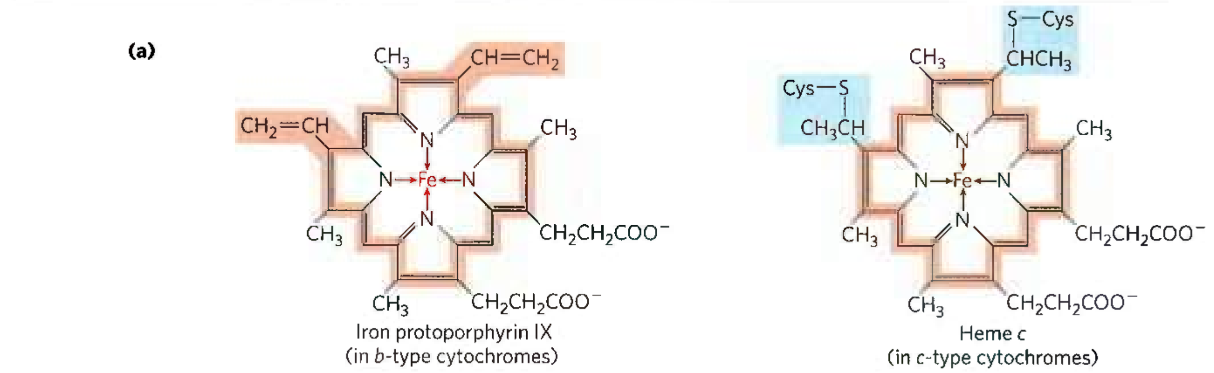


FIGURE 19-3 Ubiquinone (Q, or coenzyme Q). Complete reduction of ubiquinone requires two electrons and two protons, and occurs in two steps through the semiquinone radical intermediate.

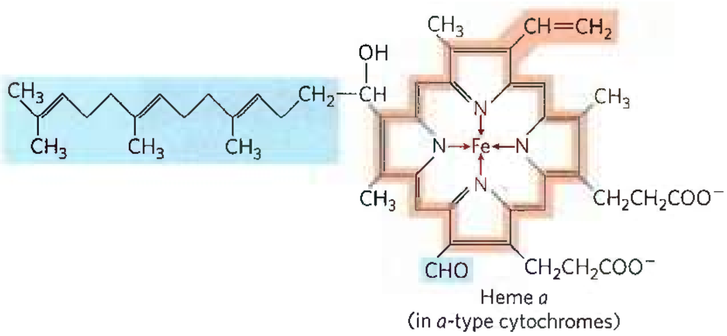
or two electrons to form ubiquinol (QH_2) and, like flavoprotein carriers, it can act at the junction between a two-electron donor and a one-electron acceptor. Small and hydrophobic, ubiquinone is not bound to proteins but instead is freely diffusible within the lipid bilayer of the inner mitochondrial membrane and can shuttle reducing equivalents between other, less mobile electron carriers in the membrane. And because it carries both electrons and protons, it plays a central role in coupling electron flow to proton movement.

The **cytochromes** are proteins with characteristic strong absorption of visible light, due to their iron-containing heme prosthetic groups (**Fig. 19-4a**). Mitochondria contain three classes of cytochromes, designated *a*, *b*, and *c*, which are distinguished by differences in their light-absorption spectra. Each type of cytochrome in its reduced (Fe^{2+}) state has three absorption bands in the visible range (Fig. 19-4b). The longest-wavelength band is near 600 nm in type *a* cytochromes, near 560 nm in type *b*, and near 550 nm in type *c*. To distinguish among closely related cytochromes of one type, the exact absorption maximum is sometimes used in the names, as in cytochrome b_{562}.



Iron protoporphyrin IX
(in *b*-type cytochromes)

Heme *c*
(in *c*-type cytochromes)



Heme *a*
(in *a*-type cytochromes)

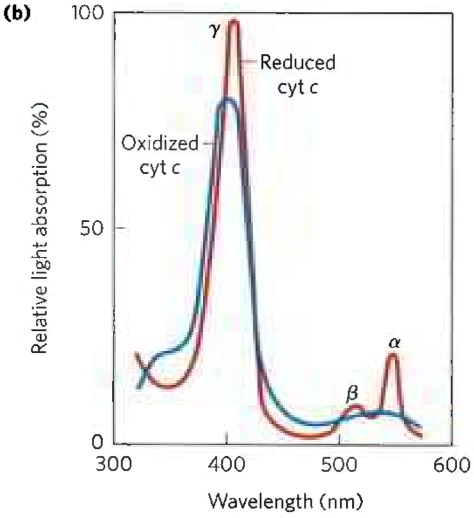


FIGURE 19-4 Prosthetic groups of cytochromes. (a) Each group consists of four five-membered, nitrogen-containing rings in a cyclic structure called a porphyrin. The four nitrogen atoms are coordinated with a central Fe ion, either Fe^{2+} or Fe^{3+}. Iron protoporphyrin IX is found in *b*-type cytochromes and in hemoglobin and myoglobin (see Fig. 5-1b). Heme *c* is covalently bound to the protein of cytochrome *c* through thioether bonds to two Cys residues. Heme *a*, found in *a*-type cytochromes, has a long isoprenoid tail attached to one of the five-membered rings. The conjugated double-bond system (shaded light red) of the porphyrin ring has delocalized π electrons that are relatively easily excited by photons with the wavelengths of visible light, which accounts for the strong absorption by hemes (and related compounds) in the visible region of the spectrum. (b) Absorption spectra of cytochrome *c* (cyt *c*) in its oxidized (blue) and reduced (red) forms. The characteristic α, β, and γ bands of the reduced form are labeled.

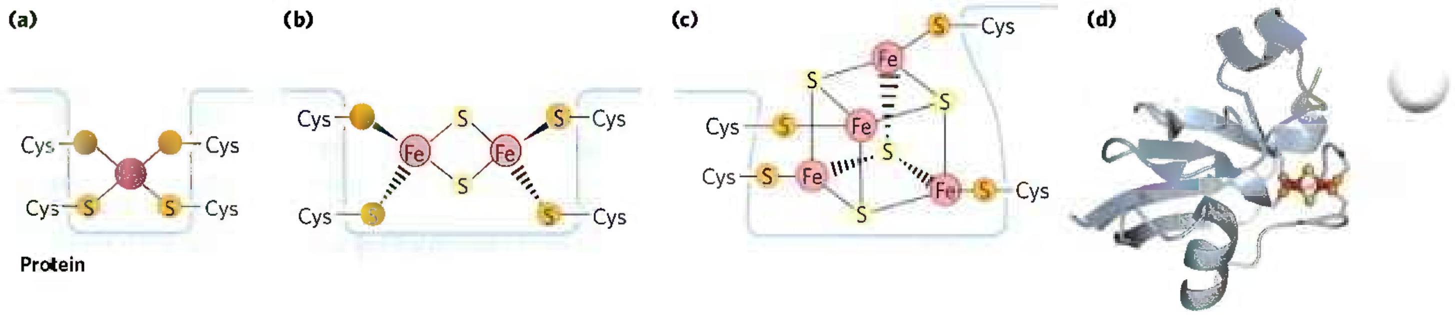


FIGURE 19-5 Iron-sulfur centers. The Fe-S centers of iron-sulfur proteins may be as simple as shown in (a), with a single Fe ion surrounded by the S atoms of four Cys residues; inorganic S is yellow and the S of Cys is orange. Other centers include both inorganic and Cys S atoms, as in (b) 2Fe-2S or (c) 4Fe-4S centers. (d) The ferredoxin of the cyanobacterium *Anabaena* 7120 has one 2Fe-2S center. (Note that in these designations, only the inorganic S atoms are counted. For example, in the 2Fe-2S center (b), each Fe ion is actually surrounded by four S atoms.) The exact standard reduction potential of the iron in these centers depends on the type of center and its interaction with the associated protein. [(d) Data from PDB ID 1FRD, B. L. Jacobson et al., *Biochemistry* 32:6788, 1993.]

The hemes of *a* and *b* cytochromes are tightly, but not covalently, bound to their associated proteins; the hemes of *c*-type cytochromes are covalently attached through Cys residues (Fig. 19-4). As with the flavoproteins, the standard reduction potential of the heme iron atom of a cytochrome depends on its interaction with protein side chains and is therefore different for each cytochrome. The cytochromes of type *a* and *b* and some of type *c* are integral proteins of the inner mitochondrial membrane. One striking exception is the soluble cytochrome *c* that associates through electrostatic interactions with the outer surface of the inner membrane.

In **iron-sulfur proteins**, the iron is present not in heme but in association with inorganic sulfur atoms or with the sulfur atoms of Cys residues in the protein, or both. These iron-sulfur (Fe-S) centers range from simple structures with a single Fe atom coordinated to four Cys —SH groups to more complex Fe-S centers with two or four Fe atoms (**Fig. 19-5**). **Rieske iron-sulfur proteins** (named after their discoverer, John S. Rieske) are a variation on this theme, in which one Fe atom is coordinated to two His residues rather than two Cys residues. All iron-sulfur proteins participate in one-electron transfers in which one iron atom of the Fe-S cluster is oxidized or reduced. At least eight Fe-S proteins function in mitochondrial electron transfer. The reduction potential of Fe-S proteins varies from –0.65 V to +0.45 V, depending on the microenvironment of the iron within the protein.

In the overall reaction catalyzed by the mitochondrial respiratory chain, electrons move from NADH, succinate, or some other primary electron donor through flavoproteins, ubiquinone, iron-sulfur proteins, and cytochromes, and finally to O_2. A look at the methods used to determine the sequence in which the carriers act is instructive, as the same general approaches have been used to study other electron-transfer chains, such as those of chloroplasts (see Fig. 20-12).

First, the standard reduction potentials of the individual electron carriers have been determined experimentally (**Table 19-2**). Electrons tend to flow spontaneously from carriers of lower $E'°$ to carriers of higher $E'°$. The

TABLE 19-2 Standard Reduction Potentials of Respiratory Chain and Related Electron Carriers

Redox reaction (half-reaction)	$E'°$ (V)
$2H^+ + 2e^- \longrightarrow H_2$	–0.414
$NAD^+ + H^+ + 2e^- \longrightarrow NADH$	–0.320
$NADP^+ + H^+ + 2e^- \longrightarrow NADPH$	–0.324
NADH dehydrogenase (FMN) + $2H^+ + 2e^- \longrightarrow$ NADH dehydrogenase ($FMNH_2$)	–0.30
Ubiquinone + $2H^+ + 2e^- \longrightarrow$ ubiquinol	0.045
Cytochrome *b* (Fe^{3+}) + $e^- \longrightarrow$ cytochrome *b* (Fe^{2+})	0.077
Cytochrome c_1 (Fe^{3+}) + $e^- \longrightarrow$ cytochrome c_1 (Fe^{2+})	0.22
Cytochrome *c* (Fe^{3+}) + $e^- \longrightarrow$ cytochrome *c* (Fe^{2+})	0.254
Cytochrome *a* (Fe^{3+}) + $e^- \longrightarrow$ cytochrome *a* (Fe^{2+})	0.29
Cytochrome a_3 (Fe^{3+}) + $e^- \longrightarrow$ cytochrome a_3 (Fe^{2+})	0.35
$\frac{1}{2}O_2 + 2H^+ + 2e^- \longrightarrow H_2O$	0.817

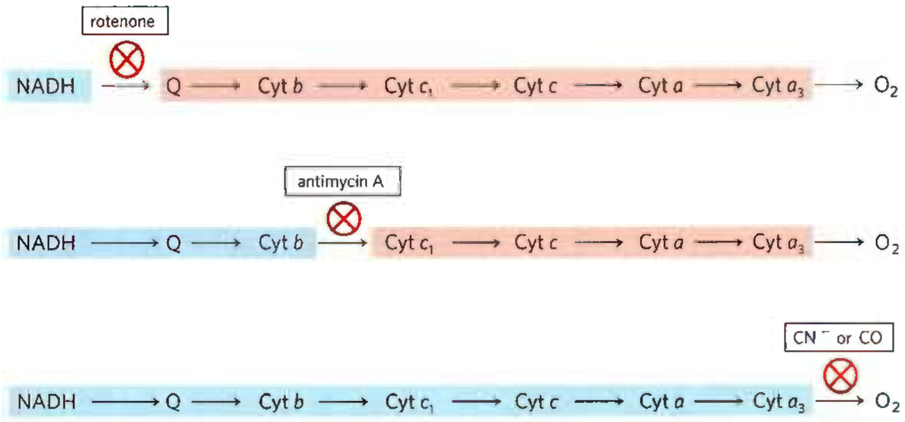


FIGURE 19-6 Method for determining the sequence of electron carriers. This method measures the effects of inhibitors of electron transfer on the oxidation state of each carrier. In the presence of an electron donor and O_2, each inhibitor causes a characteristic pattern of oxidized/reduced carriers: those before the block become reduced (blue), and those after the block become oxidized (light red).

order of carriers deduced by this method is NADH → Q → cytochrome b → cytochrome c_1 → cytochrome c → cytochrome a → cytochrome a_3 → O_2. Note, however, that the order of standard reduction potentials is not necessarily the same as the order of *actual* reduction potentials under cellular conditions, which depend on the concentrations of reduced and oxidized forms (see Eqn 13-5, p. 491).

A second method for determining the sequence of electron carriers involves reducing the entire chain of carriers experimentally by providing an electron source but no electron acceptor (no O_2). When O_2 is suddenly introduced into the system, the rate at which each electron carrier becomes oxidized, measured spectroscopically, reveals the order in which the carriers function. The carrier nearest O_2 (at the end of the chain) gives up its electrons first, the second carrier from the end is oxidized next, and so on. Such experiments have confirmed the sequence deduced from standard reduction potentials.

In a final confirmation, agents that inhibit the flow of electrons through the chain have been used in combination with measurements of the degree of oxidation of each carrier. In the presence of O_2 and an electron donor, carriers that function before the inhibited step become fully reduced, and those that function after this step are completely oxidized (**Fig. 19-6**). By using several inhibitors that block different steps in the chain, investigators have determined the entire sequence; it is the same as deduced in the first two approaches.

Electron Carriers Function in Multienzyme Complexes

The electron carriers of the respiratory chain are organized into membrane-embedded supramolecular complexes that can be physically separated. Gentle treatment of the inner mitochondrial membrane with detergents allows the resolution of four unique electron-carrier complexes, each capable of catalyzing electron transfer through a portion of the chain (**Fig. 19-7**; **Table 19-3**). **P3** Complexes I and II catalyze electron transfer to ubiquinone from two different electron donors: NADH (Complex I) and succinate (Complex II). Complex III carries electrons from reduced ubiquinone to cytochrome c, and Complex IV completes the sequence by transferring electrons from cytochrome c to O_2.

We now look in more detail at the structure and function of each complex of the mitochondrial respiratory chain.

Complex I: NADH to Ubiquinone In mammals, **Complex I,** also called **NADH:ubiquinone oxidoreductase** or

TABLE 19-3 The Protein Components of the Mitochondrial Respiratory Chain

Enzyme complex/protein	Mass (kDa)	Number of subunits[a]	Prosthetic group(s)
I NADH dehydrogenase	850	45 (14)	FMN, Fe-S
II Succinate dehydrogenase	140	4	FAD, Fe-S
III Ubiquinone:cytochrome c oxidoreductase[b]	250	11	Hemes, Fe-S
Cytochrome c[c]	13	1	Heme
IV Cytochrome oxidase[b]	204	13 (3–4)	Hemes; Cu_A, Cu_B

[a] Number of subunits in bacterial complexes is shown in parentheses.

[b] Mass and subunit data are for the monomeric form.

[c] Cytochrome c is not part of an enzyme complex; it moves between Complexes III and IV as a freely soluble protein.

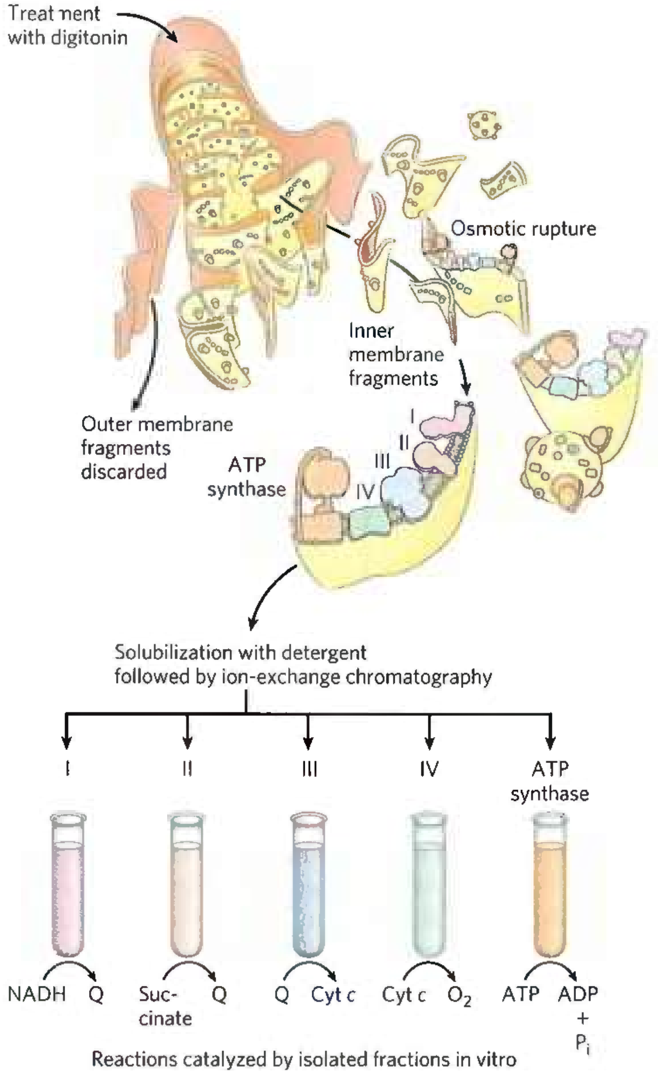

Treatment with digitonin
Osmotic rupture
Inner membrane fragments
Outer membrane fragments discarded
ATP synthase
I
II
III
IV
Solubilization with detergent followed by ion-exchange chromatography
I
II
III
IV
ATP synthase
NADH Q
Succinate Q
Q Cyt c
Cyt c O2
ATP ADP + Pi
Reactions catalyzed by isolated fractions in vitro

FIGURE 19-7 Separation of functional complexes of the respiratory chain. The outer mitochondrial membrane is first removed by treatment with the detergent digitonin. Fragments of inner membrane are then obtained by osmotic rupture of the membrane, and the fragments are gently dissolved in a second detergent. The resulting mixture of inner membrane proteins is resolved by ion-exchange chromatography into several complexes (I through IV) of the respiratory chain, each with its unique protein composition (see Table 19-3), and the enzyme ATP synthase (sometimes called Complex V). The isolated Complexes I through IV catalyze electron transfers between donors (NADH and succinate), intermediate carriers (Q and cytochrome *c*), and O_2, as shown. In vitro, isolated ATP synthase has only ATP-hydrolyzing (ATPase), not ATP-synthesizing, activity.

NADH dehydrogenase, is a large enzyme composed of 45 different polypeptide chains, including an FMN-containing flavoprotein and at least 8 iron-sulfur centers. Complex I is L-shaped, with one arm embedded in the inner membrane and the other extending into the matrix. Comparative studies of Complex I in bacteria and other organisms show that 7 polypeptides in the membrane arm and 7 in the matrix arm are conserved and essential (**Fig. 19-8**).

Complex I catalyzes two simultaneous and obligately coupled processes: (1) the exergonic transfer to ubiquinone of a hydride ion from NADH and a proton from the matrix, expressed by

$$\mathrm{NADH} + \mathrm{H}^+ + \mathrm{Q} \longrightarrow \mathrm{NAD}^+ + \mathrm{QH}_2 \qquad (19\text{-}1)$$

and (2) the endergonic transfer of four protons from the matrix to the intermembrane space. Protons are moved against a transmembrane proton gradient in this process. **P4** Complex I is therefore a proton pump driven by the energy of electron transfer, and the reaction it catalyzes is **vectorial**: it moves protons in a specific direction from one location (the matrix, which becomes negatively charged with the departure of protons) to another (the intermembrane space, which becomes positively charged). To emphasize the vectorial nature of the process, the overall reaction is often written with subscripts that indicate the location of the protons: P for the positive side of the inner membrane (the intermembrane space), N for the negative side (the matrix):

$$\mathrm{NADH} + 5\mathrm{H}^+_\mathrm{N} + \mathrm{Q} \longrightarrow \mathrm{NAD}^+ + \mathrm{QH}_2 + 4\mathrm{H}^+_\mathrm{P} \qquad (19\text{-}2)$$

Amytal (a barbiturate drug), rotenone (a plant product commonly used as an insecticide), and piericidin A (an

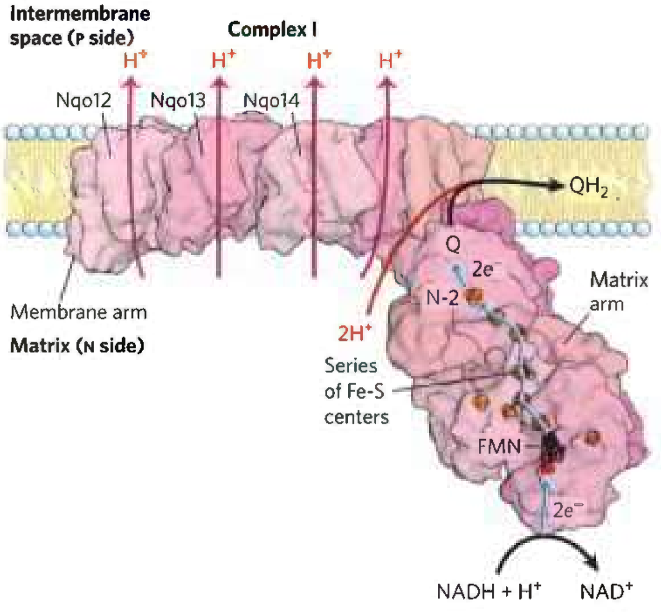

Intermembrane space (P side)
Complex I
H+
H+
H+
H+
Nqo12
Nqo13
Nqo14
QH2
Q
2e−
Matrix arm
N-2
Membrane arm
Matrix (N side)
2H+
Series of Fe-S centers
FMN
2e−
NADH + H+
NAD+

FIGURE 19-8 Structure of Complex I (NADH:ubiquinone oxidoreductase). Complex I catalyzes the transfer of a hydride ion from NADH to FMN. From the FMN, two electrons pass through a series of Fe-S centers to the Fe-S center N-2 in the matrix arm of the complex. Electron transfer from N-2 to ubiquinone on the membrane arm forms QH_2, which diffuses into the lipid bilayer. The protons travel a path dictated by subunit conformation changes triggered by the electron flow. Proton flux produces an electrochemical potential across the inner mitochondrial membrane (N side negative, P side positive). Three of the membrane subunits (subunits Nqo12, Nqo13, and Nqo14) are structurally related to a known Na^+-H^+ antiporter, and the path of proton movement may be similar in both cases. The fourth putative proton pathway is through an integral subunit closest to the Q-binding site. A long helix (not visible in this view) lying along the surface of the membrane arm may coordinate the action of all four proton pumps when Q is reduced. [Data from PDB ID 4HEA, R. Baradaran et al., *Nature* 494:443, 2013.]

TABLE 19-4 Agents That Interfere with Oxidative Phosphorylation

Type of interference	Compound[a]	Target/mode of action
Inhibition of electron transfer	Cyanide Carbon monoxide	Inhibit cytochrome oxidase
	Antimycin A	Blocks electron transfer from cytochrome *b* to cytochrome c_1
	Myxothiazol Rotenone Amytal Piericidin A	Prevent electron transfer from Fe-S center to ubiquinone
Inhibition of ATP synthase	Aurovertin	Inhibit F_1
	Oligomycin Venturicidin	Inhibit F_o
	DCCD	Blocks proton flow through F_o
Uncoupling of phosphorylation from electron transfer	FCCP DNP	Hydrophobic proton carriers
	Valinomycin	K^+ ionophore
	Uncoupling protein 1	In brown adipose tissue, forms proton-conducting pores in inner mitochondrial membrane
Inhibition of ATP-ADP exchange	Atractyloside	Inhibits adenine nucleotide translocase

[a] DCCD, dicyclohexylcarbodiimide; FCCP, cyanide-*p*-trifluoromethoxyphenylhydrazone; DNP, 2,4-dinitrophenol.

antibiotic) inhibit electron flow from the Fe-S centers of Complex I to ubiquinone (**Table 19-4**) and therefore block the overall process of oxidative phosphorylation.

Three of the seven integral protein subunits of the membrane arm are related to a Na^+-H^+ antiporter and are believed to be responsible for pumping three protons; a fourth subunit in the membrane arm, that nearest the Q-binding site, is probably responsible for pumping the fourth proton (Fig. 19-8).

How is the reduction of ubiquinone coupled to proton pumping? Reduction of Q occurs far away from the membrane arm of the protein, where proton pumping occurs, so the coupling is clearly indirect. The high-resolution view of Complex I from crystallographic and cryo-EM studies suggests that reduction of Q is coupled to a long-range conformational change conducted to all subunits along the hydrophilic core of the transmembrane arm. It seems likely that all four protons are pumped simultaneously, so that the energy from a strongly exergonic reaction (Q reduction) is broken into smaller packets, a common strategy employed by living organisms.

Complex II: Succinate to Ubiquinone We encountered **Complex II** in Chapter 16 as **succinate dehydrogenase**, the only membrane-bound enzyme in the citric acid cycle (p. 586). **P3** Complex II couples the oxidation of succinate at one site with the reduction of ubiquinone at another site about 40 Å away. Although smaller and simpler than Complex I, Complex II contains five prosthetic groups of two types and four different protein subunits (**Fig. 19-9**). Subunits C and D are integral membrane proteins, each with three transmembrane helices. They contain a heme

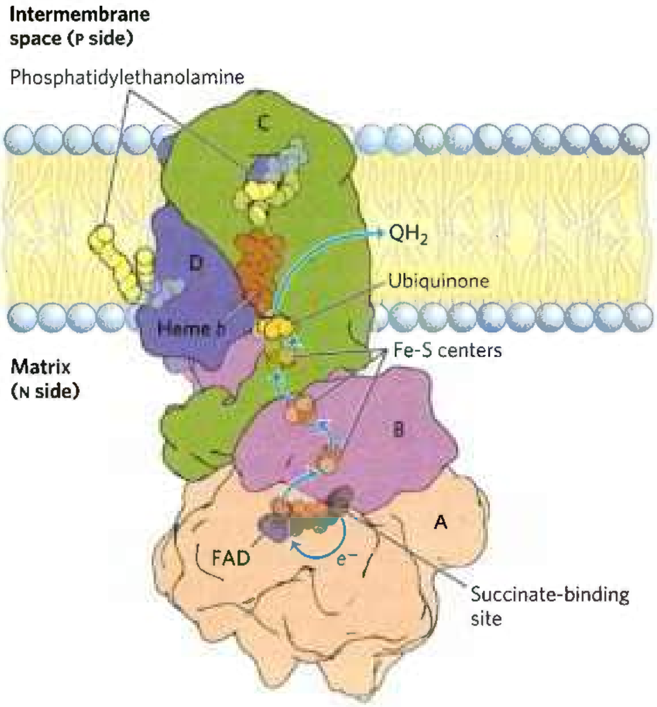


FIGURE 19-9 Structure of Complex II (succinate dehydrogenase). This complex (porcine) has two transmembrane subunits, C and D; subunits A and B extend into the matrix. Just behind the FAD in subunit A is the binding site for succinate. Subunit B has three Fe-S centers, ubiquinone is bound to subunit B, and heme *b* is sandwiched between subunits C and D. Two phosphatidylethanolamine molecules are so tightly bound to subunit D that they show up in the crystal structure. Electrons move (blue arrows) from succinate to FAD, then through the three Fe-S centers to ubiquinone. The heme *b* is not on the main path of electron transfer but protects against the formation of reactive oxygen species (ROS) by electrons that go astray. [Data from PDB ID 1ZOY, F. Sun et al., *Cell* 121:1043, 2005.]

group, heme b, and a binding site for Q, the final electron acceptor in the reaction catalyzed by Complex II. Subunits A and B extend into the matrix; they contain three 2Fe-2S centers, bound FAD, and a binding site for the substrate, succinate. Although the overall path of electron transfer is long (from the succinate-binding site to FAD, then through the Fe-S centers to the Q-binding site), none of the individual electron-transfer distances exceeds about 11 Å—a reasonable distance for rapid electron transfer (Fig. 19-9). Electron transfer through Complex II is not accompanied by proton pumping across the inner membrane, although the QH_2 produced by succinate oxidation will be used by Complex III to drive proton transfer. Because Complex II functions in the citric acid cycle, factors that affect its activity (such as the availability of oxidized Q) probably serve to coordinate that cycle with mitochondrial electron transfer.

The heme b of Complex II is apparently not in the direct path of electron transfer; it serves instead to reduce the frequency with which electrons "leak" out of the system, moving from succinate to molecular oxygen to produce the **reactive oxygen species (ROS)** hydrogen peroxide (H_2O_2) and the **superoxide radical ($\cdot O_2^-$)**, as described below. Some individuals with point mutations in Complex II subunits near heme b or the ubiquinone-binding site suffer from hereditary paraganglioma, characterized by benign tumors of the head and neck, commonly in the carotid body, an organ that senses O_2 levels in the blood. These mutations result in greater production of ROS, which cause DNA damage and genome instability that can lead to cancer. Mutations that affect the succinate-binding region in Complex II may lead to degenerative changes in the central nervous system, and some mutations are associated with tumors of the adrenal medulla. ■

Complex III: Ubiquinone to Cytochrome *c* Electrons from reduced ubiquinone (ubiquinol, QH_2) pass through two more large protein complexes in the inner mitochondrial membrane before reaching the ultimate electron acceptor, O_2. P3 P4 **Complex III** (also called **cytochrome bc_1 complex** or **ubiquinone:cytochrome *c* oxidoreductase**) couples the transfer of electrons from ubiquinol to cytochrome c with the vectorial transport of protons from the matrix to the intermembrane space. The functional unit of Complex III (**Fig. 19-10**) is a dimer. Each monomer consists of three proteins central to the action of the complex: cytochrome b, cytochrome c_1, and the Rieske iron-sulfur protein. (Several other proteins associated with Complex III in vertebrates are not conserved across the phyla and presumably play subsidiary roles.) The two cytochrome b monomers surround a cavern in the middle of the membrane, in which ubiquinone is free to move from the matrix side of the membrane (site Q_N on one monomer) to the intermembrane space (site Q_P on the other monomer) as it shuttles electrons and protons across the inner mitochondrial membrane.

To account for the role of Q in energy conservation, Mitchell proposed the Q cycle (**Fig. 19-11**). As electrons move from QH_2 through Complex III, QH_2 is oxidized with the release of protons on one side of the membrane (at Q_P), while at the other site (Q_N), Q is reduced and protons are taken up.

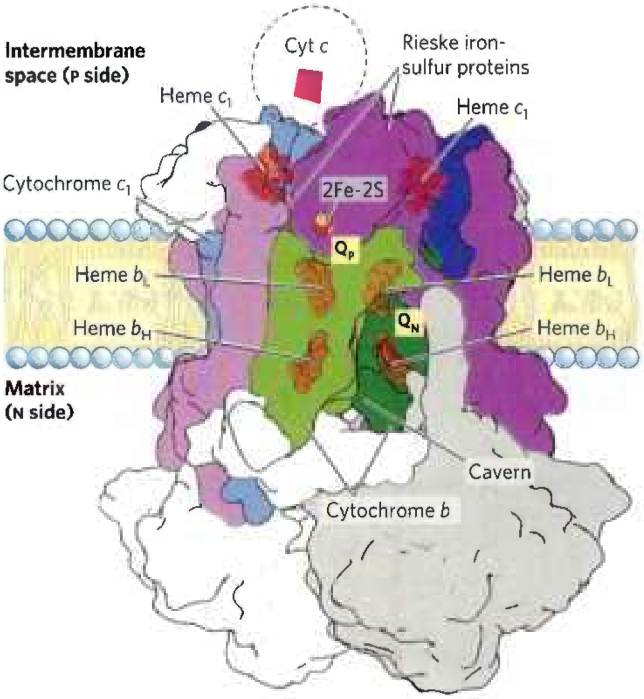
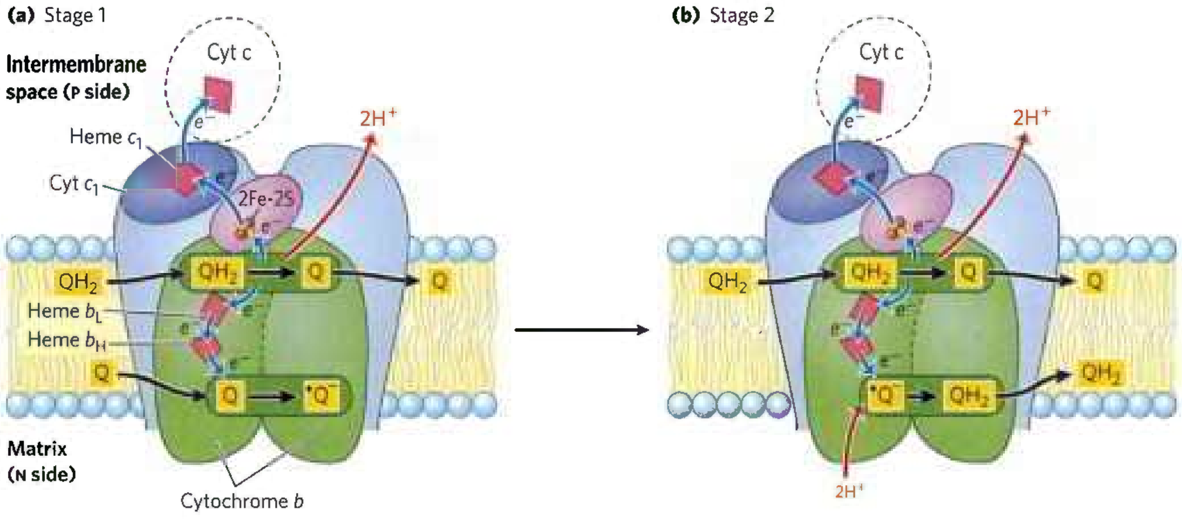


FIGURE 19-10 Structure of Complex III (cytochrome bc_1 complex). The complex (bovine) is a dimer of identical monomers, each with 11 different subunits. The functional core of each monomer consists of three subunits: cytochrome b (green), with its two hemes (b_H and b_L); the Rieske iron-sulfur protein (purple), with its 2Fe-2S centers; and cytochrome c_1 (blue), with its heme. Cytochrome c_1 and the Rieske iron-sulfur protein project from the P surface and can interact with cytochrome c (not part of the functional complex) in the intermembrane space. The complex has two distinct binding sites for ubiquinone, Q_N and Q_P, which correspond to the sites of inhibition by two drugs that block oxidative phosphorylation. Antimycin A, which blocks electron flow from cytochrome b to cytochrome c_1, specifically from heme b_H to Q, binds at Q_N, close to heme b_H on the N (matrix) side of the membrane. Myxothiazol, which prevents electron flow from QH_2 to the Rieske iron-sulfur protein, binds at Q_P, near the 2Fe-2S center and heme b_L on the P side. The dimeric structure is essential to the function of Complex III. The interface between monomers forms two caverns, each containing a Q_P site from one monomer and a Q_N site from the other. The ubiquinone intermediates move within these sheltered caverns. [Data from PDB ID 1BGY, S. Iwata et al., *Science* 281:64, 1998.]

The Q cycle is most easily understood as occurring in two stages, with two active sites where ubiquinone is either oxidized or reduced. In both stages, one QH_2 is oxidized at active site 1, shedding two H^+ and two electrons. The protons are released into the intermembrane space. The two electrons take different paths, with one reducing cytochrome c and the other reducing a molecule of Q at active site 2. Two electrons are required at active site 2 to fully reduce the Q to QH_2, one in each stage. Reducing one Q at one site while oxidizing two QH_2 at another may seem counterproductive at first glance. However, the two processes have complementary functions. The oxidation of two QH_2 is moving four protons to the intermembrane space and two



$$QH_2 + Q + \text{cyt } c\,(\text{oxidized}) \longrightarrow Q + {}^{\bullet}Q^- + 2H_P^+ + \text{cyt } c\,(\text{reduced})$$

$$QH_2 + {}^{\bullet}Q^- + 2H_N^+ + \text{cyt } c\,(\text{oxidized}) \longrightarrow Q + 2H_P^+ + QH_2 + \text{cyt } c\,(\text{reduced})$$

Net equation: $QH_2 + 2\text{ cyt } c\,(\text{oxidized}) + 2H_N^+ \longrightarrow Q + 2\text{ cyt } c\,(\text{reduced}) + 4H_P^+$

FIGURE 19-11 The Q cycle, shown in two stages. The path of electrons through Complex III is shown with blue arrows; the movement of various forms of ubiquinone, with black arrows. (a) In the first stage, Q on the N side is reduced to the semiquinone radical, which moves back into position (dotted line) to accept another electron. (b) In the second stage, the semiquinone radical is converted to QH_2. Meanwhile, on the P side of the membrane, two molecules of QH_2 are oxidized to Q, releasing two protons per Q molecule (four protons in all) into the intermembrane space. Each QH_2 donates one electron (via the Rieske Fe-S center) to cytochrome c_1, and one electron (via cytochrome *b*) to a molecule of Q near the N side, reducing it in two steps to QH_2. This reduction also consumes two protons per Q, which are taken up from the matrix (N side). Reduced cytochrome c_1 passes electrons one at a time to cytochrome *c*, which dissociates and carries electrons to Complex IV. In each cycle, one reduction of Q at the Q_N site is coupled with two oxidations of QH_2 at the Q_P site by consuming two protons from the matrix and releasing four protons into the intermembrane space.

electrons to cytochrome *c*. At the same time, the reduction of Q at the other site (using the other two electrons from the oxidation of QH_2 at site 1) is pulling protons from the matrix, creating a net movement of protons from the matrix to the intermembrane space. The QH_2 produced at active site 2 becomes a substrate for oxidation at active site 1 in subsequent turns of the cycle, and vice versa. The net equation for the redox reactions of the Q cycle is

$$QH_2 + 2\text{ cyt } c\text{ (oxidized)} + 2H_N^+ \longrightarrow Q + 2\text{ cyt } c\text{ (reduced)} + 4H_P^+ \quad (19\text{-}3)$$

The Q cycle accommodates the switch between the two-electron carrier ubiquinol (the reduced form of ubiquinone) and the one-electron carriers—hemes b_L and b_H of cytochrome *b*, and cytochromes c_1 and *c*—and results in the uptake of two protons on the N side and the release of four protons on the P side, per pair of electrons passing through Complex III to cytochrome *c*. Two of the protons released on the P side are electrogenic; the other two are electroneutral, balanced by the two charges (electrons) passed to cytochrome *c* on the P side. Although the path of electrons through this segment of the respiratory chain is complicated, the net effect of the transfer is simple: QH_2 is oxidized to Q, two molecules of cytochrome *c* are reduced, and two protons are moved from the N side to the P side of the inner mitochondrial membrane.

Cytochrome *c* is a soluble protein of the intermembrane space, which associates reversibly with the P side of the inner membrane. After its single heme accepts an electron from Complex III, cytochrome *c* moves in the intermembrane space to Complex IV to donate the electron to a binuclear copper center.

Complex IV: Cytochrome *c* to O_2 In the final step of the respiratory chain, **Complex IV**, also called **cytochrome oxidase**, carries electrons from cytochrome *c* to molecular oxygen, reducing it to H_2O. P3 P4 Complex IV is a large, dimeric enzyme of the inner mitochondrial membrane, each monomer having 13 subunits and M_r of 204,000. Bacteria contain a form that is much simpler, with only 3 or 4 subunits per monomer, but still capable of catalyzing both electron transfer and proton pumping. Comparison of the mitochondrial and bacterial complexes suggests that these 3 subunits have been conserved in evolution; in multicellular organisms, the other 10 subunits contribute to the assembly or stability of Complex IV (**Fig. 19-12**).

Subunit II of Complex IV contains two Cu ions complexed with the —SH groups of two Cys residues in a binuclear center (Cu_A; Fig. 19-12b) that resembles the 2Fe-2S centers of iron-sulfur proteins. Subunit I contains two heme groups, designated *a* and a_3, and another copper ion (Cu_B). Heme a_3 and Cu_B form a second binuclear center

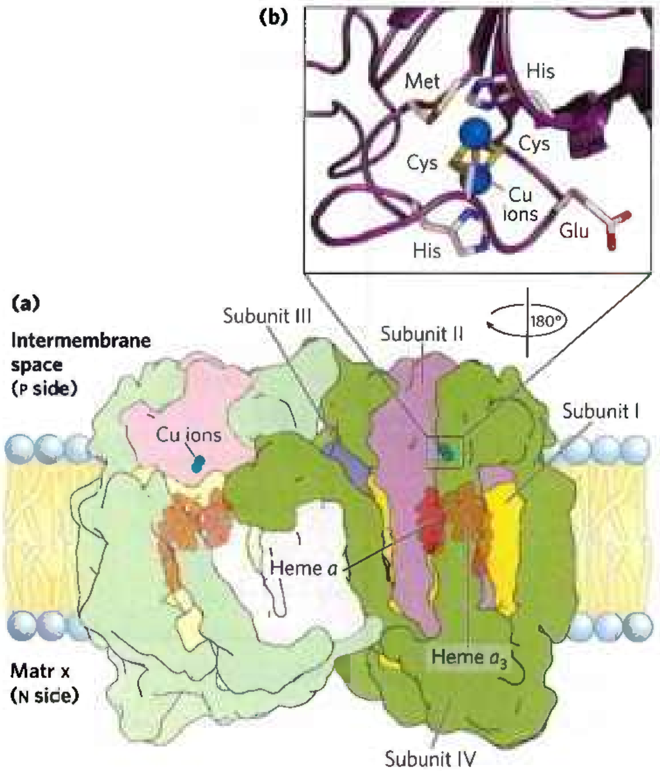

(b)
Met
His
Cys
Cys
Cu ions
Glu
His
(a)
Intermembrane space (P side)
Subunit III
Subunit II
180°
Subunit I
Cu ions
Heme a
Heme a_3
Matrix (N side)
Subunit IV

FIGURE 19-12 Structure of Complex IV (cytochrome oxidase). (a) This complex (bovine) has 13 subunits in each identical monomer of its dimeric structure. Subunit I has two heme groups, *a* and a_3, near a single copper ion, Cu_B (not visible here). Heme a_3 and Cu_B form a binuclear Fe-Cu center. Subunit II contains two Cu ions complexed with the —SH groups of two Cys residues in a binuclear center, Cu_A, that resembles the 2Fe-2S centers of iron-sulfur proteins. This binuclear center and the cytochrome *c*–binding site are located in a domain of subunit II that protrudes from the P side of the inner membrane (into the intermembrane space). Subunit III is essential for rapid proton movement through subunit II. The roles of the other 10 subunits in mammalian Complex IV are not fully understood, although some function in assembly or stabilization of the complex. (b) The binuclear center of Cu_A. The Cu ions (blue spheres) share electrons equally. When the center is reduced, the ions have the formal charges $Cu^{1+}Cu^{1+}$; when oxidized, $Cu^{1.5+}Cu^{1.5+}$. Six amino acid residues are ligands around the Cu ions: Glu, Met, two His, and two Cys. [Data from PDB ID 1OCC, T. Tsukihara et al., *Science* 272:1136, 1996.]

that accepts electrons from heme *a* and transfers them to O_2 bound to heme a_3. The detailed role of subunit III is not clear, but its presence is essential to Complex IV function.

Electron transfer through Complex IV is from cytochrome *c* to the Cu_A center, to heme *a*, to the heme a_3–Cu_B center, and finally to O_2 (**Fig. 19-13a**). For every four electrons passing through this complex, the enzyme consumes four "substrate" H^+ from the matrix (N side) in converting O_2 to two H_2O. It also uses the energy of this redox reaction to pump four protons outward into the intermembrane space (P side) for each four electrons that pass through, adding to the electrochemical potential produced by redox-driven proton transport through Complexes I and III. The overall reaction catalyzed by Complex IV is

$$4 \text{ cyt } c \text{ (reduced)} + 8H_N^+ + O_2 \longrightarrow 4 \text{ cyt } c \text{ (oxidized)} + 4H_P^+ + 2H_2O \quad (19\text{-}4)$$

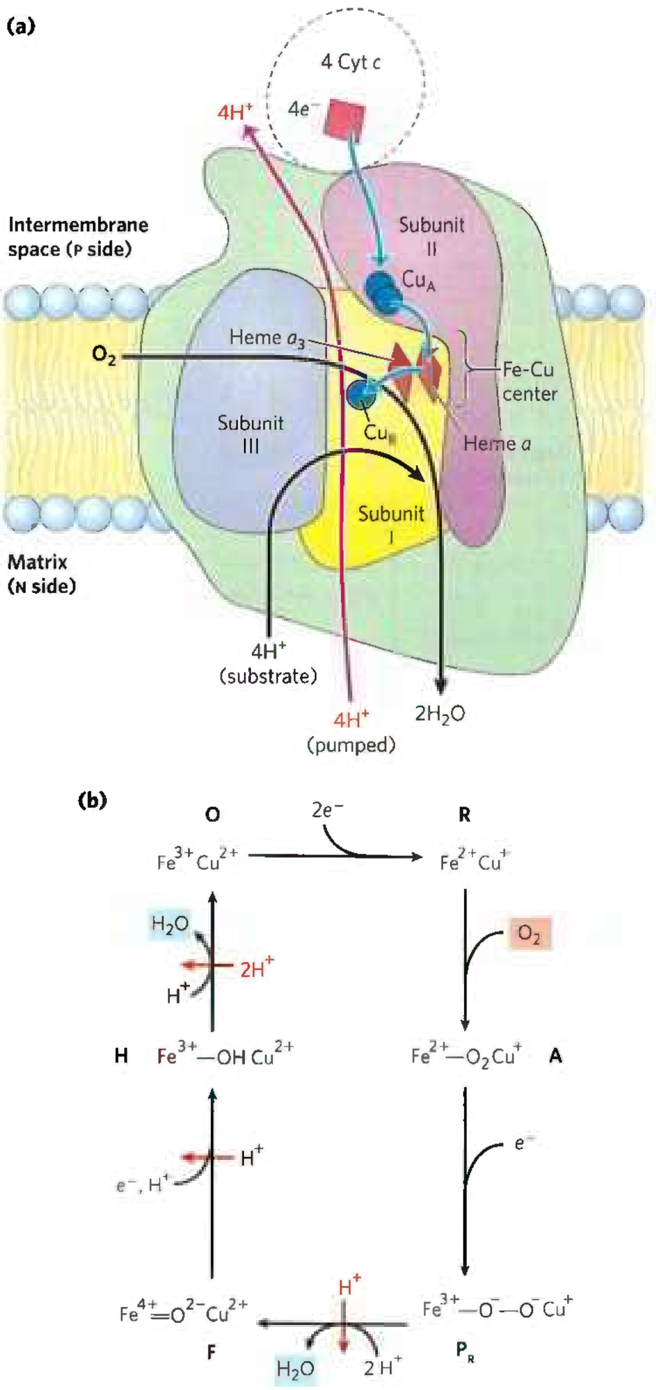

(a)
4 Cyt c
$4H^+$
$4e^-$
Intermembrane space (P side)
Subunit II
Cu_A
Heme a_3
O_2
Fe-Cu center
Subunit III
Cu_B
Heme a
Subunit I
Matrix (N side)
$4H^+$ (substrate)
$4H^+$ (pumped)
$2H_2O$
(b)
O
$2e^-$
R
$Fe^{3+}Cu^{2+}$
$Fe^{2+}Cu^+$
H_2O
$2H^+$
H^+
O_2
H
Fe^{3+}—OH Cu^{2+}
Fe^{2+}—O_2Cu^+
A
H^+
e^-, H^+
e^-
H^+
Fe^{4+}=$O^{2-}Cu^{2+}$
Fe^{3+}—O^-—O^-Cu^+
F
P_R
H_2O
$2H^+$

FIGURE 19-13 Path of electrons through Complex IV. (a) For simplicity, only one monomer of the dimeric bovine Complex IV is shown. The three proteins critical to electron flow are subunits I, II, and III. The larger green structure includes the other 10 proteins in each monomer of the dimeric complex. Electron transfer through Complex IV begins with cytochrome *c* (top). Two molecules of reduced cytochrome *c* each donate an electron to the binuclear center Cu_A. From here, electrons pass through heme *a* to the Fe-Cu center (heme a_3 and Cu_B). Oxygen now binds to heme a_3 and is reduced to its peroxy derivative (O_2^{2-}; not shown here) by two electrons from the Fe-Cu center. Delivery of two more electrons from cytochrome *c* (top), for a total of four electrons, converts the O_2^{2-} to two molecules of water, with consumption of four "substrate" protons from the matrix. At the same time, four protons are pumped from the matrix for every four electrons passing through Complex IV. A simplified reaction sequence is presented in (b). Intermediate complexes O, R, A, P_R, F, and H represent only a prominent subset of the species for which there is experimental evidence, with some steps and intermediate structures still being debated. The four electrons are introduced in separate steps, and H_2O is released in two separate steps.

Note that the O_2 in this reaction is the final acceptor for electrons originating from the many sources already described, and the stoichiometries between electron sources and O_2 molecules consumed help to define the energetics of the systems. In this chapter, stoichiometries are sometimes presented, as here, in terms of one molecule of O_2. For calculation simplicity, the stoichiometries will be presented in terms of $\frac{1}{2}O_2$ in some examples to come.

At Complex IV, O_2 is reduced at redox centers that carry only one electron at a time. A reaction scheme is presented in Figure 19-13b. Normally the incompletely reduced oxygen intermediates remain tightly bound to the complex until completely converted to water. However, a small fraction of oxygen intermediates escape. These intermediates are reactive oxygen species that can damage cellular components unless eliminated by defense mechanisms described below.

Mitochondrial Complexes Associate in Respirasomes

Although the four electron-transferring complexes can be separated in the laboratory, in the intact mitochondrion, three of the four respiratory complexes associate with each other in the inner membrane. Combinations of Complexes I and III, III and IV, and I, III, and IV are formed in organisms ranging from yeast to plants to mammals. The supercomplex containing Complexes I, III, and IV has been called the **respirasome**. Unlike the other three complexes, Complex II is generally found free-floating within the membrane. Structural characterization of the various supercomplexes has been advanced by cryo-EM (**Fig. 19-14**). The functional significance of supercomplexes has not been determined. Researchers have suggested that they may facilitate electron transfers or limit the production of reactive oxygen species. Local pools of the electron carriers cytochrome *c* and ubiquinone are not constrained within supercomplexes, but instead readily diffuse between them.

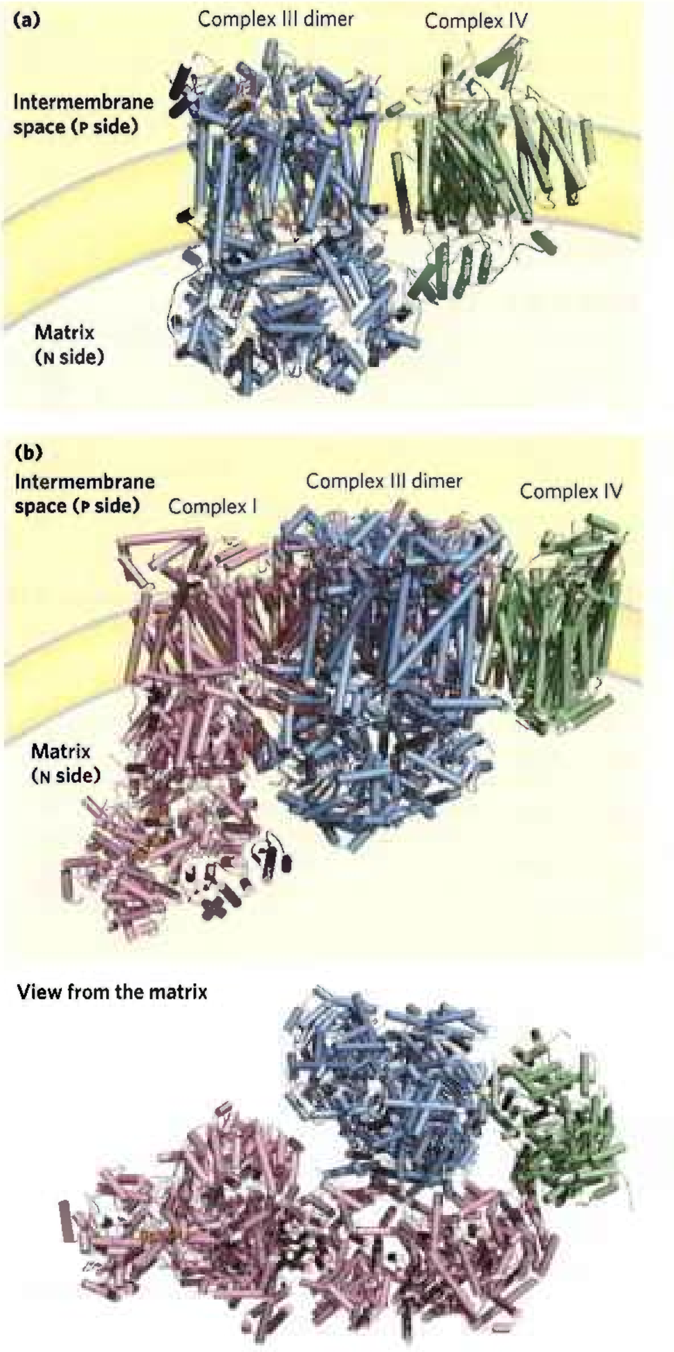


FIGURE 19-14 A respirasome composed of Complexes I, III, and IV. (a) Purified supercomplexes containing Complexes III and IV (from yeast), as determined by cryo-EM. (b) The structure of a respirasome composed of mammalian (porcine and bovine) Complexes I, III, and IV. Two views are shown. [Data from (a) PDB ID 6GIQ, S. Rathore et al., *Nat. Struct. Mol. Biol.* 26:50, 2019; (b) PDB ID 5GPN, J. Gu et al., *Nature* 537:639, 2016.]

Other Pathways Donate Electrons to the Respiratory Chain via Ubiquinone

Several other electron-transfer reactions can reduce ubiquinone in the inner mitochondrial membrane (**Fig. 19-15**). In the first step of the β oxidation of fatty acyl–CoA, catalyzed by the flavoprotein **acyl-CoA dehydrogenase** (see Fig. 17-8), electrons pass from the substrate to the FAD of the dehydrogenase, then to electron-transferring flavoprotein (ETF). ETF passes its electrons to **ETF: ubiquinone oxidoreductase**, which reduces Q in the inner mitochondrial membrane to QH_2. Glycerol 3-phosphate, formed either from glycerol released by triacylglycerol breakdown or from the reduction of dihydroxyacetone phosphate from glycolysis, is oxidized by **glycerol 3-phosphate dehydrogenase** (see Fig. 17-4), a flavoprotein located on the outer face of the inner mitochondrial membrane. The electron acceptor in this reaction is Q; the QH_2 produced enters the pool of QH_2 in the membrane. The important role of glycerol 3-phosphate dehydrogenase in shuttling reducing equivalents from cytosolic NADH into the mitochondrial matrix is described in Section 19.2 (see Fig. 19-32). **Dihydroorotate dehydrogenase**, which acts in the synthesis of pyrimidines (see Fig. 22-38), is also on the outside of the inner mitochondrial membrane and donates electrons to Q in the respiratory chain. The reduced QH_2 passes its electrons through Complex III and ultimately to O_2.

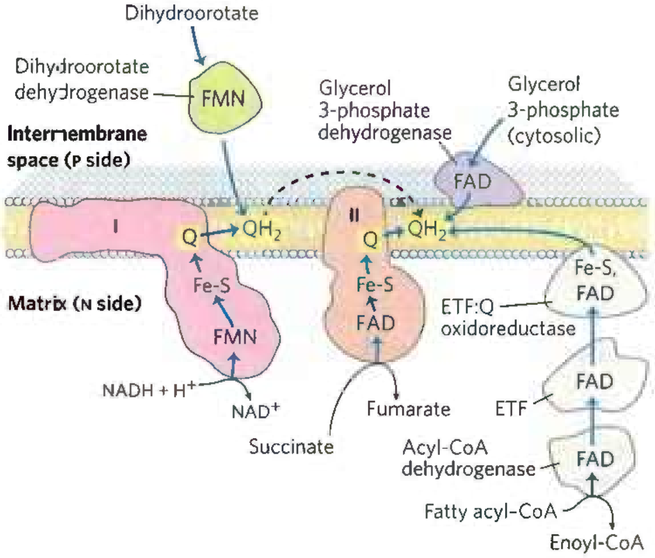

Dihydroorotate
Dihydroorotate dehydrogenase
FMN
Intermembrane space (P side)
Glycerol 3-phosphate dehydrogenase
Glycerol 3-phosphate (cytosolic)
FAD
I
Q
QH2
II
Q
QH2
Fe-S
Fe-S
FAD
Fe-S, FAD
Matrix (N side)
FMN
ETF:Q oxidoreductase
NADH + H+
NAD+
Fumarate
ETF
FAD
Succinate
Acyl-CoA dehydrogenase
FAD
Fatty acyl-CoA
Enoyl-CoA

FIGURE 19-15 Paths of electron transfer to ubiquinone in the respiratory chain. Electrons from NADH in the matrix pass through the FMN of a flavoprotein (NADH dehydrogenase) to a series of Fe-S centers (in Complex I) and then to Q. Electrons from succinate oxidation in the citric acid cycle pass through a flavoprotein with several Fe-S centers (Complex II) on the way to Q. Acyl-CoA dehydrogenase, the first enzyme of fatty acid β oxidation, transfers electrons to electron-transferring flavoprotein (ETF), from which they pass to Q via ETF:ubiquinone oxidoreductase. Dihydroorotate, an intermediate in the biosynthetic pathway to pyrimidine nucleotides, donates two electrons to Q through a flavoprotein (dihydroorotate dehydrogenase). And glycerol 3-phosphate, an intermediate of glycolysis in the cytosol, donates electrons to a flavoprotein (glycerol 3-phosphate dehydrogenase) on the outer face of the inner mitochondrial membrane, from which they pass to Q. QH_2 freely diffuses through the membrane (black dashed arrow), and can interact with several additional complexes.

The Energy of Electron Transfer Is Efficiently Conserved in a Proton Gradient

The transfer of two electrons from NADH through the respiratory chain to molecular oxygen can be summarized as

$$2\,\text{NADH} + 2\text{H}^+ + \text{O}_2 \longrightarrow 2\,\text{NAD}^+ + 2\text{H}_2\text{O} \quad (19\text{-}5)$$

This net reaction is highly exergonic. For the redox pair NAD^+/NADH, E'° is −0.320 V, and for the pair O_2/H_2O, E'° is 0.816 V. The $\Delta E'^\circ$ for this reaction is therefore 1.14 V, and the standard free-energy change (see Eqn 13-7, p. 492) is

$$\begin{aligned}\Delta G'^\circ &= -nF\,\Delta E'^\circ &(19\text{-}6)\\ &= -2(96.5\text{ kJ/V}\cdot\text{mol})(1.14\text{ V})\\ &= -220\text{ kJ/mol (of NADH)}\end{aligned}$$

This *standard* free-energy change is based on the assumption of equal concentrations (1 M) of NADH and NAD^+. In actively respiring mitochondria, the actions of many dehydrogenases keep the actual $[\text{NADH}]/[\text{NAD}^+]$ ratio well above unity, and the real free-energy change for the reaction shown in Equation 19-5 is therefore substantially greater (more negative) than −220 kJ/mol. A similar calculation for the oxidation of succinate shows that electron transfer from succinate (E'° for fumarate/succinate = 0.031 V) to O_2 has a smaller, but still negative, standard free-energy change of about −150 kJ/mol.

Much of this energy is used to pump protons out of the matrix. For each pair of electrons transferred to O_2, four protons are pumped out by Complex I, four by Complex III, and two by Complex IV (**Fig. 19-16**). The *vectorial* equation for the process is therefore

$$2\,\text{NADH} + 22\text{H}^+_\text{N} + \text{O}_2 \longrightarrow 2\,\text{NAD}^+ + 20\text{H}^+_\text{P} + 2\text{H}_2\text{O} \quad (19\text{-}7)$$

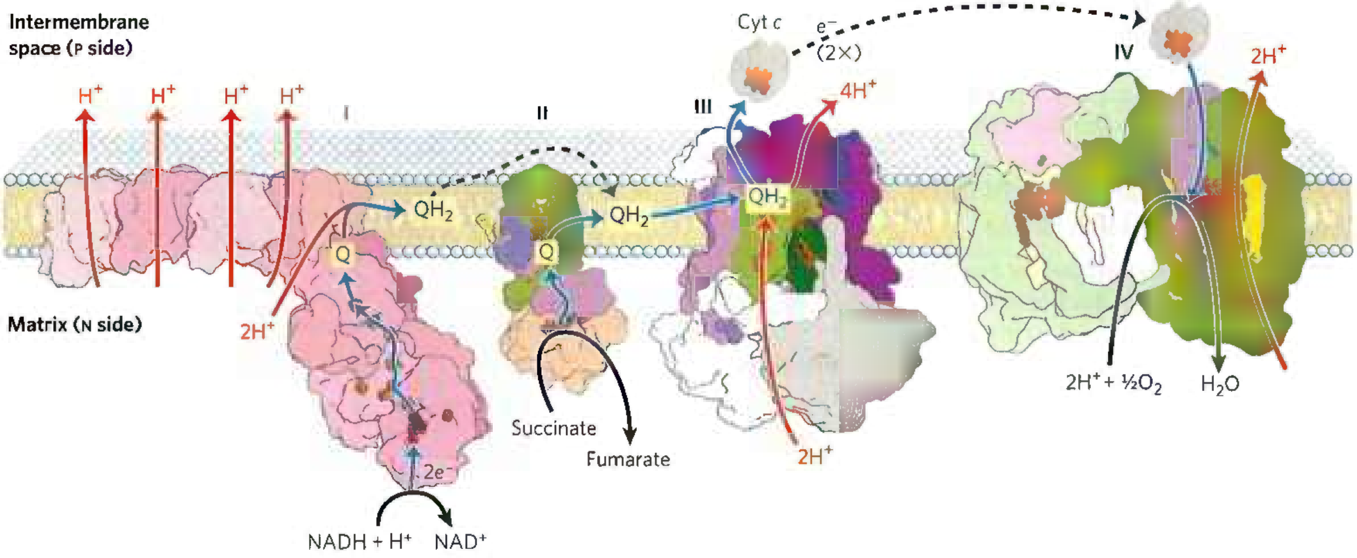
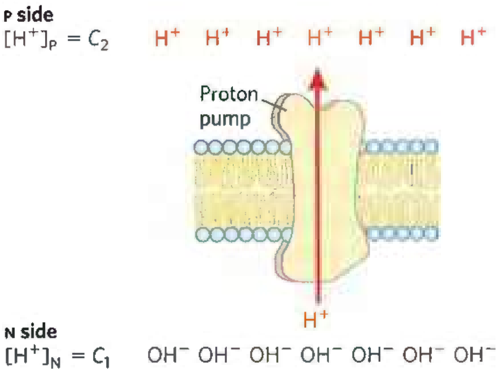

Intermembrane space (P side)
H+
H+
H+
H+
I
II
III
Cyt c
e−
(2×)
4H+
IV
2H+
QH2
QH2
QH2
Q
Q
Matrix (N side)
2H+
Succinate
Fumarate
2e−
NADH + H+
NAD+
2H+
2H+ + ½O2
H2O

FIGURE 19-16 Summary of the flow of electrons and protons through the four complexes of the respiratory chain. Electrons reach Q through Complexes I and II (as well as through several other paths shown in Fig. 19-15). Reduced Q (QH_2) serves as a mobile carrier of electrons and protons. It passes electrons to Complex III, which passes them to another mobile connecting link, cytochrome *c*. Complex IV then transfers electrons from reduced cytochrome *c* to O_2. Electron flow through Complexes I, III, and IV is accompanied by proton efflux from the matrix into the intermembrane space. In bovine heart, the approximate ratios of Complexes I:II:III:IV are 1.1:1.3:3.0:6.7. Broken lines indicate the diffusion of Q in the plane of the inner membrane, and of cytochrome *c* through the intermembrane space. [Data from Complex I: PDB ID 4HEA, R. Baradaran et al., *Nature* 494:443, 2013; Complex II: PDB ID 1ZOY, F. Sun et al., *Cell* 121:1043, 2005; Complex III: PDB ID 1BGY, S. Iwata et al., *Science* 281:64, 1998; cytochrome *c*: PDB ID 1HRC, G. W. Bushnell et al., *J. Mol. Biol.* 214:585, 1990; Complex IV: PDB ID 1OCC, T. Tsukihara et al., *Science* 272:1136, 1996.]


P side
$[H^+]_P = C_2$
H^+ H^+ H^+ H^+ H^+ H^+ H^+
Proton pump
H^+
N side
$[H^+]_N = C_1$
OH^- OH^- OH^- OH^- OH^- OH^- OH^-

$$\begin{aligned}\Delta G &= RT \ln (C_2/C_1) + ZF\Delta\psi \\ &= 2.3RT\,\Delta\text{pH} + F\Delta\psi\end{aligned}$$

FIGURE 19-17 Proton-motive force. The inner mitochondrial membrane separates two compartments of different $[H^+]$, resulting in differences in chemical concentration (ΔpH) and charge distribution ($\Delta\psi$) across the membrane. The net effect is the proton-motive force (ΔG), which can be calculated as shown here.

P4 The electrochemical energy inherent in this difference in proton concentration and separation of charge represents a temporary conservation of much of the energy of electron transfer. The energy stored in such a gradient, termed the **proton-motive force**, has two components: (1) the *chemical potential energy* due to the difference in concentration of a chemical species (H^+) in the two regions separated by the membrane, and (2) the *electrical potential energy* that results from the separation of charge when a proton moves across the membrane without a counterion (**Fig. 19-17**).

As we saw in Chapter 11, the free-energy change for the creation of an electrochemical gradient by an ion pump is

$$\Delta G = RT\ \ln(C_2/C_1) + ZF\,\Delta\psi \qquad (19\text{-}8)$$

where C_2 and C_1 are the concentrations of an ion in two regions, and $C_2 > C_1$; Z is the absolute value of its electrical charge (1 for a proton); and $\Delta\psi$ is the transmembrane difference in electrical potential, measured in volts.

For protons,

$$\begin{aligned}\ln\,(C_2/C_1) &= 2.3(\log[H^+]_P - \log[H^+]_N) \\ &= 2.3(\text{pH}_N - \text{pH}_P) = 2.3\ \Delta\text{pH}\end{aligned}$$

and Equation 19-8 reduces to

$$\Delta G = 2.3RT\ \Delta\text{pH} + F\Delta\psi \qquad (19\text{-}9)$$

In actively respiring mitochondria, the measured $\Delta\psi$ is 0.15 to 0.20 V, and the pH of the matrix is about 0.75 units more alkaline than that of the intermembrane space.

WORKED EXAMPLE 19-1 ***Energetics of Electron Transfer***

Calculate the amount of energy conserved in the proton gradient across the inner mitochondrial membrane per pair of electrons transferred through the respiratory chain from NADH to oxygen. Assume $\Delta\psi$ is 0.15 V and the pH difference is 0.75 unit at body temperature of 37 °C.

SOLUTION: Equation 19-9 gives the free-energy change when *one* mole of protons moves across the inner membrane. Substituting the values of the constants R and F, 310 K for T, and the measured values for ΔpH (0.75 unit) and $\Delta\psi$ (0.15 V) in this equation gives $\Delta G = 19$ kJ/mol (of protons). Because the transfer of two electrons from NADH to O_2 is accompanied by the outward pumping of 10 protons (Eqn 19-7), roughly 190 kJ (of the 220 kJ released by oxidation of 1 mol of NADH) is conserved in the proton gradient.

When protons flow spontaneously *down* their electrochemical gradient, energy is made available to do work. In mitochondria, chloroplasts, and aerobic bacteria, the electrochemical energy in the proton gradient drives the synthesis of ATP from ADP and P_i. We return to the energetics and stoichiometry of ATP synthesis driven by the electrochemical potential of the proton gradient in Section 19.2.

Reactive Oxygen Species Are Generated during Oxidative Phosphorylation

Several steps in the path of oxygen reduction in mitochondria have the potential to produce reactive oxygen species (superoxide, hydrogen peroxide, and hydroxyl radicals) that can damage cells. Some intermediates in the electron-transfer system, such as the partially reduced ubisemiquinone, can react directly with oxygen to form the superoxide radical ($^\bullet Q^-$) as an intermediate. The $^\bullet Q^-$ radical is formed when a single electron is passed to O_2 in the reaction

$$O_2 + e^- \longrightarrow {}^\bullet O_2^-$$

Successive reduction of the superoxide radical with additional electrons produces H_2O_2, hydroxyl radicals ($^\bullet$OH), and finally H_2O. The very reactive hydroxyl radical can be especially damaging (**Fig. 19-18**).

Reactive oxygen species (ROS) can wreak havoc, reacting with and damaging enzymes, membrane lipids, and nucleic acids. In actively respiring mitochondria, 0.2% to as much as 2% of the O_2 used in respiration forms $^\bullet O_2^-$—more than enough to have lethal effects unless the free radical is quickly disposed of. Factors that slow the flow of electrons through the respiratory chain increase the formation of superoxide, perhaps by prolonging the lifetime of $^\bullet O_2^-$ generated in the Q cycle. The formation of ROS is favored when two conditions are met: (1) mitochondria are not making ATP (for lack of ADP or O_2) and therefore have a large proton-motive force and a high QH_2/Q ratio, and (2) there is a high $NADH/NAD^+$ ratio in the matrix. In these situations, the mitochondrion is under oxidative stress—more electrons are available to enter the respiratory chain than can be immediately passed through to oxygen. When the supply of electron donors (NADH) is matched with that of electron acceptors, there is less oxidative stress, and ROS production is much reduced. Although overproduction of ROS is clearly detrimental, *low* levels of ROS are

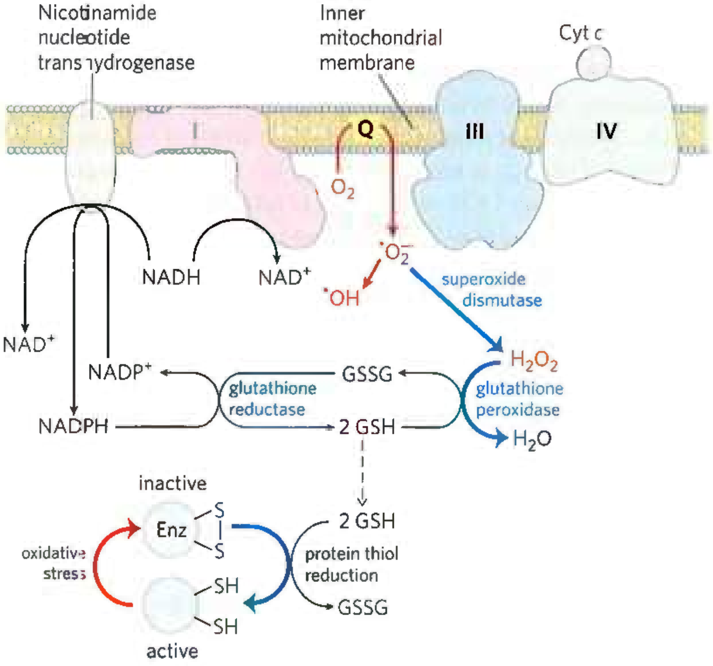


FIGURE 19-18 ROS formation in mitochondria and mitochondrial defenses. When the rate of electron entry into the respiratory chain and the rate of electron transfer through the chain are mismatched, superoxide radical ($^{\bullet}O_2^-$) production increases at Complexes I and III as the partially reduced ubiquinone radical ($^{\bullet}Q^-$) donates an electron to O_2. Superoxide acts on aconitase, a 4Fe-4S protein (not shown), to release Fe^{2+}. Fe^{2+} in turn will react with hydrogen peroxide (in a process called the Fenton reaction) to produce the highly reactive hydroxyl free radical ($^{\bullet}OH$). The reactions shown in blue defend the cell against the damaging effects of superoxide. Reduced glutathione (GSH; see Fig. 22-29) donates electrons for the reduction of H_2O_2 and of the oxidized Cys residues (—S—S—) of enzymes and other proteins; GSH is regenerated from the oxidized form (GSSG) by reduction with NADPH.

used by the cell as a signal reflecting the insufficient supply of oxygen (hypoxia), triggering metabolic adjustments (see Fig. 19-34).

To prevent oxidative damage by $^{\bullet}O_2^-$, cells have the enzyme **superoxide dismutase**, which catalyzes the reaction

$$2\,^{\bullet}O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

The hydrogen peroxide (H_2O_2) thus generated is rendered harmless by **glutathione peroxidase** (Fig. 19-18). Glutathione reductase recycles the oxidized glutathione to its reduced form, using electrons from the NADPH generated by nicotinamide nucleotide transhydrogenase (in the mitochondrion) or by the pentose phosphate pathway (in the cytosol; see Fig. 14-30). Reduced glutathione also serves to keep protein sulfhydryl groups in their reduced state, preventing some of the deleterious effects of oxidative stress.

SUMMARY 19.1 *The Mitochondrial Respiratory Chain*

- Chemiosmotic theory provides the intellectual framework for understanding many biological energy transductions, including oxidative phosphorylation and photophosphorylation. The energy of electron flow is conserved by the concomitant pumping of protons across the membrane, producing an electrochemical gradient, the proton-motive force.
- In mitochondria, hydride ions removed from substrates (such as α-ketoglutarate and malate) by NAD-linked dehydrogenases donate electrons to the respiratory chain, which transfers the electrons to molecular O_2, reducing it to H_2O.
- Reducing equivalents from NADH are passed through a series of carrier types that include ubiquinone, Fe-S centers, and cytochromes.
- Electron carriers are arranged in multiprotein complexes, which couple electron transfer to the generation of proton gradients. Complex I transfers electrons from NADH to ubiquinone. Complex II harvests electrons from the oxidation of succinate, also transferring them to ubiquinone. Ubiquinone diffuses through the inner mitochondrial membrane, and transfers the electrons to cytochrome *b*, the first carrier in Complex III. In this complex, electrons move first to an Fe-S center. The Fe-S center passes electrons, one at a time, through cytochrome *c* and into Complex IV, cytochrome oxidase. This copper-containing enzyme, which also contains cytochromes *a* and a_3, accumulates electrons, then passes them to O_2, reducing it to H_2O.
- Complexes I, III, and IV form supercomplexes that facilitate electron flow between them.
- Some electrons enter this chain of carriers through alternative paths. Electrons derived from the oxidation of fatty acids pass to ubiquinone via the electron-transferring flavoprotein. The oxidation of glycerol phosphate and of dihydroorotate also sends electrons into the respiratory chain at the level of QH_2.
- Much of the free energy generated by electron transfer and the reduction of oxygen to form water is recovered and stored in the form of an electrochemical proton gradient across the mitochondrial inner membrane.
- Potentially harmful reactive oxygen species produced in mitochondria are inactivated by a set of protective enzymes, including superoxide dismutase and glutathione peroxidase. Low levels of ROS serve as signals coordinating mitochondrial oxidative phosphorylation with other metabolic pathways.

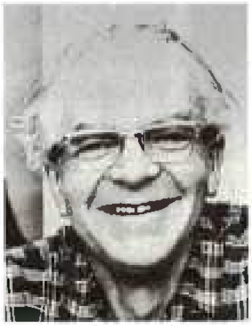
19.2 ATP Synthesis

How is a concentration gradient of protons transformed into ATP? We have seen that electron transfer releases, and the proton-motive force conserves, more than enough free energy (about 190 kJ) per "mole" of electron pairs to drive the formation of a mole of ATP, which requires about 50 kJ (p. 480). Mitochondrial oxidative phosphorylation therefore poses no thermodynamic problem. But what is the chemical mechanism that couples proton flux with phosphorylation?

In the Chemiosmotic Model, Oxidation and Phosphorylation Are Obligately Coupled

The **chemiosmotic model**, proposed by Peter Mitchell, is the paradigm for energy coupling. According to the model (**Fig. 19-19**), the electrochemical energy inherent in the difference in proton concentration and the separation of charge across the inner mitochondrial membrane—the proton-motive force—drives the synthesis of ATP as protons flow passively back into the matrix through a proton pore in **ATP synthase**. To emphasize this crucial role of the proton-motive force, the equation for ATP synthesis is sometimes written

$$\text{ADP} + \text{P}_i + n\text{H}^+_\text{P} \longrightarrow \text{ATP} + \text{H}_2\text{O} + n\text{H}^+_\text{N} \qquad (19\text{-}10)$$

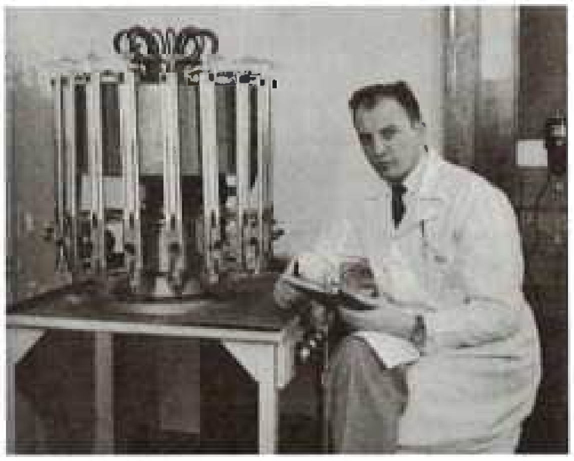


Peter Mitchell, 1920–1992 [AP Photos/AP Images]

Mitchell used the term "chemiosmotic" to describe enzymatic reactions that involve, simultaneously, a chemical reaction and a transport process, and the overall process is sometimes referred to as "chemiosmotic coupling." Here, "coupling" refers to the *obligate* connection between mitochondrial ATP synthesis and electron flow through the respiratory chain; neither of the two processes can proceed without the other. The operational definition of *coupling* is shown in **Figure 19-20**.

P5 When isolated mitochondria are suspended in a buffer containing ADP, P_i, and an oxidizable substrate such as succinate, three easily measured processes occur: (1) the substrate is oxidized (succinate yields fumarate), (2) O_2 is consumed, and (3) ATP is synthesized. Oxygen consumption and ATP synthesis depend on the presence of an oxidizable substrate (succinate in this case) as well as ADP and P_i.

Because substrate oxidation drives ATP synthesis, inhibitors of electron transfer block ATP synthesis (Fig. 19-20a). The converse is also true: inhibition of ATP synthesis blocks electron transfer in intact mitochondria. When isolated mitochondria are given O_2 and oxidizable substrates, but not ADP (Fig. 19-20b), no ATP synthesis can occur and electron transfer to O_2 does not proceed. Henry Lardy, who pioneered the use



Henry Lardy, 1917–2010 [© Courtesy David L. Nelson, Ph.D.]

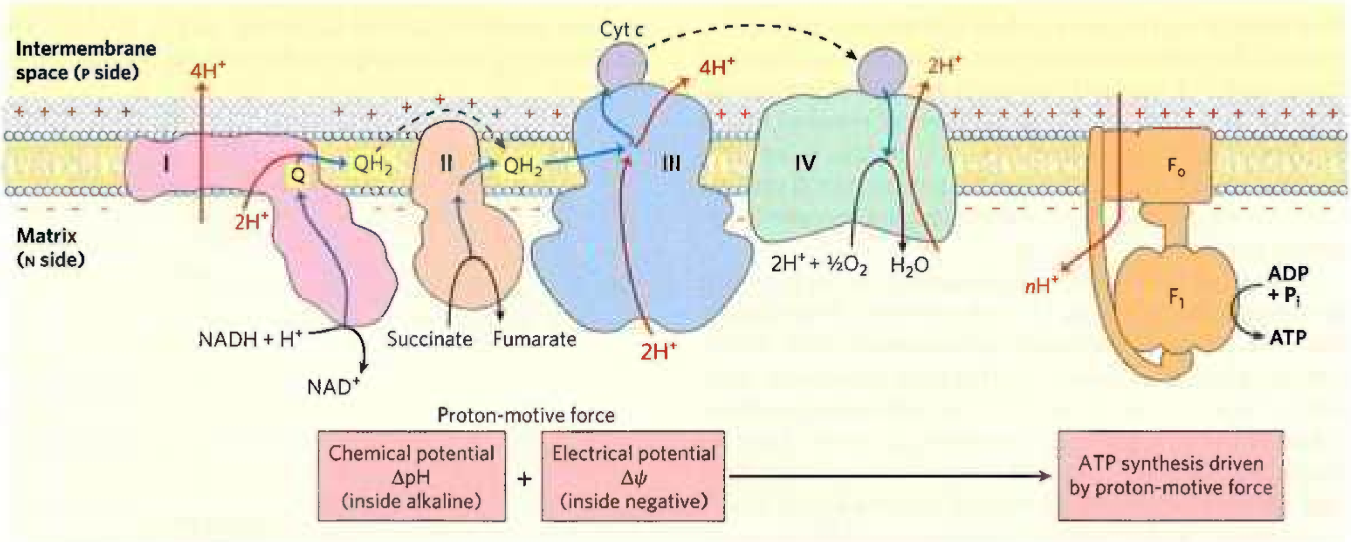

Intermembrane space (P side)
4H⁺
Cyt c
4H⁺
2H⁺
I
Q
QH₂
II
QH₂
III
IV
Fo
Matrix (N side)
2H⁺
2H⁺ + ½O₂
H₂O
nH⁺
F₁
ADP + Pi
ATP
NADH + H⁺
Succinate
Fumarate
2H⁺
NAD⁺
Proton-motive force
Chemical potential ΔpH (inside alkaline)
+
Electrical potential Δψ (inside negative)
ATP synthesis driven by proton-motive force

FIGURE 19-19 Chemiosmotic model. In this simple representation of the chemiosmotic theory applied to mitochondria, electrons from NADH and other oxidizable substrates pass through a chain of carriers arranged asymmetrically in the inner membrane. Electron flow is accompanied by proton transfer across the membrane, producing both a chemical gradient (ΔpH) and an electrical gradient (Δψ), which, combined, create the proton-motive force. The inner mitochondrial membrane is impermeable to protons; protons can reenter the matrix only through proton-specific channels (F_o). The proton-motive force that drives protons back into the matrix provides the energy for ATP synthesis, catalyzed by the F_1 complex associated with F_o.

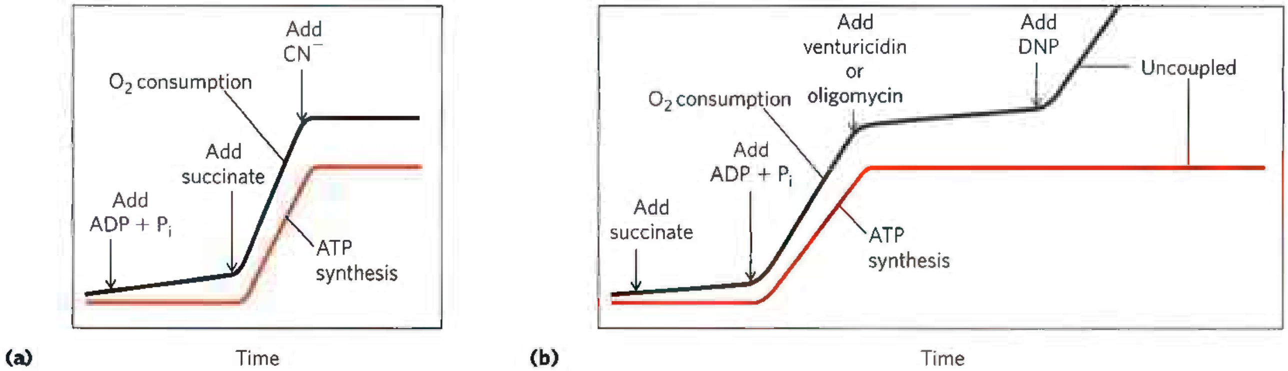


FIGURE 19-20 Coupling of electron transfer and ATP synthesis in mitochondria. In experiments to demonstrate coupling, mitochondria are suspended in a buffered medium, and an O_2 electrode monitors O_2 consumption. At intervals, samples are removed and assayed for the presence of ATP. (a) Addition of ADP and P_i alone results in little or no increase in either respiration (O_2 consumption; black) or ATP synthesis (red). When succinate is added, respiration begins immediately, and ATP is synthesized. Addition of cyanide (CN^-), which blocks electron transfer between cytochrome oxidase (Complex IV) and O_2, inhibits both respiration and ATP synthesis. (b) Mitochondria provided with succinate respire and synthesize ATP only when ADP and P_i are added. Subsequent addition of venturicidin or oligomycin, inhibitors of ATP synthase, blocks both ATP synthesis and respiration. Dinitrophenol (DNP) is an uncoupler, allowing respiration to continue without ATP synthesis.

of antibiotics to explore mitochondrial function, showed coupling of oxidation and phosphorylation by using oligomycin and venturicidin. These toxic antibiotics bind to the ATP synthase in mitochondria, inhibiting both ATP synthesis *and* the transfer of electrons through the chain of carriers to O_2 (Fig. 19-20b). As oligomycin does not interact with the electron carriers, it follows that electron transfer and ATP synthesis are obligately coupled: neither reaction occurs without the other.

Chemiosmotic theory readily explains the dependence of electron transfer on ATP synthesis in mitochondria. When the flow of protons into the matrix through the proton channel of ATP synthase is blocked (with oligomycin, for example), no path exists for the return of protons to the matrix, and the continued extrusion of protons driven by the activity of the respiratory chain generates a large proton gradient. The proton-motive force builds up until the cost (free energy) of pumping protons out of the matrix against this gradient equals or exceeds the energy released by the transfer of electrons from NADH to O_2. At this point electron flow must stop; the free energy for the overall process of electron flow coupled to proton pumping becomes zero, and the system is at equilibrium.

Certain conditions and reagents, however, can uncouple oxidation from phosphorylation. When intact mitochondria are disrupted by treatment with detergent or by physical shear, the resulting membrane fragments can still catalyze electron transfer from succinate or NADH to O_2, but no ATP synthesis is coupled to this respiration. Certain chemical compounds cause uncoupling without physically disrupting mitochondrial structure. Chemical uncouplers include 2,4-dinitrophenol (DNP) and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) (Table 19-4; **Fig. 19-21**), weak acids with hydrophobic properties that permit them to diffuse readily across mitochondrial membranes. After entering the matrix in the protonated form, they can release a proton, thus dissipating the proton gradient. Resonance stabilization delocalizes the charge on the anionic forms, making them sufficiently hydrophobic to diffuse back across the membrane, where they can pick up a proton and repeat the process. Ionophores such as valinomycin (see Fig. 11-43) allow inorganic ions to pass easily through membranes. Ionophores uncouple electron transfer from oxidative phosphorylation by dissipating the electrical contribution to the electrochemical gradient across the mitochondrial membrane.

A prediction of the chemiosmotic theory is that, because the role of electron transfer in mitochondrial ATP synthesis is simply to pump protons to create the electrochemical potential of the proton-motive force, an artificially created proton gradient should be able to replace electron transfer in driving ATP synthesis. This has been experimentally confirmed (**Fig. 19-22**). *In the*

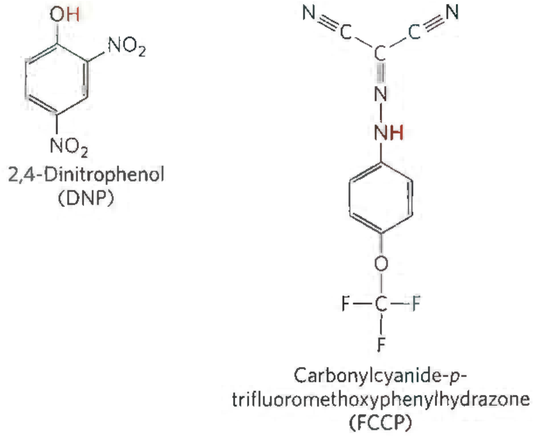


FIGURE 19-21 Two chemical uncouplers of oxidative phosphorylation. Both DNP and FCCP have a dissociable proton (red) and are very hydrophobic. They carry protons across the inner mitochondrial membrane, dissipating the proton gradient. Both also uncouple photophosphorylation.

absence of an oxidizable substrate, the proton-motive force alone suffices to drive ATP synthesis.

ATP Synthase Has Two Functional Domains, F_o and F_1

P2 Mitochondrial ATP synthase is an F-type ATPase (see Fig. 11-40b) similar in structure and mechanism to the ATP synthases of bacteria and (as we will see in Chapter 20) chloroplasts. This large enzyme complex of the inner mitochondrial membrane catalyzes the formation of ATP from ADP and P_i, driven by the flow of protons from the P to the N side of the membrane (Eqn 19-10). ATP synthase, also called Complex V to relate it to the electron-transfer complexes described in the last section, has two distinct components. These are F_1, a peripheral membrane protein, and F_o (*o* denoting oligomycin-sensitive), which is integral to the membrane. F_1, the first factor recognized as essential for oxidative phosphorylation, was identified and purified by Efraim Racker and his colleagues in the early 1960s.

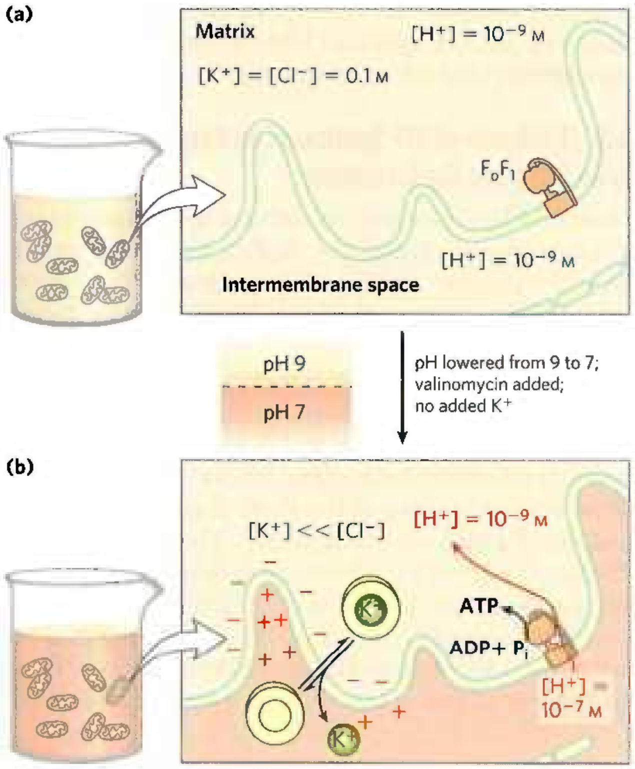


FIGURE 19-22 Evidence for the role of a proton gradient in ATP synthesis. An artificially imposed electrochemical gradient can drive ATP synthesis in the absence of an oxidizable substrate as electron donor. In this two-step experiment, (a) isolated mitochondria are first incubated in a pH 9 buffer containing 0.1 M KCl. Slow leakage of buffer and KCl into the mitochondria eventually brings the matrix into equilibrium with the surrounding medium. No oxidizable substrates are present. (b) Mitochondria are now removed from the pH 9 buffer and resuspended in pH 7 buffer containing valinomycin but no KCl. The change in buffer creates a difference of two pH units across the inner mitochondrial membrane. The outward flow of K^+, carried by valinomycin down the K^+ ion concentration gradient without a counterion, creates a charge imbalance across the membrane (matrix negative). The sum of the chemical potential provided by the pH difference and the electrical potential provided by the separation of charges is a proton-motive force large enough to support ATP synthesis in the absence of an oxidizable substrate.

In the laboratory, small membrane vesicles formed from inner mitochondrial membranes carry out ATP synthesis coupled to electron transfer. When F_1 is gently extracted, the "stripped" vesicles still contain intact respiratory chains and the F_o portion of ATP synthase. **P5** The vesicles can catalyze electron transfer from NADH to O_2 but cannot produce a proton gradient: F_o has a proton pore through which protons leak as fast as they are pumped by electron transfer, and without a proton gradient the F_1-depleted vesicles cannot make ATP. Isolated F_1 catalyzes ATP hydrolysis (the reversal of synthesis) and was therefore originally called **F_1 ATPase**. When purified F_1 is added back to the depleted vesicles, it reassociates with F_o, plugging its proton pore and restoring the membrane's capacity to couple electron transfer and ATP synthesis.

ATP Is Stabilized Relative to ADP on the Surface of F_1

Isotope exchange experiments using purified F_1 reveal an extraordinary fact about the enzyme's catalytic mechanism: on the enzyme surface, the reaction $ADP + P_i \rightleftharpoons ATP + H_2O$ is readily reversible—the free-energy change for ATP synthesis is close to zero. When ATP is hydrolyzed by F_1 in the presence of ^{18}O-labeled water, the P_i released contains an ^{18}O atom. Careful measurement of the ^{18}O content of P_i formed in vitro by F_1-catalyzed hydrolysis of ATP reveals that the P_i has not one but three or four ^{18}O atoms (**Fig. 19-23**). This indicates that the terminal pyrophosphate bond in ATP is cleaved and re-formed repeatedly before P_i leaves the enzyme surface. This exchange reaction occurs in unenergized F_oF_1 complexes (with no proton gradient) and with isolated F_1—the exchange does not require the input of energy.

Kinetic studies of the initial rates of ATP synthesis and hydrolysis confirm the conclusion that $\Delta G'^{\circ}$ for ATP synthesis on the enzyme is near zero. From the measured rates of hydrolysis ($k_1 = 10\ s^{-1}$) and synthesis ($k_{-1} = 24\ s^{-1}$), the calculated equilibrium constant for the reaction

$$\text{Enz-ATP} \rightleftharpoons \text{Enz-(ADP} + P_i)$$

is

$$K'_{eq} = \frac{k_{-1}}{k_1} = \frac{24\ s^{-1}}{10\ s^{-1}} = 2.4$$

From this K'_{eq}, the calculated apparent $\Delta G'^{\circ}$ is close to zero. This is much different from the K'_{eq} of about 10^5 ($\Delta G'^{\circ} = -30.5$ kJ/mol) for the hydrolysis of ATP free in solution (i.e., not on the enzyme surface).

What accounts for the huge difference? ATP synthase stabilizes ATP relative to ADP + P_i by binding ATP more tightly, releasing enough energy to counterbalance the cost of making ATP. Careful measurements of the binding constants show that F_oF_1 binds ATP with very high affinity ($K_d \leq 10^{-12}$ M) and ADP with much lower affinity ($K_\text{d} \approx 10^{-5}$ M). The difference in K_d corresponds to a difference of about 40 kJ/mol in binding energy, and this binding energy drives the equilibrium toward formation of the product ATP.

The Proton Gradient Drives the Release of ATP from the Enzyme Surface

Although ATP synthase equilibrates ATP with ADP + P_i, in the absence of a proton gradient the newly synthesized ATP does not leave the surface of the enzyme. Effectively, the enzyme cannot turn over and synthesize a second molecule of ATP. It is the proton gradient that causes the enzyme to release the ATP formed on its surface. The reaction coordinate diagram of the process (**Fig. 19-24**) illustrates the difference between the mechanism of ATP synthase and that of many other enzymes that catalyze endergonic reactions.

For the continued synthesis of ATP, the enzyme must cycle between a form that binds ATP very tightly and a form that releases ATP. Chemical and crystallographic studies of the ATP synthase have revealed the structural basis for this alternation in function.

Each β Subunit of ATP Synthase Can Assume Three Different Conformations

Mitochondrial F_1 has nine subunits of five different types, with the composition $\alpha_3\beta_3\gamma\delta\varepsilon$. Each of the three β subunits has one catalytic site for ATP synthesis. The crystallographic determination of the F_1 structure by John E. Walker and colleagues revealed structural details that help explain the catalytic mechanism of the enzyme. The knoblike portion of F_1 is a flattened sphere, 8 nm by 10 nm, consisting of alternating α and β subunits arranged like the sections of an orange (**Fig. 19-25a–d**). Although the amino acid sequences of the three β subunits are identical, *their conformations differ*. The conformational differences extend to differences in their ATP/ADP-binding sites. When the protein is crystallized in the presence of ADP and App(NH)p, a close structural analog of ATP that cannot be hydrolyzed by the ATPase activity of F_1, the binding site of one of the three β subunits is filled with

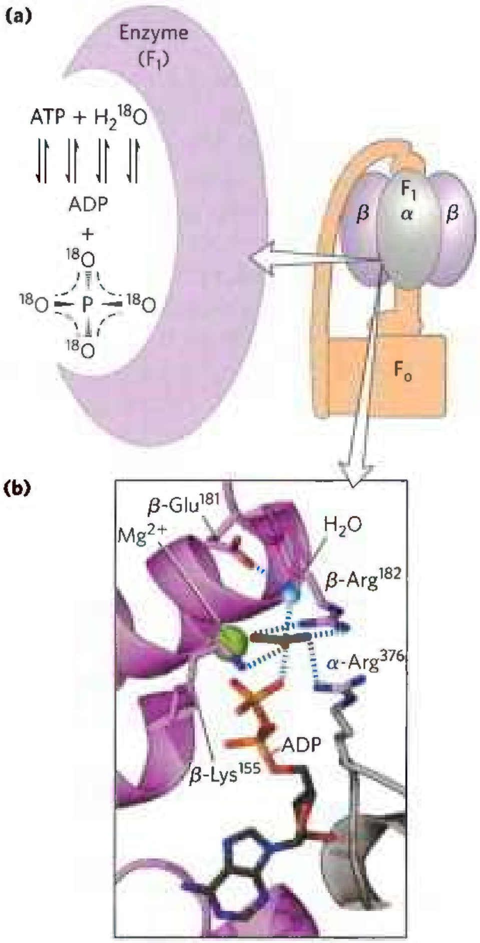


FIGURE 19-23 Catalytic mechanism of F_1. (a) An ^{18}O-exchange experiment. F_1 solubilized from mitochondrial membranes is incubated with ATP in the presence of ^{18}O-labeled water. At intervals, a sample of the solution is withdrawn and analyzed for the incorporation of ^{18}O into the P_i produced from ATP hydrolysis. In minutes, the P_i contains three or four ^{18}O atoms, indicating that both ATP hydrolysis and ATP synthesis have occurred several times during the incubation. (b) The likely transition state complex for ATP hydrolysis and synthesis by ATP synthase. The α subunit is shown in gray, β in purple. The positively charged residues β-Arg^{182} and α-Arg^{376} coordinate two oxygens of the pentavalent phosphate intermediate; β-Lys^{155} interacts with a third oxygen, and the Mg^{2+} ion further stabilizes the intermediate. The blue sphere represents the leaving group (H_2O). These interactions result in ready equilibration of ATP and ADP + P_i in the active site. [(b) Data from PDB ID 1BMF, J. P. Abrahams et al., *Nature* 370:621, 1994.]

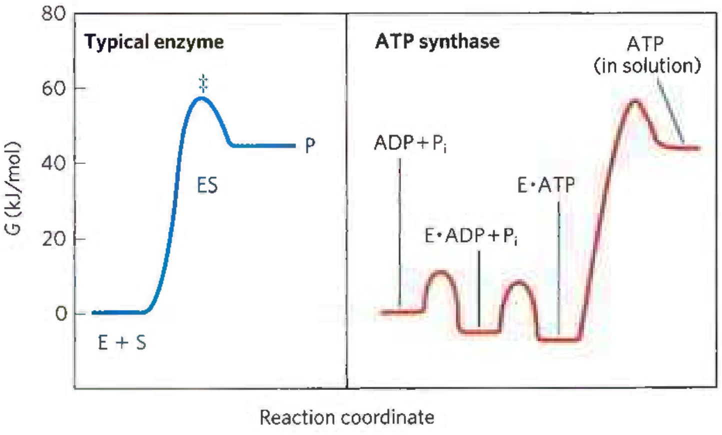
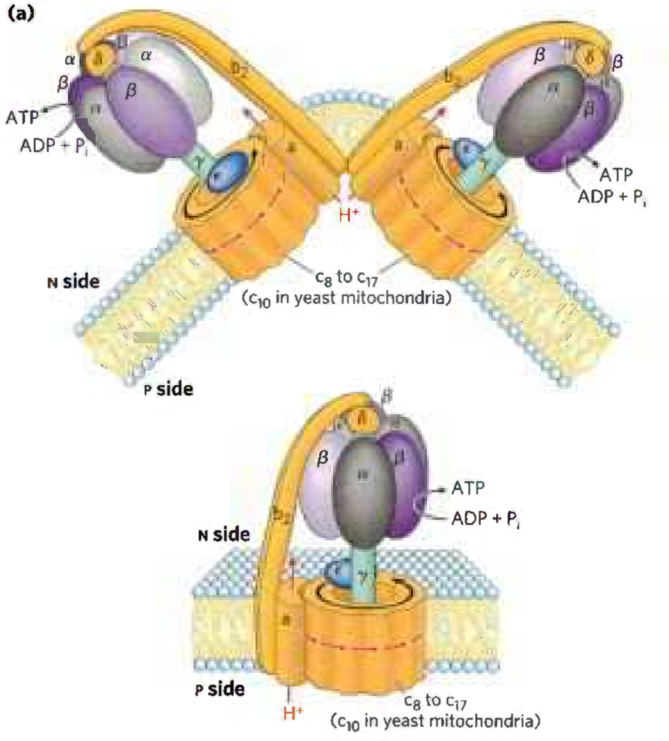
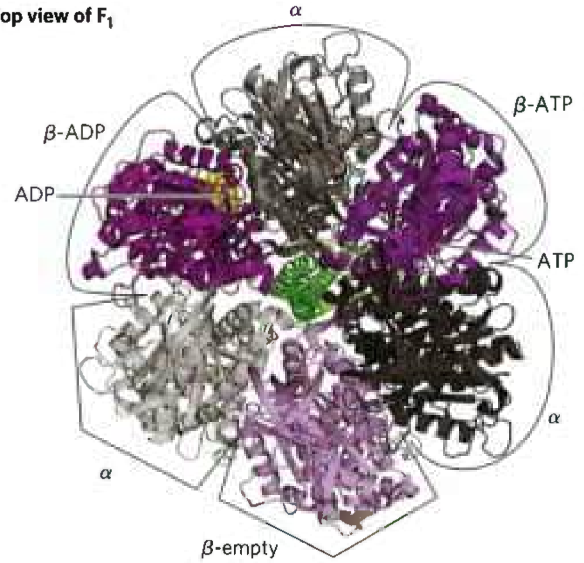


FIGURE 19-24 Reaction coordinate diagrams for ATP synthase and for a more typical enzyme. In a typical enzyme-catalyzed reaction (left), reaching the transition state (‡) between substrate and product is the major energy barrier to overcome. In the reaction catalyzed by ATP synthase (right), release of ATP from the enzyme, not formation of ATP, is the major energy barrier. The free-energy change for the formation of ATP from ADP and P_i in aqueous solution is large and positive, but on the enzyme surface, the very tight binding of ATP provides sufficient binding energy to bring the free energy of the enzyme-bound ATP close to that of ADP + P_i, so the reaction is readily reversible. The equilibrium constant is near 1. The free energy required for the release of ATP is provided by the proton-motive force.

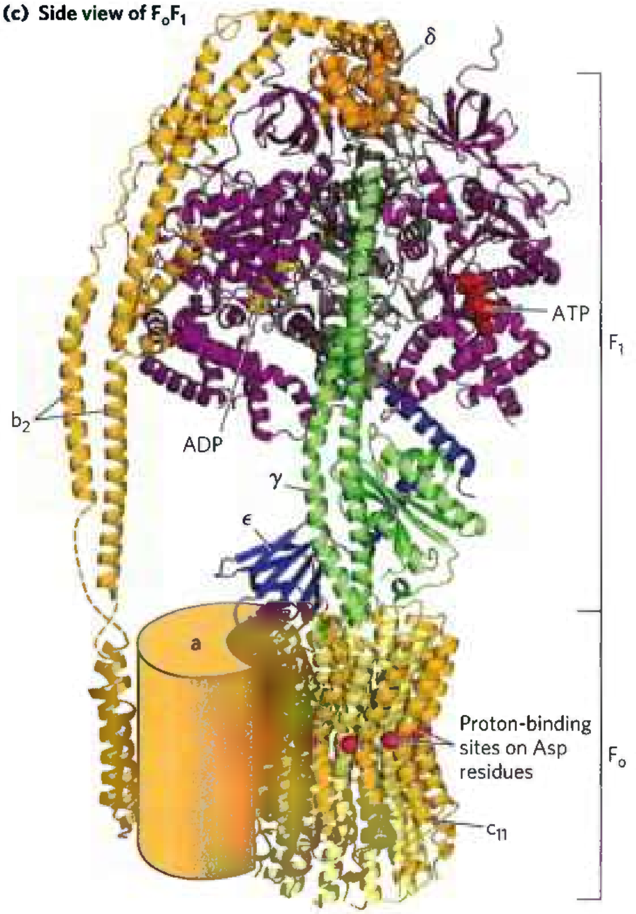
FIGURE 19-25 Mitochondrial ATP synthase complex. (a) A cartoon representation of the F_oF_1 complex. The dimeric form is found in eukaryotic mitochondria. The monomeric form is observed in bacteria. (b) F_1 viewed from above (that is, from the N side of the membrane), showing the three β (shades of purple) and three α (shades of gray) subunits and the central shaft (γ subunit, green). Each β subunit, near its interface with the neighboring α subunit, has a nucleotide-binding site critical to the catalytic activity. The single γ subunit associates primarily with one of the three $\alpha\beta$ pairs, forcing each of the three β subunits into slightly different conformations, with different nucleotide-binding sites. In the crystalline enzyme, one subunit, β-ADP, has ADP (yellow) in its binding site; the next, β-ATP, has ATP (red); and the third, β-empty, has no bound nucleotide. (c) The entire enzyme viewed from the side (in the plane of the membrane). The F_1 portion has three α subunits and three β subunits arranged like the segments of an orange around a central shaft, the γ subunit (green). (Two α subunits and one β subunit have been omitted to reveal the γ subunit and the binding sites for ATP and ADP on the β subunits.) The δ subunit confers oligomycin sensitivity on the ATP synthase, and the ε subunit may serve to inhibit the enzyme's ATPase activity under some circumstances. The F_o subunit consists of one a subunit and two b subunits, which anchor the F_oF_1 complex in the membrane and act as a stator (the stationary part of a rotary system), holding the α and β subunits in place. F_o also includes the c ring, made up of a number (8 to 17, depending on the species) of identical c subunits, small, hydrophobic proteins. The c ring and the a subunit interact to provide a transmembrane path for protons. Each of the c subunits in F_o has a critical Asp residue near the middle of the membrane, which undergoes protonation/deprotonation during the catalytic cycle of the ATP synthase. Shown here is the homologous c_{11} ring of the Na^+-ATPase of *Ilyobacter tartaricus*, for which the structure is well established. The Na^+-binding sites, which correspond to the proton-binding sites of the F_oF_1 complex, are shown with their bound Na^+ ions (red spheres). (d) A view of F_o perpendicular to the membrane. As in (c), red spheres represent the Na^+- or proton-binding sites in Asp residues. [(a) Information from W. Kühlbrandt and K. M. Davies, *Trends Biochem. Sci.* 41:106, 2016. (b, c, d) Data from F_1: PDB ID 1BMF, J. P. Abrahams et al., *Nature* 370:621, 1994; PDB ID 1JNV, A. C. Hausrath et al., *J. Biol. Chem.* 276:47,227, 2001; PDB ID 2A7U, S. Wilkens et al., *Biochemistry* 44:11,786, 2005; PDB ID 2CLY, V. Kane Dickson et al., *EMBO J.* 25:2911, 2006; F_o: PDB ID 1B9U, O. Dmitriev et al., *J. Biol. Chem.* 274:15,598, 1999; c ring: PDB ID 1YCE, T. Meier et al., *Science* 308:659, 2005.]



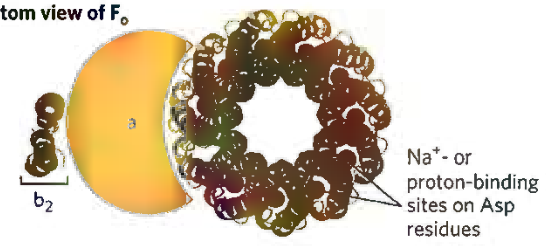
(b) Top view of F_1



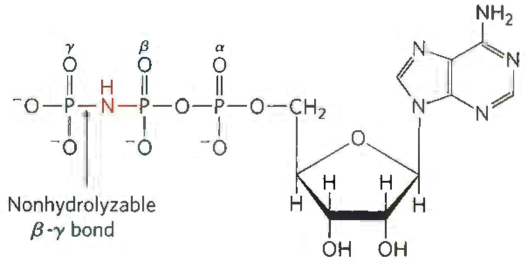
(c) Side view of F_oF_1



(d) Bottom view of F_o



App(NH)p, the second is filled with ADP, and the third is empty. The corresponding β subunit conformations are designated β-ATP, β-ADP, and β-empty (Fig. 19-25b). This difference in nucleotide binding among the three subunits is critical to the mechanism of the complex. The polypeptides that make up the stalk in the F_1 crystal structure are asymmetrically arranged. One domain of the single γ subunit makes up a central shaft that passes through F_1. Another globular domain of γ helps to stabilize the β-empty conformation in a β subunit it is transiently associated with (Fig. 19-25c).



App(NH)p (β,γ-imidoadenosine 5′-triphosphate)

The F_o complex, with its proton pore, is composed of three subunits, a, b, and c, in the proportion ab_2c_n, where n ranges from 8 to 17, depending on the species. Subunit c is a small (M_r 8,000), very hydrophobic polypeptide, consisting almost entirely of two transmembrane helices, with a small loop extending from the matrix side of the membrane. The crystal structure of the yeast F_oF_1 shows 10 c subunits, each with two transmembrane helices roughly perpendicular to the plane of the membrane and arranged in two concentric circles to create the **c ring**. The inner circle is made up of the amino-terminal helices of each c subunit; the outer circle, about 55 Å in diameter, is made up of the carboxyl-terminal helices. *The c subunits in the c ring rotate together as a unit around an axis perpendicular to the membrane.* The ε and γ subunits of F_1 form a leg-and-foot that projects from the bottom (membrane) side of F_1 and stands firmly on the ring of c subunits. The a subunit consists of several hydrophobic helices that span the membrane in close association with one of the c subunits in the c ring.

Rotational Catalysis Is Key to the Binding-Change Mechanism for ATP Synthesis

On the basis of detailed kinetic and binding studies of the reactions catalyzed by F_oF_1, Paul Boyer proposed a **rotational catalysis** mechanism in which the three active sites of F_1 take turns catalyzing ATP synthesis (**Fig. 19-26**). A given β subunit starts in the β-ADP conformation, which binds ADP and P_i from the surrounding medium. The subunit now changes conformation, assuming the β-ATP form that tightly binds and stabilizes ATP, bringing about the ready equilibration of ADP + P_i with ATP on the enzyme surface. Finally, the subunit changes to the β-empty conformation, which has very low affinity for ATP, and the newly synthesized ATP leaves the enzyme surface. Another round of catalysis begins when this subunit again assumes the β-ADP form and binds ADP and P_i.

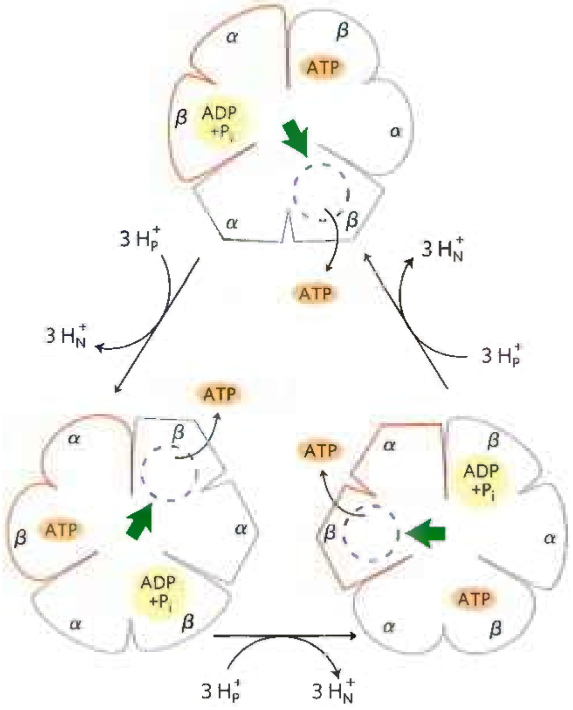


FIGURE 19-26 Binding-change model for ATP synthase. The F_1 complex has three nonequivalent adenine nucleotide–binding sites, one for each pair of α and β subunits. At any given moment, one of these sites is in the β-ATP conformation (which binds ATP tightly), a second is in the β-ADP (loose-binding) conformation, and a third is in the β-empty (very-loose-binding) conformation. In this view from the N side, the proton-motive force causes rotation of the central shaft — the γ subunit, shown as a green arrowhead — which comes into contact with each $\alpha\beta$ subunit pair in succession. This produces a cooperative conformational change in which the β-ATP site is converted to the β-empty conformation, and ATP dissociates; the β-ADP site is converted to the β-ATP conformation, which promotes condensation of bound ADP + P_i to form ATP; and the β-empty site becomes a β-ADP site, which loosely binds ADP + P_i entering from the solvent. Note that the direction of rotation reverses when the ATP synthase is acting as an ATPase, as in the experiment depicted in Figure 19-27.

P5 The conformational changes central to this mechanism are driven by the passage of protons through the F_o portion of ATP synthase. The streaming of protons through the F_o pore causes the c ring and the attached γ subunit to rotate about the long axis of γ, which is perpendicular to the plane of the membrane. The γ subunit passes through the center of the $\alpha_3\beta_3$ spheroid, which is held stationary relative to the membrane surface by the b_2 and δ subunits (Fig. 19-25a). With each rotation of 120°, γ comes into contact with a different β subunit, and the contact forces that β subunit into the β-empty conformation.

The three β subunits interact in such a way that when one assumes the β-empty conformation, its neighbor to one side *must* assume the β-ADP form, and the other neighbor the β-ATP form. Thus, one complete rotation of the γ subunit causes each β subunit to cycle through all three of its possible conformations, and for each rotation, three ATP are synthesized and released from the enzyme surface.

One strong prediction of this **binding-change model** is that the γ subunit should rotate in one direction when F_oF_1 is synthesizing ATP and in the opposite direction when the enzyme is hydrolyzing ATP. This prediction of rotation with ATP hydrolysis was confirmed in elegant experiments in the laboratories of Masasuke Yoshida and Kazuhiko Kinosita, Jr. The rotation of γ in a single F_1 molecule was observed microscopically by attaching a long, thin, fluorescent actin polymer to γ and watching it move relative to $\alpha_3\beta_3$ immobilized on a microscope slide as ATP was hydrolyzed. (The expected reversal of the rotation when ATP is being synthesized could not be tested in this experiment; there is no proton gradient to drive ATP synthesis.) When the entire F_oF_1 complex (not just F_1) was used in a similar experiment, the entire ring of c subunits rotated with γ (**Fig. 19-27**). The "shaft" rotated in the predicted direction through 360°. The rotation was not smooth but occurred in three discrete steps of 120°. As calculated from the known rate of ATP hydrolysis by one F_1 molecule and from the frictional drag on the long actin polymer, the efficiency of this mechanism in converting chemical energy into motion is close to 100%. It is, in Boyer's words, "a splendid molecular machine!"

P5 A model that illustrates how proton flow and rotary motion are coupled in the F_o complex is shown in **Figure 19-28**. The a subunit is stationary, while the c ring

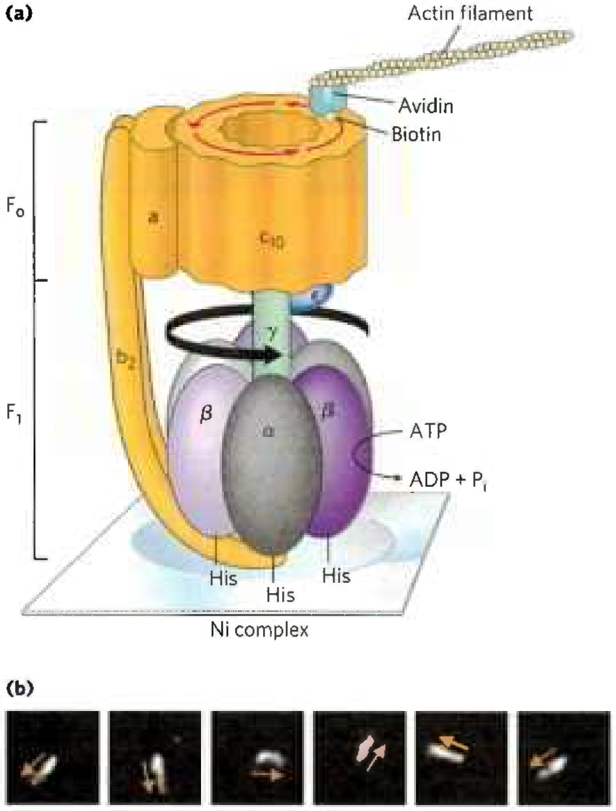


FIGURE 19-27 Experimental demonstration of rotation of F_o and γ. This fundamental property of the ATP synthase reaction was demonstrated in several creative ways. (a) In one experiment, illustrated in this cartoon, F_1, genetically engineered to contain a run of His residues, was tightly adhered to a microscope slide coated with a Ni complex; biotin was covalently attached to a c subunit of F_o. The protein avidin, which binds biotin very tightly, was covalently attached to long filaments of actin labeled with a fluorescent probe. Biotin-avidin binding then attached the actin filaments to the c subunit. When ATP was provided as substrate for the ATPase activity of F_1, the labeled filament rotated in one direction, proving that the F_o cylinder of c subunits rotates. (b) In another experiment, a fluorescent actin filament was attached directly to the γ subunit. The series of fluorescence micrographs (read left to right) shows the position of the actin filament at intervals of 133 ms. Note that as the filament rotated, it made discrete jumps rather than smooth rotation about the circle. The cylinder and shaft move as one unit. [(a) Information from Y. Sambongi et al., *Science* 286:1722, 1999. (b) Courtesy Hiroyuki Noji.]

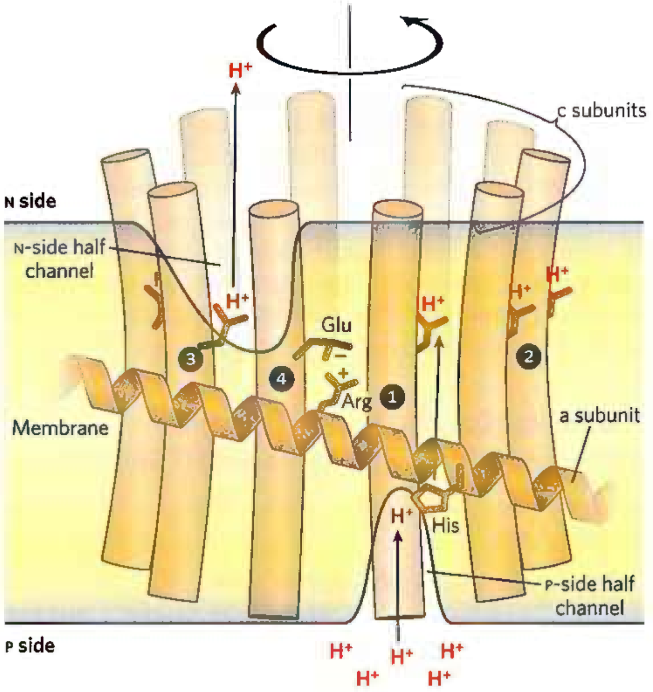


FIGURE 19-28 A model for proton-driven rotation of the c ring. The a subunit of the F_o complex of the ATP synthase (see Fig. 19-25a) has two hydrophilic half-channels for protons, one leading from the P side to the middle of the membrane, the other leading from the middle of the membrane to the N side (matrix). The function of the stationary a subunit is to conduct protons to and from the c ring subunits to drive c ring rotational motion. Individual c subunits in F_o (the total number varies from 8 to 17 in different species) are arranged in a circle about a central core. Each c subunit has a critical Glu residue (an Asp in some species) about midway across the membrane, with a perturbed pK_a allowing it to donate or accept a proton (red H^+) at pH near neutrality. The cycle that c subunits go through is illustrated. One c subunit ① is initially positioned so that a proton that enters the half-channel on the P side (where the proton concentration is relatively high) encounters and protonates a conserved His residue in the a subunit, transferring it to the c subunit Glu residue. This triggers a rotation-facilitating conformational change in the protonated c subunit as the Glu loses its negative charge. The now neutral Glu residue is sequestered in the hydrophobic membrane layer as it rotates as part of the c ring ②. As the c ring rotates, the c subunit we are following eventually makes contact with the channel to the N side of the membrane, where the environment is relatively alkaline, and the proton is released ③. As the Glu reacquires its negative charge, another rotation-facilitating conformational change occurs such that the Glu interacts transiently with a conserved Arg residue in the immobile a subunit ④. The interaction with the Arg is disrupted as the Glu is again protonated by the His residue, in motions that again facilitate rotation. The c subunits positioned near the half channels are providing the rotational driving force at any given moment, as they are the ones undergoing conformation changes associated with protonation and deprotonation. The orientation of the proton gradient dictates the direction of proton flow and makes rotation of the c ring essentially unidirectional. [Information from W. Kühlbrandt and K. M. Davies, *Trends Biochem. Sci.* 41:106, 2016.]

rotates. Critical interactions occur between conserved amino acids in the a and c subunits. The individual subunits in the c ring are arranged in a circle with only a few in contact with the a subunit at any moment. Protons diffuse across the membrane through a path made up of both a and c subunits. Transient protonation of a key Glu residue in each c subunit elicits conformation changes that drive rotation and transmit protons between hydrophilic half channels positioned on each side of the membrane. The rotary movement of the c ring is made unidirectional by the large difference in proton concentration across the membrane. The number of protons that must be transferred to produce one complete rotation of the c ring is equal to the number of c subunits in the ring. Structural studies of the c ring have shown that the number of c subunits differs in different organisms (**Fig. 19-29**). In bovine mitochondria the number is 8, in yeast mitochondria and in *Escherichia coli* it is 10, and the number of c subunits can range as high as 17, as is seen in the soil bacterium *Burkholderia pseudomallei*. The rate of rotation in intact mitochondria has been estimated at about 6,000 rpm—100 rotations per second.

Chemiosmotic Coupling Allows Nonintegral Stoichiometries of O_2 Consumption and ATP Synthesis

The overall reaction equation for ATP synthesis has the following form:

$$x\ \text{ADP} + x\text{P}_\text{i} + \tfrac{1}{2}\text{O}_2 + \text{H}^+ + \text{NADH} \longrightarrow x\ \text{ATP} + \text{H}_2\text{O} + \text{NAD}^+ \qquad (19\text{-}11)$$

The value of x is sometimes called the **P/O ratio** or the **P/2e^- ratio**. When a proton gradient is coupled to ATP synthesis as described above, there is no theoretical requirement for P/O to be integral. The relevant questions about stoichiometry become these: How many protons are pumped outward by electron transfer from one NADH to O_2? and How many protons must flow inward through the F_oF_1 complex to drive the synthesis of one ATP? The measurement of proton fluxes is technically complicated; the investigator must take into account the buffering capacity of mitochondria, nonproductive leakage of protons across the inner membrane, and use of the proton gradient for functions other than ATP synthesis, such as driving the transport of substrates across the inner mitochondrial membrane (described below). When NADH or succinate (which sends electrons into the respiratory chain at the level of ubiquinone) is the oxidizable substrate, the consensus experimental values for number of protons pumped out per pair of electrons are 10 and 6, respectively. The most widely accepted experimental value for number of protons required to drive the synthesis of an ATP molecule is 4, of which 1 is used in transporting P_i, ATP, and ADP across the mitochondrial membrane (see below). If 10 protons are pumped out per NADH and 4 must flow in to produce 1 ATP, the proton-based P/O ratio is 2.5 for NADH as the electron donor and 1.5 (6/4) for succinate. However, as we will see in Worked Example 19-2, the proton stoichiometry of ATP synthesis by ATP synthase depends upon the number of c units in F_o, which ranges from 8 to 17, depending on the species.

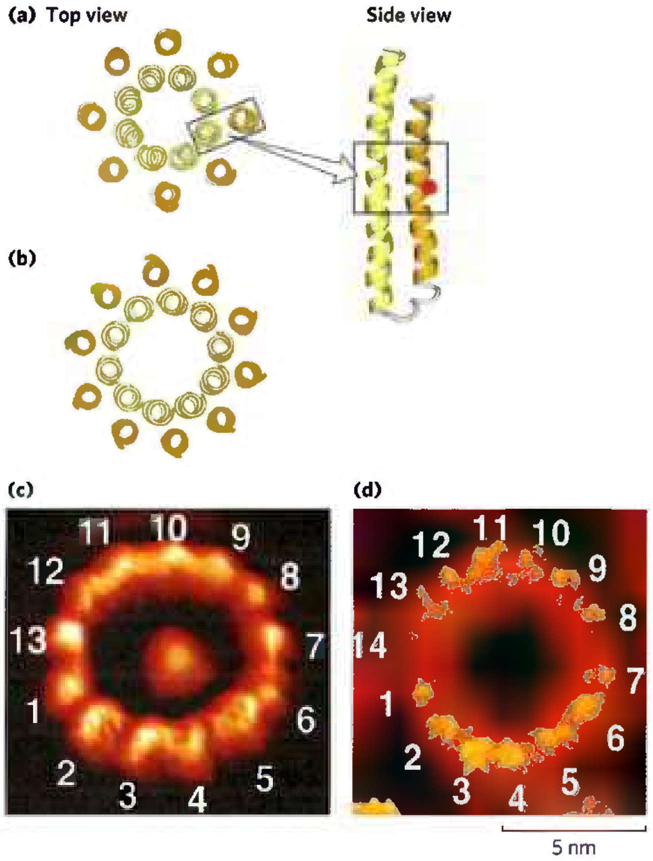


FIGURE 19-29 Species differences in number of c subunits in the c ring of the F_o complex. The structures of the c rings from several species have been determined by x-ray crystallography. Each helix in the inner ring is half of a hairpin-shaped c subunit; the outer ring of helices forms the other half of the hairpin structure. The essential Glu residue (Asp in some species) is shown as a red dot. Views of the c ring perpendicular to the membrane show the number of c subunits for (a) bovine mitochondria (8 subunits) and (b) yeast mitochondria (10). Atomic force microscopy has been used to visualize the c rings of (c) a thermophilic bacterium, *Bacillus* species TA2.A1 (13 subunits), and (d) spinach (14). According to the model in Figure 19-28, different numbers of c subunits in the c ring should result in different ratios of ATP formed per pair of electrons passing through the respiratory chain (i.e., different P/O ratios). [(a) Data from PDB ID 1OHH, E. Cabezon et al., *Nat. Struct. Biol.* 10:744, 2003. (c) Republished with permission of Elsevier from *J. Mol. Biol*, Matthies, et al., Vol. 388(3), ©2009; permission conveyed through Copyright Clearance Center, Inc. (d) H. Seelert et al. Structural biology: Proton-powered turbine of a plant motor. *Nature* 405, 418–419. Reprinted by permission from Macmillan Publishers Ltd.]

WORKED EXAMPLE 19-2 *Stoichiometry of ATP Production: Effect of c Ring Size*

(a) If the ATP synthase of *bovine* mitochondria has 8 c subunits per c ring, what is the predicted ratio of ATP formed per NADH oxidized? (b) What is the predicted value for *yeast* mitochondria, with 10 c subunits per ATP synthase? (c) What are the comparable values for electrons entering the respiratory chain from $FADH_2$?

SOLUTION: (a) Here we are asked to determine how many ATP molecules are produced per NADH. This is another way of asking us to calculate the P/O ratio, or x, in Equation 19-11. If the c ring has 8 c subunits, then one full rotation will transfer 8 protons to the matrix and produce 3 ATP molecules. But this synthesis also requires the transport of 3 P_i into the matrix, at a cost of 1 proton each, adding 3 more protons to the total number required. This brings the total cost to (11 protons)/(3 ATP) = 3.7 protons/ATP. The generally agreed value for the number of protons pumped out per pair of electrons transferred from NADH is 10 (Eqn 19-7). So, oxidizing 1 NADH produces (10 protons)/(3.7 protons/ATP) = 2.7 ATP.

(b) If the c ring has 10 c subunits, then one full rotation will transfer 10 protons to the matrix and produce 3 ATP molecules. Adding in the 3 protons to transport the 3 P_i into the matrix brings the total cost to (13 protons)/(3 ATP) = 4.3 protons/ATP. Oxidizing 1 NADH produces (10 protons)/(4.3 protons/ATP) = 2.3 ATP.

(c) When electrons enter the respiratory chain from $FADH_2$ (at ubiquinone), only 6 protons are available to drive ATP synthesis. This changes the calculation for bovine mitochondria to (6 protons)/(3.7 protons/ATP) = 1.6 ATP per pair of electrons from $FADH_2$. For yeast mitochondria, the calculation is (6 protons)/(4.3 protons/ATP) = 1.4 ATP per pair of electrons from $FADH_2$.

These calculated values of x, or the P/O ratio, define a range that includes the experimental values of 2.5 ATP/NADH and 1.5 ATP/$FADH_2$, and we therefore use these values throughout this book.

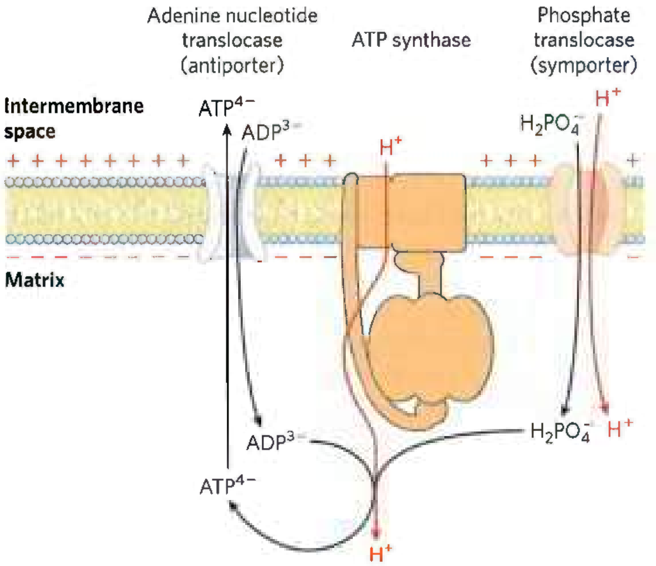


FIGURE 19-30 Adenine nucleotide and phosphate translocases. Transport systems of the inner mitochondrial membrane carry ADP and P_i into the matrix and newly synthesized ATP into the cytosol. The adenine nucleotide translocase is an antiporter; the same protein moves ADP into the matrix and ATP out. The effect of replacing ATP^{4-} with ADP^{3-} in the matrix is the net efflux of one negative charge, which is favored by the charge difference across the inner membrane (outside positive). At pH 7, P_i is present as both HPO_4^{2-} and $H_2PO_4^-$; the phosphate translocase is specific for $H_2PO_4^-$. There is no net flow of charge during symport of $H_2PO_4^-$ and H^+, but the relatively low proton concentration in the matrix favors the inward movement of H^+. Thus the proton-motive force is responsible both for providing the energy for ATP synthesis and for transporting substrates (ADP and P_i) into and product (ATP) out of the mitochondrial matrix. All three of these transport systems can be isolated as a single membrane-bound complex (ATP synthasome).

The Proton-Motive Force Energizes Active Transport

Although the primary role of the proton gradient in mitochondria is to furnish energy for the synthesis of ATP, the proton-motive force also drives several transport processes essential to oxidative phosphorylation. The inner mitochondrial membrane is generally impermeable to charged species, but two specific systems transport ADP and P_i into the matrix and ATP out to the cytosol (**Fig. 19-30**).

The **adenine nucleotide translocase**, integral to the inner membrane, binds ADP^{3-} in the intermembrane space and transports it into the matrix in exchange for an ATP^{4-} molecule simultaneously transported outward (see Fig. 13-11 for the ionic forms of ATP and ADP). Because this antiporter moves four negative charges out for every three moved in, its activity is favored by the transmembrane electrochemical gradient, which gives the matrix a net negative charge; the proton-motive force drives ATP-ADP exchange. Adenine nucleotide translocase is specifically inhibited by atractyloside, a toxic glycoside produced by a species of thistle. If the transport of ADP into and ATP out of mitochondria is inhibited, cytosolic ATP cannot be regenerated from ADP, explaining the toxicity of atractyloside.

A second membrane transport system essential to oxidative phosphorylation is the **phosphate translocase**, which promotes symport of one $H_2PO_4^-$ and one H^+ into the matrix. This transport process, too, is favored by the transmembrane proton gradient (Fig. 19-30). Notice that the process requires movement of one proton from the P side to the N side of the inner membrane, consuming some of the energy of electron transfer. A complex of the ATP synthase and both translocases, the **ATP synthasome**, can be isolated from mitochondria by gentle dissection with detergents, suggesting that the functions of these three proteins are very tightly integrated.

ATP and ADP cross the outer mitochondrial membrane via the voltage-dependent anion channel (VDAC), a 19-stranded β barrel with an opening about 27 Å wide, connecting the cytosol and the intermembrane space. Each VDAC, when open, can move 10^5 ATP molecules per second. The opening is gated by voltage, as its name indicates, and under some conditions VDAC is closed to ATP.

Shuttle Systems Indirectly Convey Cytosolic NADH into Mitochondria for Oxidation

The NADH dehydrogenase of the inner mitochondrial membrane of animal cells can accept electrons only from NADH in the matrix. Given that the inner membrane is not permeable to NADH, how can the NADH generated by glycolysis in the cytosol be reoxidized to NAD^+ by O_2 via the respiratory chain? Special shuttle systems carry reducing equivalents from cytosolic NADH into mitochondria by an indirect route. The most active NADH shuttle, which functions in liver, kidney, and heart mitochondria, is the **malate-aspartate shuttle** (**Fig. 19-31**). The reducing equivalents of cytosolic NADH are first transferred to cytosolic oxaloacetate to yield malate, catalyzed by cytosolic malate dehydrogenase. The malate thus formed passes through the inner membrane via the malate–α-ketoglutarate transporter. Within the matrix, the reducing equivalents are passed to NAD^+ by the action of matrix malate dehydrogenase, forming NADH; this NADH can pass electrons directly to the respiratory chain. About 2.5 molecules of ATP are generated as this pair of electrons passes to O_2. Cytosolic oxaloacetate must be regenerated by transamination reactions and the activity of membrane transporters to start another cycle of the shuttle.

Skeletal muscle and brain use a different NADH shuttle, the **glycerol 3-phosphate shuttle** (**Fig. 19-32**). It differs from the malate-aspartate shuttle in that it delivers the reducing equivalents from NADH through FAD in glycerol 3-phosphate dehydrogenase to ubiquinone and thus into Complex III, not Complex I (Fig. 19-15), providing enough energy to synthesize only 1.5 ATP molecules per pair of electrons.

The mitochondria of plants have an *externally* oriented NADH dehydrogenase that can transfer electrons directly from cytosolic NADH into the respiratory chain at the level of ubiquinone. Because this pathway bypasses the NADH dehydrogenase of Complex I and the associated proton movement, the yield of ATP from cytosolic NADH is less than that from NADH generated in the matrix (**Box 19-1**).

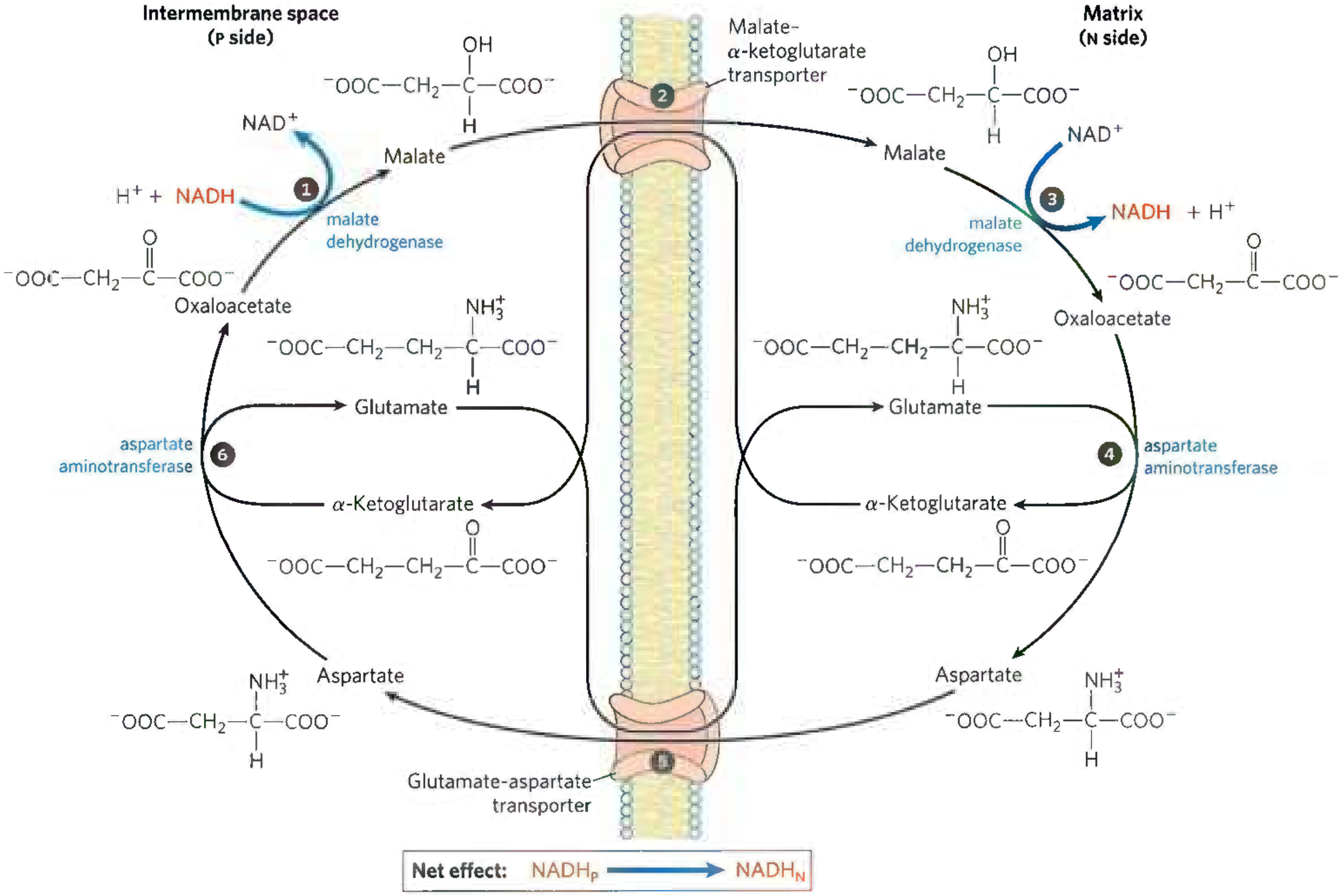

Intermembrane space
(P side)
Matrix
(N side)
Malate-
α-ketoglutarate
transporter
Glutamate-aspartate
transporter
⁻OOC—CH₂—C(OH)(H)—COO⁻
Malate
NAD⁺
H⁺ + NADH
①
malate
dehydrogenase
⁻OOC—CH₂—C(=O)—COO⁻
Oxaloacetate
②
③
NADH + H⁺
④
⑤
⑥
⁻OOC—CH₂—CH₂—C(NH₃⁺)(H)—COO⁻
Glutamate
α-Ketoglutarate
⁻OOC—CH₂—CH₂—C(=O)—COO⁻
aspartate
aminotransferase
Aspartate
⁻OOC—CH₂—C(NH₃⁺)(H)—COO⁻
Net effect: NADH_P → NADH_N

FIGURE 19-31 Malate-aspartate shuttle. This shuttle for transporting reducing equivalents from cytosolic NADH into the mitochondrial matrix is used in liver, kidney, and heart. ① NADH in the cytosol enters the intermembrane space through openings in the outer membrane (porins), then passes two reducing equivalents to oxaloacetate, producing malate. ② Malate crosses the inner membrane via the malate–α-ketoglutarate transporter. ③ In the matrix, malate passes two reducing equivalents to NAD^+, and the resulting NADH is oxidized by the respiratory chain; the oxaloacetate formed from malate cannot pass directly into the cytosol. ④ Oxaloacetate is first transaminated to aspartate, and ⑤ aspartate can leave via the glutamate-aspartate transporter. ⑥ Oxaloacetate is regenerated in the cytosol, completing the cycle, and glutamate produced in the same reaction enters the matrix via the glutamate-aspartate transporter.

BOX 19-1

Hot, Stinking Plants and Alternative Respiratory Pathways

Many flowering plants attract insect pollinators by releasing odorant molecules that mimic an insect's natural food sources or potential egg-laying sites. Plants pollinated by flies or beetles that normally feed on or lay their eggs in dung or carrion sometimes use foul-smelling compounds to attract these insects.

One family of stinking plants is the Araceae, which includes philodendrons, arum lilies, and skunk cabbages. These plants have tiny flowers densely packed on an erect structure, the spadix, surrounded by a modified leaf, the spathe. The spadix releases odors of rotting flesh or dung. Before pollination the spadix also heats up, in some species to as much as 20 to 40 °C above ambient temperatures. Heat production (thermogenesis) helps evaporate odorant molecules for better dispersal, and because rotting flesh and dung are usually warm from the hyperactive metabolism of scavenging microbes, the heat itself might also attract insects. In the case of the eastern skunk cabbage (Fig. 1), which flowers in late winter or early spring when snow still covers the ground, thermogenesis allows the spadix to grow up through the snow.

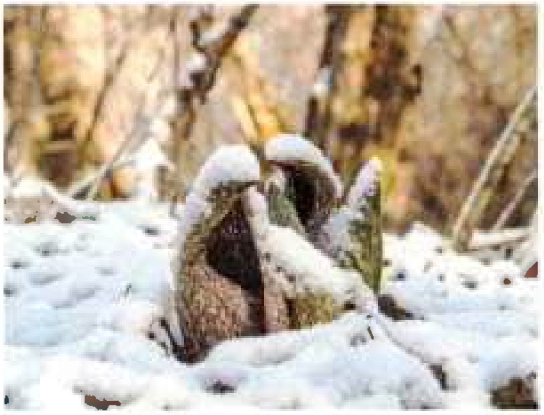


FIGURE 1 Eastern skunk cabbage. [Colin Purrington.]

How does a skunk cabbage heat its spadix? The mitochondria of plants, fungi, and unicellular eukaryotes have respiratory chains that are essentially the same as those in animals, but they also have an alternative respiratory pathway. A QH_2 oxidase transfers electrons from the ubiquinone pool directly to oxygen, bypassing the two proton-translocating steps of Complexes III and IV (Fig. 2). Energy that might have been conserved as ATP is instead released as heat. Plant mitochondria also have an alternative NADH dehydrogenase, insensitive to the Complex I inhibitor rotenone (see Table 19-4), that transfers electrons from NADH in the matrix directly to ubiquinone, bypassing Complex I and its associated proton pumping. And plant mitochondria have yet another NADH dehydrogenase, on the external face of the inner membrane, that transfers electrons from NADPH or NADH in the intermembrane space to ubiquinone, again bypassing Complex I. Thus when electrons enter the alternative respiratory pathway through the rotenone-insensitive NADH dehydrogenase, the external NADH dehydrogenase, or succinate dehydrogenase (Complex II), and pass to O_2 via the cyanide-resistant alternative oxidase, energy is not conserved as ATP but is released as heat. A skunk cabbage can use the heat to melt snow, produce a foul stench, or attract beetles or flies.

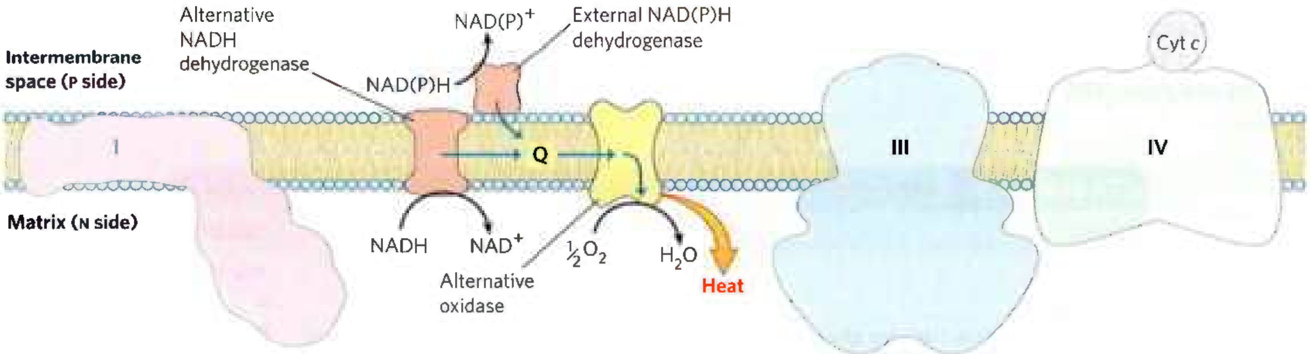


FIGURE 2 Electron carriers of the inner membrane of plant mitochondria. Electrons can flow through Complexes I, III, and IV, as in animal mitochondria, or through plant-specific alternative carriers by the paths shown with blue arrows.

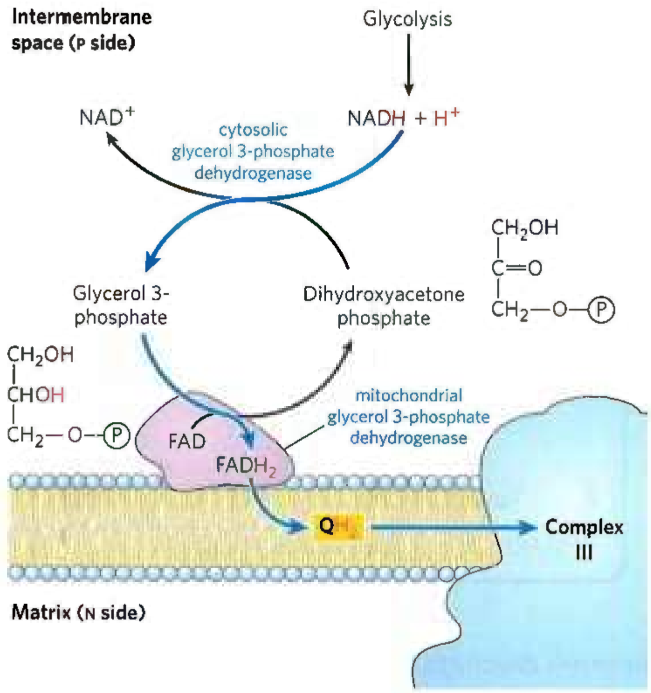

Intermembrane space (P side)
Glycolysis
NAD$^+$
NADH + H$^+$
cytosolic glycerol 3-phosphate dehydrogenase
CH$_2$OH
C=O
CH$_2$—O—Ⓟ
Glycerol 3-phosphate
Dihydroxyacetone phosphate
CH$_2$OH
CHOH
CH$_2$—O—Ⓟ
FAD
FADH$_2$
mitochondrial glycerol 3-phosphate dehydrogenase
Q
Complex III
Matrix (N side)

FIGURE 19-32 Glycerol 3-phosphate shuttle. This alternative means of moving reducing equivalents from the cytosol to the respiratory chain operates in skeletal muscle and the brain. In the cytosol, dihydroxyacetone phosphate accepts two reducing equivalents from NADH in a reaction catalyzed by cytosolic glycerol 3-phosphate dehydrogenase. An isozyme of glycerol 3-phosphate dehydrogenase bound to the outer face of the inner membrane then transfers two reducing equivalents from glycerol 3-phosphate in the intermembrane space to ubiquinone. Note that this shuttle does not involve membrane transport systems.

SUMMARY 19.2 *ATP Synthesis*

- The chemiosmotic theory describes the coupling of ATP synthesis to an electrochemical proton gradient. The flow of electrons through Complexes I, III, and IV results in pumping of protons across the inner mitochondrial membrane, making the matrix alkaline relative to the intermembrane space. This proton gradient provides the energy, in the form of the proton-motive force, for ATP synthesis from ADP and P_i.
- ATP synthase has two major components, called F_o and F_1. Both components have multiple subunits. The overall complex spans the inner mitochondrial membrane.
- ATP synthesis is reversible within the active site on the β subunits of the F_1 complex. Very tight binding to ATP offsets the negative ΔG for ATP hydrolysis in solution.
- Release of ATP from ATP synthase is promoted by the transmembrane proton gradient.
- The subunits of the F_1 complex cycle from (ADP + P_i)–bound to ATP-bound to empty conformations.
- ATP synthase carries out "rotational catalysis," in which the flow of protons through F_o causes the c ring to rotate and in turn trigger the subunit conformational changes in F_1.
- The ratio of ATP synthesized per $\frac{1}{2}O_2$ reduced to H_2O (the P/O ratio) is about 2.5 when electrons enter the respiratory chain at Complex I, and 1.5 when electrons enter at ubiquinone. This ratio varies among species, depending on the number of c subunits in the F_o complex.
- Energy conserved in a proton gradient can drive solute transport uphill across a membrane.
- The inner mitochondrial membrane is impermeable to NADH and NAD$^+$, but NADH equivalents are moved from the cytosol to the matrix by either of two shuttles. NADH equivalents moved in by the malate-aspartate shuttle enter the respiratory chain at Complex I and yield a P/O ratio of 2.5; those moved in by the glycerol 3-phosphate shuttle enter at ubiquinone and give a P/O ratio of 1.5.

19.3 Regulation of Oxidative Phosphorylation

Oxidative phosphorylation produces most of the ATP made in aerobic cells. Complete oxidation of a molecule of glucose to CO_2 yields 30 or 32 ATP (**Table 19-5**). By

TABLE 19-5 ATP Yield from Complete Oxidation of Glucose

Process	Direct product	Final ATP
Glycolysis	2 NADH (cytosolic) 2 ATP	3 or 5[a] 2
Pyruvate oxidation (two per glucose)	2 NADH (mitochondrial matrix)	5
Acetyl-CoA oxidation in citric acid cycle (two per glucose)	6 NADH (mitochondrial matrix) 2 $FADH_2$ 2 ATP or 2 GTP	15 3 2
Total yield per glucose		30 or 32

[a] If the malate/aspartate shuttle is used to transfer reducing equivalents into the mitochondrion, the yield is 5 ATP. If the glycerol 3-phosphate shuttle is used, the yield is 3 ATP.

comparison, glycolysis under anaerobic conditions (lactate fermentation) yields only 2 ATP per glucose. Clearly, the evolution of oxidative phosphorylation provided a tremendous increase in the energy efficiency of catabolism. Complete oxidation to CO_2 of the coenzyme A derivative of palmitate (16:0), which also occurs in the mitochondrial matrix, yields 108 ATP per palmitoyl-CoA (see Table 17-1). A similar calculation can be made for the ATP yield from oxidation of each of the amino acids (Chapter 18). Aerobic oxidative pathways that result in electron transfer to O_2 accompanied by oxidative phosphorylation therefore account for the vast majority of the ATP produced in catabolism, so the regulation of ATP production by oxidative phosphorylation to match the cell's fluctuating needs for ATP is absolutely essential.

Oxidative Phosphorylation Is Regulated by Cellular Energy Needs

The rate of respiration (O_2 consumption) in mitochondria is generally limited by the availability of ADP as a substrate for phosphorylation. Dependence of the rate of O_2 consumption on the availability of the P_i acceptor, ADP (Fig. 19-20b), the **acceptor control** of respiration, can be remarkable. In some animal tissues, the **acceptor control ratio**, the ratio of the maximal rate of ADP-induced O_2 consumption to the basal rate in the absence of ADP, is at least 10.

The intracellular concentration of ADP is one measure of the energy status of cells. Another, related measure is the **mass-action ratio** of the ATP-ADP system, $[ATP]/([ADP][P_i])$. Usually this ratio is very high, so the ATP-ADP system is almost fully phosphorylated. When the rate of some energy-requiring process (protein synthesis, for example) increases, the rate of breakdown of ATP to ADP and P_i increases, lowering the mass-action ratio. With more ADP available for oxidative phosphorylation, the rate of respiration increases, causing regeneration of ATP. This continues until the mass-action ratio returns to its normal high level, at which point respiration slows again. The rate of oxidation of cellular fuels is regulated with such sensitivity and precision that the $[ATP]/([ADP][P_i])$ ratio fluctuates only slightly in most tissues, even during extreme variations in energy demand. In short, ATP is formed only as fast as it is used in energy-requiring cellular activities.

An Inhibitory Protein Prevents ATP Hydrolysis during Hypoxia

We have already encountered ATP synthase as an ATP-driven proton pump (see Fig. 11-40), catalyzing the reverse of ATP synthesis under some experimental conditions. When a cell is hypoxic (deprived of oxygen), as in a heart attack or a stroke, electron transfer to oxygen slows, and so does the pumping of protons. The proton-motive force soon collapses. **P5** Under these conditions, the ATP synthase could operate in reverse, hydrolyzing ATP made by glycolysis to pump protons outward and causing a disastrous drop in ATP levels. This is prevented by a small (84 amino acids) protein inhibitor, IF_1. IF_1 simultaneously binds to two ATP synthase molecules, inhibiting the enzyme's activity in both directions (**Fig. 19-33**). IF_1 is inhibitory only in its dimeric form, which is favored at pH lower than 6.5. In a cell starved for oxygen, the main source of ATP becomes glycolysis, and the pyruvic or lactic acid thus formed lowers the pH in the cytosol and the mitochondrial matrix. This favors IF_1 dimerization, leading to inhibition of ATP synthase and thereby preventing any wasteful hydrolysis of ATP. When aerobic metabolism resumes, production of pyruvic acid slows, the pH of the cytosol rises, the IF_1 dimer is destabilized, and the inhibition of ATP synthase is lifted. IF_1 is an intrinsically disordered protein (p. 117); it acquires a favored conformation only on interaction with ATP synthase. In many tumors and cancer cell lines, which rely more heavily on glycolysis for ATP generation, IF_1 is expressed at unusually high levels.

Hypoxia Leads to ROS Production and Several Adaptive Responses

In hypoxic cells there is an imbalance between the input of electrons from fuel oxidation in the mitochondrial matrix and transfer of electrons to molecular oxygen, leading to increased formation of reactive oxygen species. In addition to the glutathione peroxidase system (Fig. 19-18), cells have two other lines of defense against ROS (**Fig. 19-34**). One is regulation of pyruvate dehydrogenase

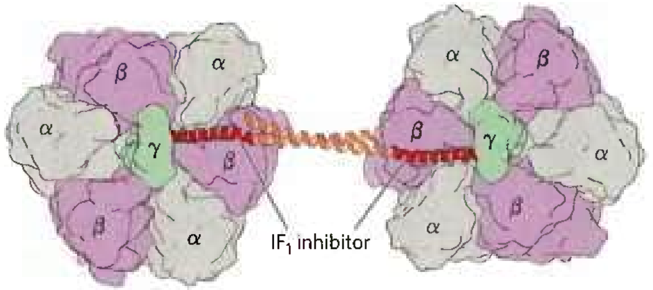


FIGURE 19-33 Structure of bovine F_1-ATPase in a complex with its regulatory protein IF_1. Two F_1 molecules are viewed here from the N side, as in Figure 19-25b. The inhibitor IF_1 (red) binds to the $\alpha\beta$ interface of the subunits in the diphosphate (ADP) conformation (α-ADP and β-ADP), freezing the two F_1 complexes and thereby blocking ATP hydrolysis (and synthesis). (Parts of the IF_1 α-helices that link the two F_1 molecules failed to resolve in the crystals of F_1 and are modeled based on the crystal structure of isolated IF_1.) [Data from PDB ID 1OHH, E. Cabezon et al., *Nat. Struct. Biol.* 10:744, 2003.]

(PDH), the enzyme that delivers acetyl-CoA to the citric acid cycle (Chapter 16). Under hypoxic conditions, PDH kinase phosphorylates mitochondrial PDH, inactivating it and slowing the delivery of $FADH_2$ and NADH from the citric acid cycle to the respiratory chain. A second means of preventing ROS formation is the replacement of one subunit of Complex IV, known as COX4-1, with another subunit, COX4-2, that is better suited to hypoxic conditions. With COX4-1, the catalytic properties of Complex IV are optimal for respiration at normal oxygen concentrations; with COX4-2, Complex IV is optimized for operation under hypoxic conditions.

The changes in PDH activity and the COX4-2 content of Complex IV are both mediated by HIF-1, the hypoxia-inducible factor. HIF-1 (another intrinsically disordered protein) accumulates in hypoxic cells and, acting as a transcription factor, triggers increased synthesis of PDH kinase, COX4-2, and a protease that degrades COX4-1. HIF-1 is a master regulator of O_2 homeostasis. Recall that it also mediates the changes in glucose transport and glycolytic enzymes that produce the Warburg effect, the dependence on glycolysis (not mitochondrial respiration) for ATP production, even in the presence of sufficient oxygen (see Box 14-1).

When these mechanisms for dealing with ROS are insufficient, either due to genetic mutation affecting one of the protective proteins or under conditions of very high rates of ROS production, mitochondrial function is compromised. Mitochondrial damage is thought to be involved in aging, heart failure, certain rare cases of diabetes (described below), and several maternally inherited genetic diseases that affect the nervous system. ■

ATP-Producing Pathways Are Coordinately Regulated

The major catabolic pathways have overlapping and concerted regulatory mechanisms that allow them to function together in an economical and self-regulating manner to produce ATP and biosynthetic precursors. The relative concentrations of ATP and ADP control not only the rates of electron transfer and oxidative phosphorylation but also the rates of the citric acid cycle, pyruvate oxidation, and glycolysis (**Fig. 19-35**). Whenever ATP consumption increases, the rate of electron transfer and oxidative phosphorylation increases. Simultaneously, the rate of pyruvate oxidation via the citric acid cycle increases, increasing the flow of electrons into

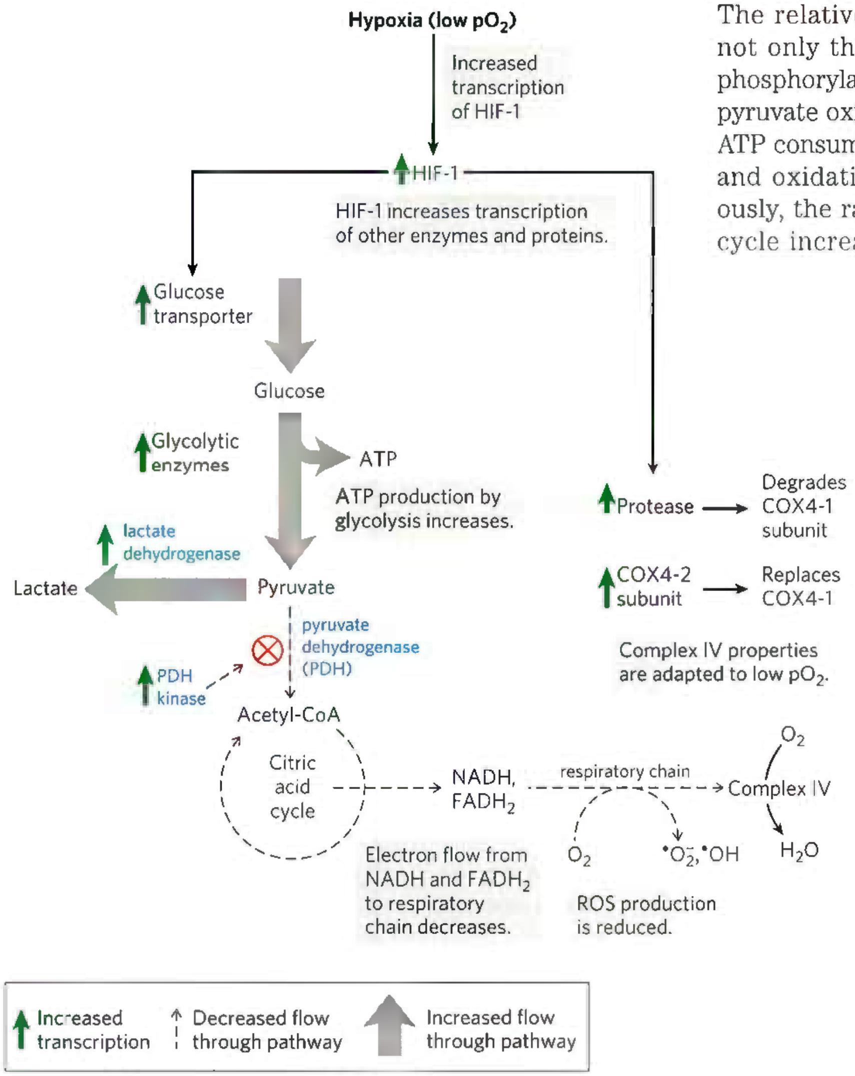

Hypoxia (low pO2)
Increased transcription of HIF-1
HIF-1
HIF-1 increases transcription of other enzymes and proteins.
Glucose transporter
Glucose
Glycolytic enzymes
ATP
ATP production by glycolysis increases.
lactate dehydrogenase
Lactate
Pyruvate
pyruvate dehydrogenase (PDH)
PDH kinase
Acetyl-CoA
Citric acid cycle
NADH, FADH2
respiratory chain
Complex IV
O2
H2O
O2
•O2−, •OH
Electron flow from NADH and FADH2 to respiratory chain decreases.
ROS production is reduced.
Protease
Degrades COX4-1 subunit
COX4-2 subunit
Replaces COX4-1
Complex IV properties are adapted to low pO2.
Increased transcription
Decreased flow through pathway
Increased flow through pathway

FIGURE 19-34 Regulation of gene expression by hypoxia-inducible factor (HIF-1) to reduce ROS formation. Under conditions of low oxygen (hypoxia), HIF-1 is synthesized in greater amounts and acts as a transcription factor, increasing synthesis of the glucose transporter, glycolytic enzymes, pyruvate dehydrogenase kinase (PDH kinase), lactate dehydrogenase, a protease that degrades the cytochrome oxidase subunit COX4-1, and cytochrome oxidase subunit COX4-2. These changes counter the formation of ROS by decreasing the supply of NADH and $FADH_2$ and making cytochrome oxidase of Complex IV more effective. [Information from D. A. Harris, *Bioenergetics at a Glance*, p. 36, Blackwell Science, 1995.]

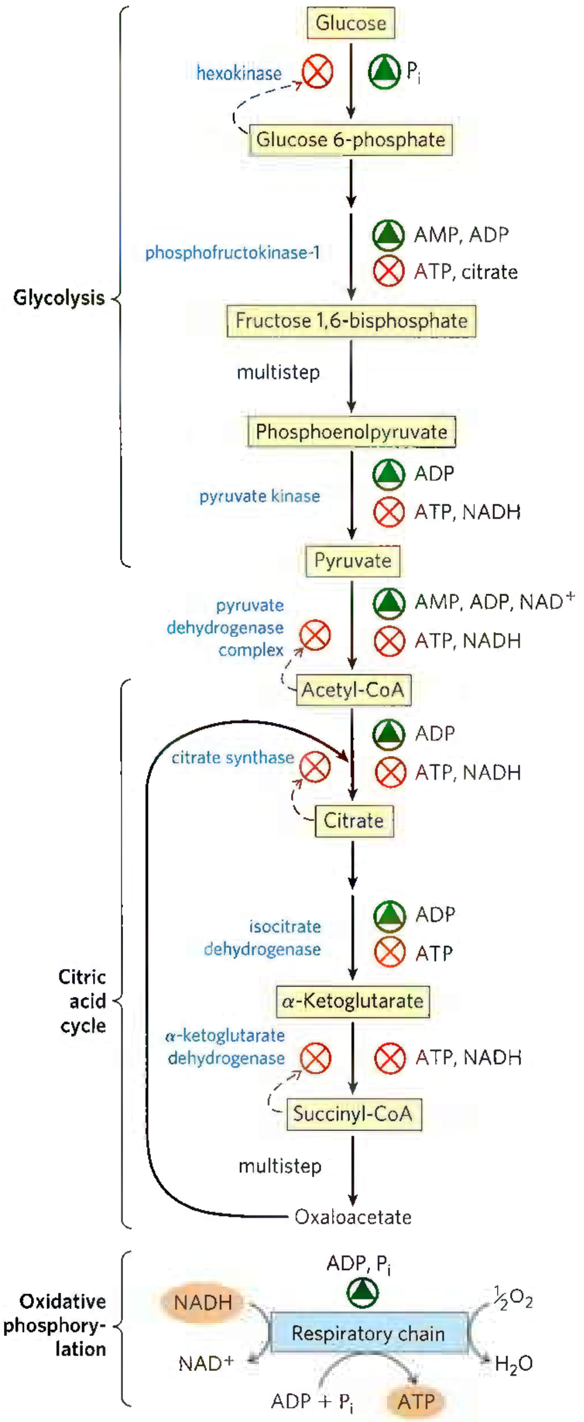

Glycolysis
Glucose
hexokinase
P_i
Glucose 6-phosphate
phosphofructokinase-1
AMP, ADP
ATP, citrate
Fructose 1,6-bisphosphate
multistep
Phosphoenolpyruvate
pyruvate kinase
ADP
ATP, NADH
Pyruvate
pyruvate dehydrogenase complex
AMP, ADP, NAD^+
ATP, NADH
Citric acid cycle
Acetyl-CoA
citrate synthase
ADP
ATP, NADH
Citrate
isocitrate dehydrogenase
ADP
ATP
α-Ketoglutarate
α-ketoglutarate dehydrogenase
ATP, NADH
Succinyl-CoA
multistep
Oxaloacetate
Oxidative phosphorylation
ADP, P_i
NADH
$\frac{1}{2}O_2$
Respiratory chain
NAD^+
H_2O
ADP + P_i
ATP

FIGURE 19-35 Regulation of ATP-producing pathways. This diagram shows the coordinated regulation of glycolysis, pyruvate oxidation, the citric acid cycle, and oxidative phosphorylation by the relative concentrations of ATP, ADP, and AMP, and by NADH. High [ATP] (or low [ADP] and [AMP]) produces low rates of glycolysis, pyruvate oxidation, acetate oxidation via the citric acid cycle, and oxidative phosphorylation. All four pathways are accelerated when the use of ATP and the formation of ADP, AMP, and P_i increase. The ability of citrate to inhibit both glycolysis and the citric acid cycle reinforces the action of the adenine nucleotide system. In addition, increased [NADH] and [acetyl-CoA] also inhibit the oxidation of pyruvate to acetyl-CoA, and a high [NADH]/[NAD^+] ratio inhibits the dehydrogenase reactions of the citric acid cycle (see Fig. 16-18).

the respiratory chain. These events, in turn, can evoke an increased rate of glycolysis, increasing the rate of pyruvate formation. When conversion of ADP to ATP lowers the ADP concentration, acceptor control slows electron transfer and thus oxidative phosphorylation. Glycolysis and the citric acid cycle are also slowed, because ATP is an allosteric inhibitor of the glycolytic enzyme phosphofructokinase-1 (see Fig. 14-23) and of pyruvate dehydrogenase (see Fig. 16-18).

Phosphofructokinase-1 is also inhibited by citrate, the first intermediate of the citric acid cycle. When the cycle is "idling," citrate accumulates within mitochondria, then is transported into the cytosol. When the cytosolic concentrations of both ATP and citrate rise, they produce a concerted allosteric inhibition of phosphofructokinase-1 that is greater than the sum of their individual effects, slowing glycolysis.

SUMMARY 19.3 ***Regulation of Oxidative Phosphorylation***

- Oxidative phosphorylation is regulated by cellular energy demands. Intracellular [ADP] and the mass-action ratio [ATP]/([ADP][P_i]) are measures of a cell's energy status.
- In hypoxic (oxygen-deprived) cells, a protein inhibitor blocks ATP hydrolysis by the reverse activity of ATP synthase, preventing a drastic drop in [ATP].
- The adaptive responses to hypoxia, mediated by HIF-1, slow electron transfer into the respiratory chain and modify Complex IV to act more efficiently under low-oxygen conditions.
- ATP and ADP concentrations set the rate of electron transfer through the respiratory chain via a series of coordinated controls on respiration, glycolysis, and the citric acid cycle.

19.4 Mitochondria in Thermogenesis, Steroid Synthesis, and Apoptosis

Although ATP production is a central role for the mitochondrion, this organelle has other functions that, in specific tissues or under specific circumstances, are also crucial. In adipose tissue, mitochondria generate heat to protect vital organs from low ambient temperature; in the adrenal glands and the gonads, mitochondria are the sites of steroid hormone synthesis; and in most or all tissues, they are key participants in apoptosis (programmed cell death).

Uncoupled Mitochondria in Brown Adipose Tissue Produce Heat

We noted above that respiration slows when the cell is adequately supplied with ATP. There is a remarkable and instructive exception to this general rule. Most newborn mammals,

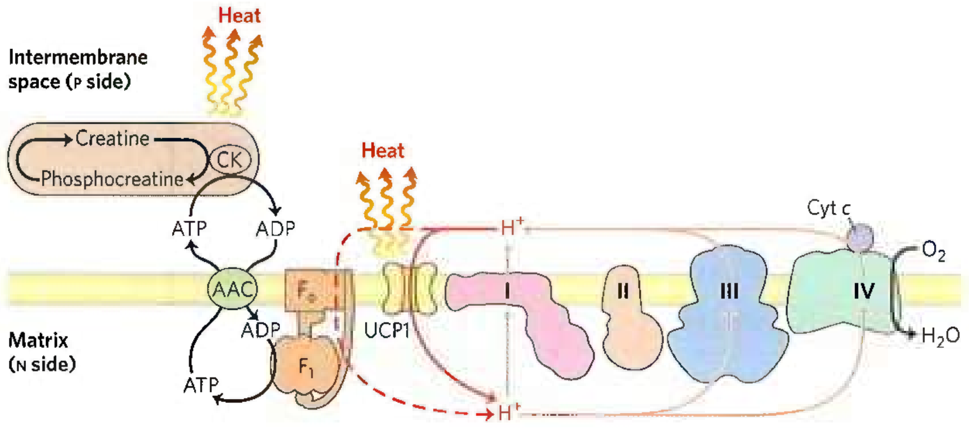


FIGURE 19-36 Two mechanisms of thermogenesis in mitochondria. UCP1, an uncoupling protein in the mitochondria of brown adipose tissue, causes the energy conserved by proton pumping to be dissipated as heat by providing an alternative route for protons to reenter the mitochondrial matrix. A futile cycle in which creatine is phosphorylated by creatine kinase (CK), using ATP and producing ADP transported by the ATP/ADP carrier (AAC), also generates heat.

including humans, have a type of adipose tissue called **brown adipose tissue** (**BAT**; p. 852), in which P1 fuel oxidation serves not to produce ATP but to generate heat to keep the newborn warm. This specialized adipose tissue is brown because of the presence of large numbers of mitochondria and thus high concentrations of cytochromes, with heme groups that are strong absorbers of visible light.

There are at least two mechanisms of thermogenesis. The mitochondria of brown adipocytes are much like those of other mammalian cells, except in having a unique protein in their inner membrane. **Uncoupling protein 1 (UCP1)**, a long-chain fatty acid/H^+ symporter, provides a path for protons to return to the matrix without passing through the F_oF_1 complex (**Fig. 19-36**). P5 As a result of this short-circuiting of protons, the energy of oxidation is not conserved by ATP formation but is dissipated as heat, which contributes to maintaining body temperature. However, UCP1 is only part of the story, and heat generation occurs in mammals even when it is absent. The thermogenic action of UCP1 is supplemented by a futile cycle involving creatine and phosphocreatine (Fig. 19-36). ATP synthesized in the mitochondrial matrix is exported to the intermembrane space via the adenine nucleotide translocator, an ADP/ATP antiporter. There, the ATP is used to phosphorylate creatine to create phosphocreatine and ADP. The ADP is transported back into the matrix. Hydrolysis of phosphocreatine completes a futile cycle that liberates heat.

Hibernating animals also depend on the activity of uncoupled BAT mitochondria to generate heat during their long dormancy (see Box 17-1). We will return to the role of UCP1 when we discuss the regulation of body mass in Chapter 23 (pp. 867–869).

Mitochondrial P-450 Monooxygenases Catalyze Steroid Hydroxylations

P1 Mitochondria are the site of biosynthetic reactions that produce steroid hormones, including the sex hormones, glucocorticoids, mineralocorticoids, and vitamin D hormone. These compounds are synthesized from cholesterol or a related sterol in a series of hydroxylations catalyzed by enzymes of the **cytochrome P-450** family (see Box 21-1), all of which have a critical heme group (its absorption at 450 nm gives this family its name). In the hydroxylation reactions, one atom of molecular oxygen is incorporated into the substrate and the second is reduced to H_2O, making cytochrome P-450 enzymes monooxygenases:

$$\text{R—H} + O_2 + \text{NADPH} + H^+ \longrightarrow \text{R—OH} + H_2O + \text{NADP}^+$$

In this reaction, two species are oxidized: NADPH and R—H.

There are dozens of P-450 enzymes, all situated in the inner mitochondrial membrane with their catalytic site exposed to the matrix. Steroidogenic cells are packed with mitochondria specialized for steroid synthesis; the mitochondria are generally larger than those in other tissues and have more extensive and highly convoluted inner membranes (**Fig. 19-37**).

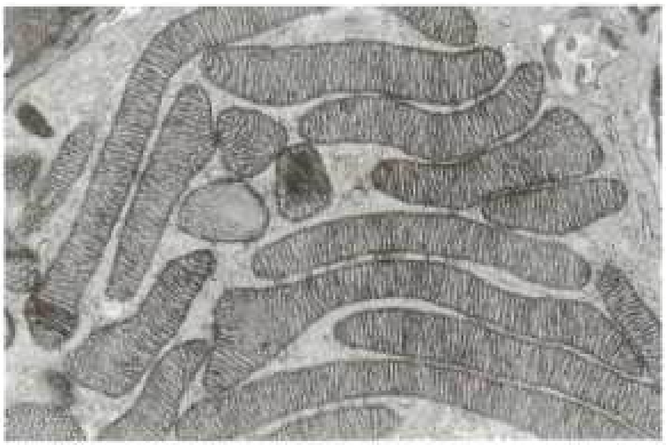


FIGURE 19-37 Mitochondria of adrenal gland, specialized for steroid synthesis. As seen in this electron micrograph of a thin section of adrenal gland, mitochondria are profuse and have extensive cristae, providing a large surface for the P-450 enzymes of the inner membrane. [Don Fawcett/Science Source.]

The path of electron flow in the mitochondrial P-450 system is complex, involving a flavoprotein and an iron-sulfur protein that carry electrons from NADPH to the P-450 heme (**Fig. 19-38**). All P-450 enzymes have a heme that interacts with O_2 and a substrate-binding site that confers specificity.

Another large family of P-450 enzymes is found in the endoplasmic reticulum of hepatocytes. These enzymes catalyze reactions similar to the mitochondrial P-450 reactions, but their substrates include a wide variety of hydrophobic compounds, many of which are **xenobiotics** — compounds not found in nature but synthesized industrially. The P-450 enzymes of the ER have very broad and overlapping substrate specificities. Hydroxylation of the hydrophobic compounds makes them more water-soluble, and they can then be cleared by the kidneys and excreted in urine. Among the substrates for these P-450 oxygenases are many commonly used prescription drugs. Metabolism by P-450 enzymes limits a drug's lifetime in the bloodstream and thus its therapeutic effects. Humans differ in their genetic complement of P-450 enzymes in the ER, as well as in the extent to which certain P-450 enzymes have been induced, such as by a history of ethanol ingestion. In principle, therefore, an individual's genetics and personal history should be considered in determining therapeutic drug doses. In practice, this precise tailoring of dosage is not yet economically feasible, but it may become so. ■

Mitochondria Are Central to the Initiation of Apoptosis

Apoptosis, also called **programmed cell death**, is a process in which individual cells die for the good of the organism, such as in the course of normal embryonic development, and the organism conserves the cells' molecular components (amino acids, nucleotides, and so forth). Apoptosis may be triggered by an external signal, acting at a plasma membrane receptor, or by internal events such as DNA damage, viral infection, oxidative stress from the accumulation of ROS, or other stress such as a heat shock.

P1 Mitochondria play a critical role in triggering apoptosis. When a stressor gives the signal for cell death, one early consequence is an increase in the permeability of the outer mitochondrial membrane, allowing cytochrome *c* to escape from the intermembrane space into the cytosol (**Fig. 19-39**). The increased permeability is due to the opening of the **permeability transition pore complex (PTPC)**, a multisubunit protein in the outer membrane; its opening and closing are affected by several proteins that stimulate or suppress apoptosis. When released into the cytosol, cytochrome *c* interacts with monomers of the protein **Apaf-1 (apoptosis protease activating factor-1)**, causing the formation of an **apoptosome** composed of seven Apaf-1 and seven cytochrome *c* molecules. The apoptosome provides the platform on which the proenzyme procaspase-9 is activated to caspase-9, a member of a family of highly specific proteases, called the **caspases**, involved in apoptosis. These cysteine proteases cleave proteins only on the carboxyl-terminal side of *Asp* residues, thus the name "caspases." Caspase-9 initiates a cascade of proteolytic activations, with one caspase activating a second, and this in turn activating a third, and so forth (see Fig. 12-42). Note that this role of cytochrome *c* in apoptosis is a clear case of "moonlighting," in that one protein plays two very different roles in the cell (see Box 16-1).

SUMMARY 19.4 *Mitochondria in Thermogenesis, Steroid Synthesis, and Apoptosis*

- In the brown adipose tissue of newborns, electron transfer is uncoupled from ATP synthesis, and the energy of fuel oxidation is dissipated as heat. Hibernating animals use this strategy to avoid freezing.
- Hydroxylation reaction steps in the synthesis of steroid hormones in steroidogenic tissues (adrenal gland, gonads, liver, and kidney) take place in specialized mitochondria. Key reactions are catalyzed by a family of P-450 monooxygenases.

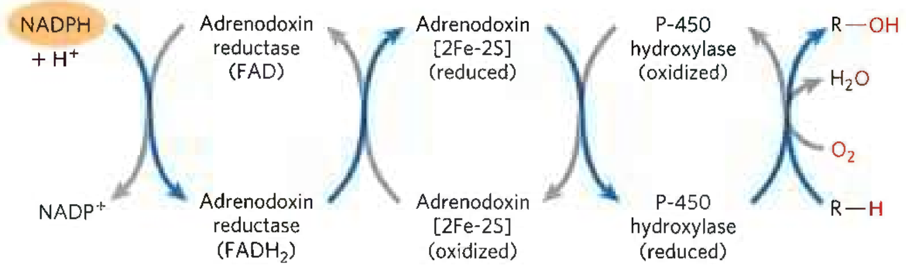

NADPH
+ H⁺
NADP⁺
Adrenodoxin reductase (FAD)
Adrenodoxin reductase (FADH₂)
Adrenodoxin [2Fe-2S] (reduced)
Adrenodoxin [2Fe-2S] (oxidized)
P-450 hydroxylase (oxidized)
P-450 hydroxylase (reduced)
R—OH
H₂O
O₂
R—H

FIGURE 19-38 Path of electron flow in mitochondrial cytochrome P-450 reactions in adrenal gland. Two electrons are transferred from NADPH to the FAD-containing flavoprotein adrenodoxin reductase, which passes the electrons, one at a time, to adrenodoxin, a small, soluble 2Fe-2S protein. Adrenodoxin passes single electrons to the cytochrome P-450 hydroxylase, which interacts directly with O_2 and the substrate (R—H) to form the products, H_2O and R—OH.

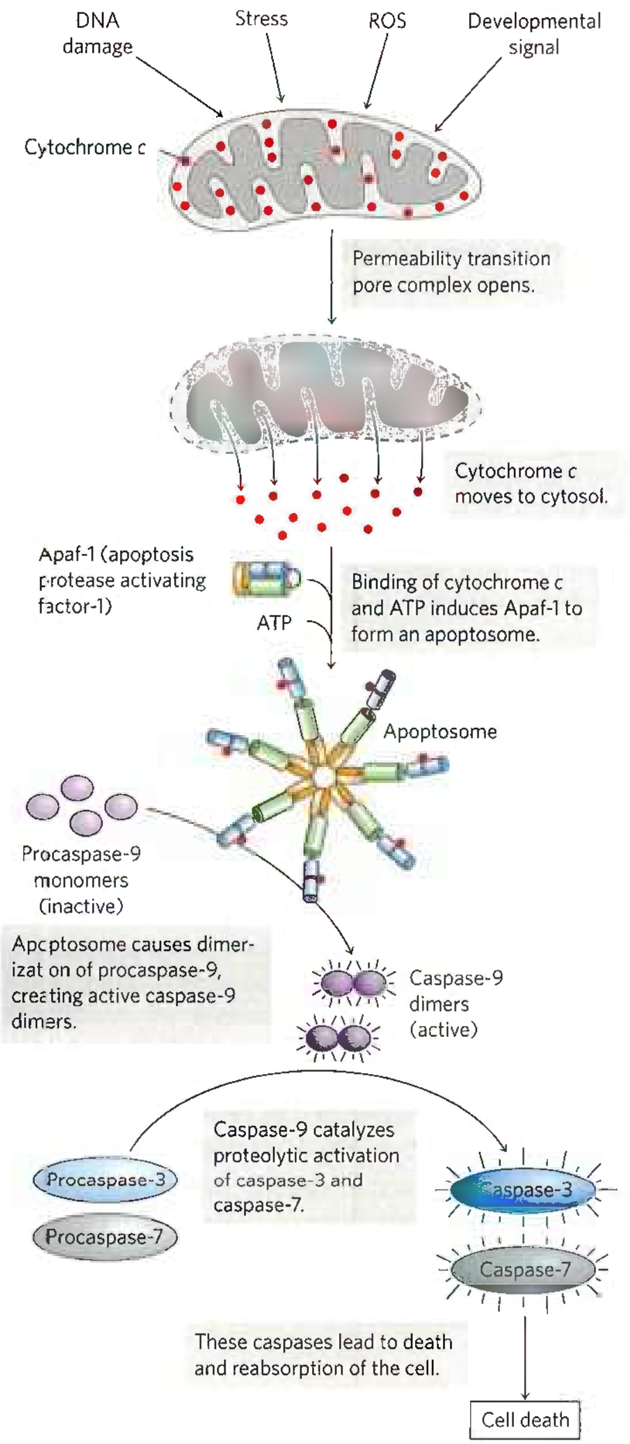

DNA damage
Stress
ROS
Developmental signal
Cytochrome c
Permeability transition pore complex opens.
Cytochrome c moves to cytosol.
Apaf-1 (apoptosis protease activating factor-1)
ATP
Binding of cytochrome c and ATP induces Apaf-1 to form an apoptosome.
Apoptosome
Procaspase-9 monomers (inactive)
Apoptosome causes dimerization of procaspase-9, creating active caspase-9 dimers.
Caspase-9 dimers (active)
Caspase-9 catalyzes proteolytic activation of caspase-3 and caspase-7.
Procaspase-3
Procaspase-7
Caspase-3
Caspase-7
These caspases lead to death and reabsorption of the cell.
Cell death

FIGURE 19-39 Role of cytochrome *c* in apoptosis. Cytochrome *c* is a small, soluble, mitochondrial protein, located in the intermembrane space, that carries electrons between Complex III and Complex IV during respiration. In a completely separate role, as outlined here, it acts as a trigger for apoptosis by stimulating the activation of a family of proteases called caspases. [Information from S. J. Riedl and G. S. Salvesen, *Nat. Rev. Mol. Cell Biol.* 8:409, 2007, Fig. 3.]

■ Mitochondria play a central role in apoptosis. Mitochondrial cytochrome *c*, released into the cytosol, participates in activation of caspase-9, one of the proteases involved in apoptosis.

19.5 Mitochondrial Genes: Their Origin and the Effects of Mutations

Mitochondria contain their own genome, a circular, double-stranded DNA (mtDNA) molecule. Each of the hundreds or thousands of mitochondria in a typical cell has about five copies of this genome. The human mitochondrial chromosome (**Fig. 19-40**) contains 37 genes (16,569 bp), including 13 that encode subunits of proteins of the respiratory chain (**Table 19-6**); the remaining genes code for rRNA and tRNA molecules essential to the protein-synthesizing machinery of mitochondria. To synthesize these 13 protein subunits, mitochondria have their own ribosomes, distinctly different from those in the cytoplasm. The great majority of mitochondrial proteins—about 1,200 different types—are encoded by nuclear genes, synthesized on cytoplasmic ribosomes, then imported into and assembled in the mitochondria (Chapter 27).

Mitochondria Evolved from Endosymbiotic Bacteria

The existence of mitochondrial DNA, ribosomes, and tRNAs supports the theory of the endosymbiotic origin of mitochondria (see Fig. 1-37), which holds that the first organisms capable of aerobic metabolism, including respiration-linked ATP production, were bacteria. **P2** Primitive eukaryotes that lived anaerobically (by fermentation) acquired the ability to carry out oxidative phosphorylation when they established a symbiotic relationship with bacteria living in their cytosol. After a long period of evolution and the movement of many bacterial

TABLE 19-6 Respiratory Proteins Encoded by Mitochondrial Genes in Humans

Complex	Number of subunits	Number of subunits encoded by mtDNA
I NADH dehydrogenase	45	7
II Succinate dehydrogenase	4	0
III Ubiquinone:cytochrome *c* oxidoreductase	11	1
IV Cytochrome oxidase	13	3
V ATP synthase	8	2

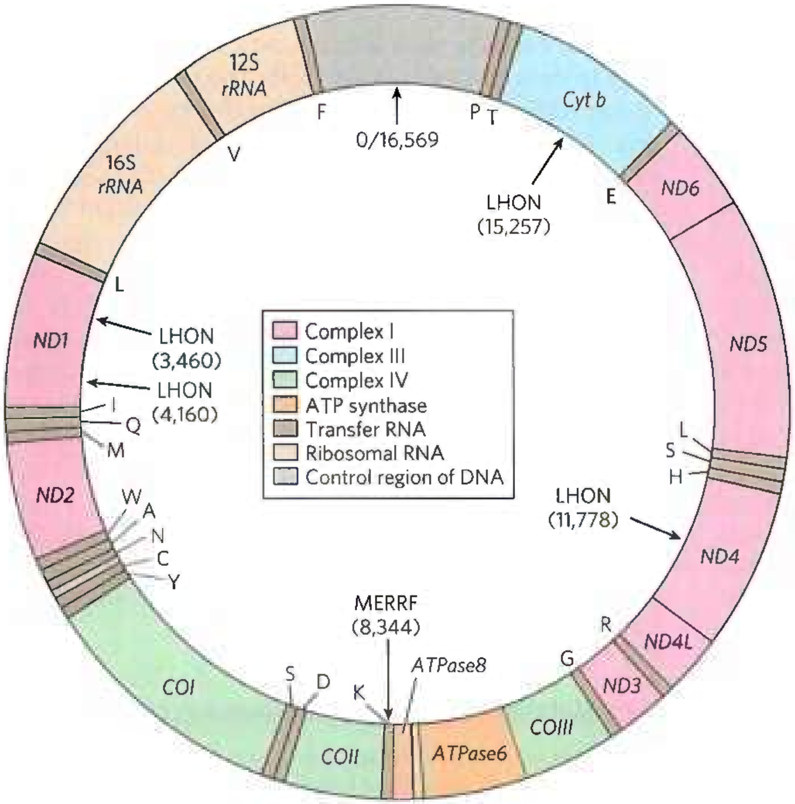

12S rRNA
16S rRNA
F
V
0/16,569
P
T
Cyt b
LHON (15,257)
E
ND6
L
ND1
LHON (3,460)
LHON (4,160)
I
Q
M
Complex I
Complex III
Complex IV
ATP synthase
Transfer RNA
Ribosomal RNA
Control region of DNA
ND5
L
S
H
LHON (11,778)
ND4
ND2
W
A
N
C
Y
MERRF (8,344)
R
ND4L
G
ND3
ATPase8
S
D
K
COI
COII
ATPase6
COIII

FIGURE 19-40 Mitochondrial genes and mutations. A map of human mitochondrial DNA, showing the genes that encode proteins of Complex I, the NADH dehydrogenase (*ND1* to *ND6*); the cytochrome *b* of Complex III (*Cyt b*); the subunits of cytochrome oxidase, Complex IV (*COI* to *COIII*); and two subunits of ATP synthase (*ATPase6* and *ATPase8*). The colors of the genes correspond to those of the complexes shown in Figure 19-7. Also included here are the genes for ribosomal RNAs (*rRNA*) and for some mitochondrion-specific transfer RNAs; tRNA specificity is indicated by the one-letter codes for amino acids. Arrows indicate the positions of mutations that cause Leber hereditary optic neuropathy (LHON) and myoclonic epilepsy with ragged-red fibers (MERRF) syndrome. Numbers in parentheses indicate the position of the altered nucleotides (nucleotide 1 is at the top of the circle, and numbering proceeds counterclockwise). [Information from M. A. Morris, *J. Clin. Neuroophthalmol.* 10:159, 1990.]

genes into the nucleus of the "host" eukaryote, the endosymbiotic bacteria eventually became mitochondria.

This hypothesis presumes that early free-living bacteria had the enzymatic machinery for oxidative phosphorylation. And it predicts that their modern bacterial descendants must have respiratory chains closely similar to those of modern eukaryotes. They do. Aerobic bacteria carry out NAD-linked electron transfer from substrates to O_2, coupled to the phosphorylation of cytosolic ADP. The dehydrogenases are located in the bacterial cytosol, and the respiratory chain in the plasma membrane. The electron carriers translocate protons outward across the plasma membrane as electrons are transferred to O_2. Bacteria such as *E. coli* have F_oF_1 complexes in their plasma membranes; the F_1 portion protrudes into the cytosol and catalyzes ATP synthesis from ADP and P_i as protons flow back into the cell through the proton channel of F_o.

The respiration-linked extrusion of protons across the bacterial plasma membrane also provides the driving force for other processes. Certain bacterial transport systems bring about uptake of extracellular nutrients (lactose, for example) against a concentration gradient, in symport with protons. And the rotary motion of bacterial flagella is provided by "proton turbines," molecular rotary motors driven not by ATP but directly by the transmembrane electrochemical potential generated by respiration-linked proton pumping (**Fig. 19-41**). **P2** It seems likely that the chemiosmotic mechanism evolved early, before the emergence of eukaryotes.

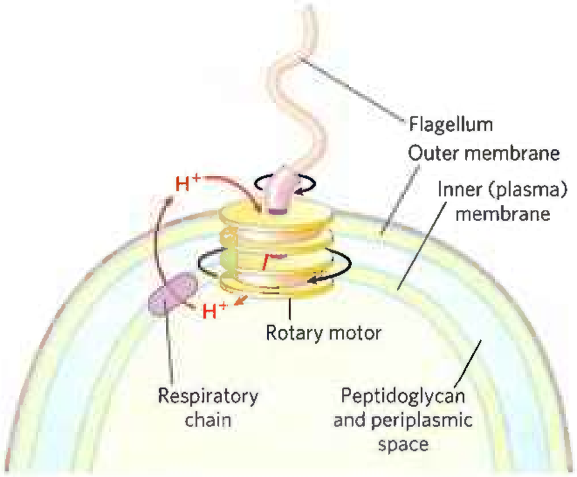

Flagellum
Outer membrane
Inner (plasma) membrane
H⁺
H⁺
Rotary motor
Respiratory chain
Peptidoglycan and periplasmic space

FIGURE 19-41 Rotation of bacterial flagella by proton-motive force. The shaft and rings at the base of the flagellum make up a rotary motor that has been called a "proton turbine." Protons ejected by electron transfer flow back into the cell through the turbine, causing rotation of the shaft of the flagellum. This motion differs fundamentally from the motion of muscle and of eukaryotic flagella and cilia, for which ATP hydrolysis is the energy source.

Mutations in Mitochondrial DNA Accumulate throughout the Life of the Organism

The respiratory chain is the major producer of reactive oxygen species in cells, so mitochondrial contents, including the mitochondrial genome, suffer the greatest exposure to, and damage by, ROS. Moreover, the mitochondrial DNA replication system is less effective than the nuclear system at correcting mistakes made during replication and at repairing DNA damage. As a consequence, defects in mtDNA accumulate over time. One theory of aging is that this gradual accumulation of defects is the primary cause of many of the "symptoms" of aging, which include, for example, progressive weakening of skeletal and heart muscle.

A unique feature of mitochondrial inheritance is the variation among individual cells, and between one individual organism and another, in the effects of a mtDNA mutation. A typical cell has hundreds or thousands of mitochondria, each with multiple copies of its own genome (Fig. 19-2b). Animals inherit essentially all of their mitochondria from the female parent. Eggs are large and contain 10^5 or 10^6 mitochondria; sperm are much smaller and contain far fewer mitochondria—perhaps 100 to 1,000. Furthermore, there is an active mechanism for targeting sperm-derived mitochondria for degradation in the fertilized egg. Just after fertilization, maternal phagosomes migrate to the site of sperm entry, engulf sperm mitochondria, and degrade them.

Suppose that, in a female organism, damage to one mitochondrial genome occurs in a germ cell from which oocytes develop, such that the germ cell contains mainly mitochondria with wild-type genes but one mitochondrion with a mutant gene. During the course of oocyte maturation, as this germ cell and its descendants repeatedly divide, the defective mitochondrion replicates and its progeny, all defective, are randomly distributed to daughter cells. Eventually, the mature egg cells contain different proportions of the defective mitochondria. When an egg cell is fertilized and undergoes the many divisions of embryonic development, the resulting somatic cells differ in their proportion of mutant mitochondria (Fig. 19-42a). This **heteroplasmy** (in contrast to **homoplasmy**, in which every mitochondrial genome in every cell is the same) results in mutant phenotypes of varying degrees of severity. Cells (and tissues) containing mostly wild-type mitochondria have the wild-type phenotype; they are essentially normal. Other heteroplasmic cells have intermediate phenotypes, some almost normal, others (with a high proportion of mutant mitochondria) abnormal (Fig. 19-42b). If the abnormal phenotype is associated with a disease, individuals with the same mtDNA mutation may have disease symptoms of differing severity—depending on the number and distribution of affected mitochondria.

Some Mutations in Mitochondrial Genomes Cause Disease

About 1 in 5,000 people have a disease-causing mutation in a mitochondrial protein that reduces the cell's capacity to produce ATP. A growing number of these diseases have been attributed to mutations in mitochondrial genes. Some tissues and cell types—neurons,

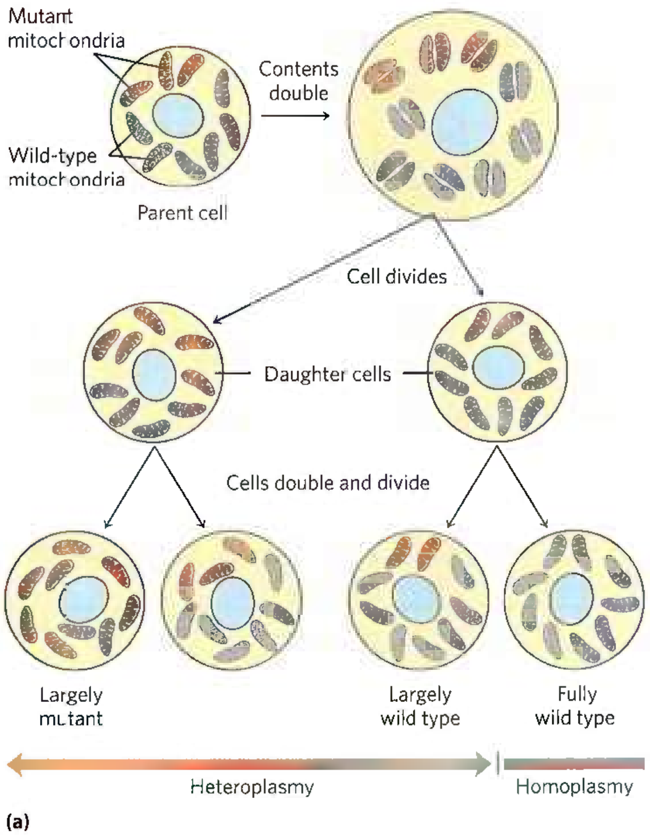
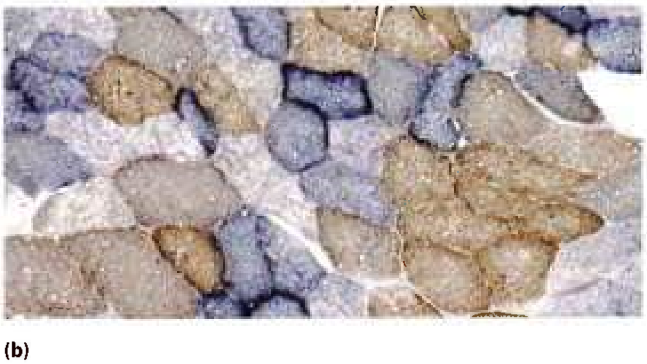


FIGURE 19-42 Heteroplasmy in mitochondrial genomes. (a) When a mature egg cell is fertilized, all of the mitochondria in the resulting diploid cell (zygote) are maternal; none come from the sperm. If some fraction of the maternal mitochondria have a mutant gene, the random distribution of mitochondria during subsequent cell divisions yields some daughter cells with mostly mutant mitochondria, some with mostly wild-type mitochondria, and some in between. Thus daughter cells show a varying degree of heteroplasmy. (b) Different degrees of heteroplasmy produce different cellular phenotypes. This section of human muscle tissue is from an individual with defective cytochrome oxidase. The cells were stained so that wild-type cells are blue and cells with mutant cytochrome oxidase are brown. As the micrograph shows, different cells in the same tissue are affected to different degrees by the mitochondrial mutation. [(b) Courtesy of Rob Taylor. Reprinted with permission from R. W. Taylor and D. M. Turnbull, *Nat. Rev. Genet.* 6:389, 2005, Fig. 2a.]



myocytes of both skeletal and cardiac muscle, and β cells of the pancreas—are less able than others to tolerate lowered ATP production and are therefore more affected by mutations in mitochondrial proteins.

A group of genetic diseases known as the **mitochondrial encephalomyopathies** affect primarily the brain and skeletal muscle. These diseases are invariably inherited from the mother, because, as noted above, a developing embryo derives all its mitochondria from the egg. The rare disease **Leber hereditary optic neuropathy (LHON)** affects the central nervous system, including the optic nerves, causing bilateral loss of vision in early adulthood. A single base change in the mitochondrial gene *ND4* (Fig. 19-40) changes an Arg residue to a His residue in a polypeptide of Complex I, and the result is mitochondria partially defective in electron transfer from NADH to ubiquinone. Although these mitochondria can produce some ATP by electron transfer from succinate, they apparently cannot supply sufficient ATP to support the very active metabolism of neurons, including the optic nerve. A single base change in the mitochondrial gene for cytochrome *b*, a component of Complex III, also produces LHON, demonstrating that the pathology results from a general reduction of mitochondrial function, not specifically from a defect in electron transfer through Complex I.

A mutation in the mitochondrial gene *ATP6* affects the proton pore in ATP synthase, leading to low rates of ATP synthesis while leaving the respiratory chain intact. Oxidative stress due to the continued supply of electrons from NADH increases the production of ROS, and the damage to mitochondria caused by ROS sets up a vicious cycle. Half of individuals with this mutant gene die within days or months of birth.

Myoclonic epilepsy with ragged-red fibers (MERRF) syndrome is caused by a mutation in the mitochondrial gene that encodes a tRNA specific for lysine (tRNA^{Lys}). This disease, characterized by uncontrollable muscular jerking, results from defective production of several of the proteins that require mitochondrial tRNAs for their synthesis. Skeletal muscle fibers of individuals with MERRF syndrome have abnormally shaped mitochondria that sometimes contain paracrystalline structures (**Fig. 19-43**). Other mutations in mitochondrial genes are believed to be responsible for the progressive muscular weakness that characterizes mitochondrial myopathy and for enlargement and deterioration of the heart muscle in hypertrophic cardiomyopathy.

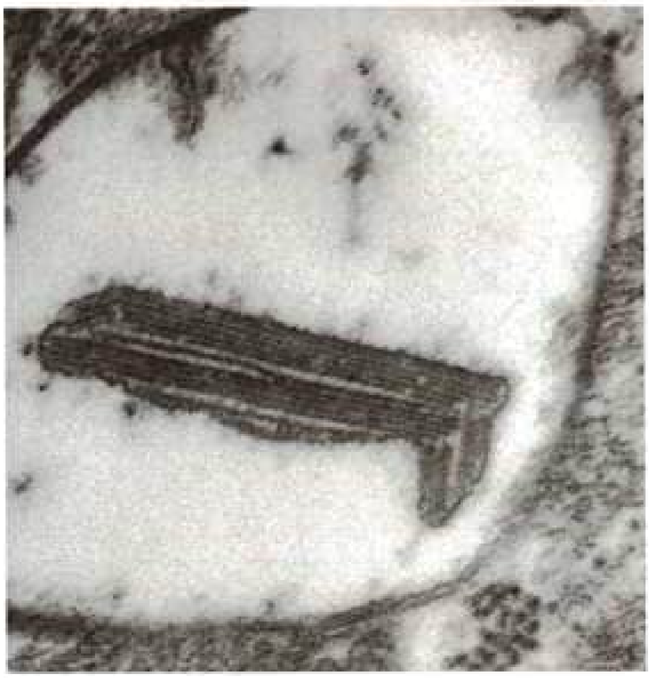


FIGURE 19-43 Paracrystalline inclusions in MERRF syndrome mitochondrion. Electron micrograph of an abnormal mitochondrion from the muscle of an individual with MERRF syndrome, showing the paracrystalline protein inclusions sometimes present in the mutant mitochondria. [From Regionalized Pathology Correlates with Augmentation of mtDNA Copy Numbers in a Patient with Myoclonic Epilepsy with Ragged-Red Fibers (MERRF-Syndrome). *PLOS ONE*, Anja Brinckmann et al., October 20, 2010. https://doi.org/10.1371/journal.pone.0013513]

If a prospective mother is known to carry a pathogenic mitochondrial gene, the technique of mitochondrial donation can circumvent the passage of that mutant gene to her offspring. A prospective mother's nuclear genes are microscopically transplanted into an enucleated ovum from a donor with healthy mitochondria, then the ovum is fertilized in vitro and the resulting embryo is transplanted into the mother's uterus. This and similar "three-parent baby" procedures, which were approved in the United Kingdom in 2015, raise ethical issues that are being vigorously debated.

Mitochondrial disease can also result from mutations in any of the ~1,200 nuclear genes that encode mitochondrial proteins. For example, a mutation in one of the nuclear-encoded proteins of Complex IV, COX6B1, results in severe defects in brain development and thickened walls of the heart muscle. Other nuclear genes encode proteins essential for the *assembly* of mitochondrial complexes. Mutations in these genes can also lead to serious mitochondrial disease. ■

A Rare Form of Diabetes Results from Defects in the Mitochondria of Pancreatic β Cells

The insulin so important to glucose homeostasis in all humans is produced and exported from pancreatic β cells. Insulin export hinges on the ATP concentration in those cells. When blood glucose is high, β cells take up glucose and oxidize it by glycolysis and the citric acid cycle, raising [ATP] above a threshold level (**Fig. 19-44**). When [ATP] exceeds this threshold, an ATP-gated K^+ channel in the plasma membrane closes, depolarizing the membrane and triggering insulin release (see Fig. 23-24).

Normal insulin release can be compromised in several ways. Pancreatic β cells with defects in any aspect of oxidative phosphorylation may not be able to increase

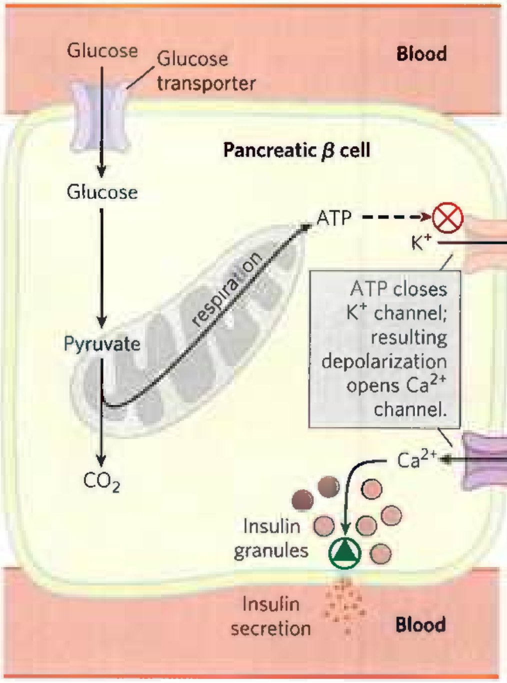


FIGURE 19-44 A mitochondrial defect prevents insulin secretion. In the normal situation, as depicted here, when the blood glucose level rises, production of ATP in β cells increases. ATP, by blocking K^+ channels, depolarizes the plasma membrane and thus opens voltage-gated Ca^{2+} channels. The resulting influx of Ca^{2+} triggers exocytosis of insulin-containing secretory vesicles, releasing insulin. When oxidative phosphorylation in β cells is defective, [ATP] is never sufficient to trigger this process, and insulin is not released.

[ATP] above this threshold, and the resulting failure of insulin release effectively produces diabetes. For example, defects in the gene for glucokinase, the hexokinase IV isozyme present in β cells, lead to a rare form of diabetes called MODY2 (*m*aturity *o*nset *d*iabetes of the *y*oung); low glucokinase activity prevents the generation of ATP concentrations above the threshold needed to trigger insulin secretion. Mutations in the mitochondrial tRNA^{Lys} or tRNA^{Leu} genes also compromise mitochondrial ATP production by limiting the expression of electron transfer components encoded in the mitochondrial DNA. Type 2 diabetes mellitus is common among individuals with these defects (although such cases make up a very small fraction of all cases of diabetes).

When nicotinamide nucleotide transhydrogenase, which is part of the mitochondrial defense against ROS (Fig. 19-18), is genetically defective, the accumulation of ROS damages mitochondria, slowing ATP production and blocking insulin release by β cells (Fig. 19-44). Damage caused by ROS, including damage to mtDNA, may also underlie other human diseases; there is some evidence for its involvement in Alzheimer, Parkinson, and Huntington diseases and in heart failure, as well as in aging. ■

SUMMARY 19.5 *Mitochondrial Genes: Their Origin and the Effects of Mutations*

- A small proportion of human mitochondrial proteins, 13 in all, are encoded by the mitochondrial genome and synthesized in mitochondria. About 1,200 mitochondrial proteins are encoded by nuclear genes and imported into mitochondria after their synthesis.
- Mitochondria arose from aerobic bacteria that entered into an endosymbiotic relationship with ancestral eukaryotes.
- Mutations in the mitochondrial genome accumulate over the life of the organism. Mutations in nuclear or mitochondrial genes that encode components of the respiratory chain, ATP synthase, and the ROS-scavenging system, and even in tRNA genes, can cause a variety of human diseases, which often most severely affect muscle, heart, pancreatic β cells, and brain.
- It is possible to combine the mitochondria from one woman with the nuclear genes of another to create an ovum free of a mutation that would have led to a mitochondrial disease.
- Mitochondrial defects in pancreatic β cells that limit ATP production when glucose levels are high can compromise normal insulin release and give rise to a form of type 2 diabetes.

KEY TERMS

Terms in bold are defined in the glossary.

chemiosmotic theory	659
cristae	660
respiratory chain	661
flavoprotein	662
reducing equivalent	662
ubiquinone (coenzyme Q, Q)	662
cytochromes	663
iron-sulfur protein	664
Rieske iron-sulfur protein	664
Complex I	665
NADH dehydrogenase	666
vectorial	666
Complex II	667
succinate dehydrogenase	667
reactive oxygen species (ROS)	668
superoxide radical ($^\bullet O_2^-$)	668
Complex III	668
cytochrome *bc*$_1$ complex	668
Q cycle	668
Complex IV	669
cytochrome oxidase	669
respirasome	671
proton-motive force	673
ATP synthase	675
F_1 ATPase	677
c ring	680
rotational catalysis	680
binding-change model	681
P/O ratio	682
$P/2e^-$ ratio	682
malate-aspartate shuttle	684
glycerol 3-phosphate shuttle	684
acceptor control	687
mass-action ratio (*Q*)	687
brown adipose tissue (BAT)	690
uncoupling protein 1 (UCP1)	690
cytochrome P-450	690
xenobiotics	691
apoptosis	691
apoptosome	691
caspase	691
heteroplasmy	694
homoplasmy	694

PROBLEMS

1. Oxidation-Reduction Reactions Complex I, the NADH dehydrogenase complex of the mitochondrial respiratory chain, promotes the following series of oxidation-reduction reactions, in which Fe^{3+} and Fe^{2+} represent the iron in iron-sulfur centers, Q is ubiquinone, QH_2 is ubiquinol, and E is the enzyme:

(1) $NADH + H^+ + E\text{-}FMN \longrightarrow NAD^+ + E\text{-}FMNH_2$

(2) $E\text{-}FMNH_2 + 2Fe^{3+} \longrightarrow E\text{-}FMN + 2Fe^{2+} + 2H^+$

(3) $2Fe^{2+} + 2H^+ + Q \longrightarrow 2Fe^{3+} + QH_2$

Sum: $NADH + H^+ + Q \longrightarrow NAD^+ + QH_2$

For each of the three reactions catalyzed by Complex I, identify (a) the electron donor, (b) the electron acceptor, (c) the conjugate redox pair, (d) the reducing agent, and (e) the oxidizing agent.

2. All Parts of Ubiquinone Have a Function In electron transfer, only the quinone portion of ubiquinone undergoes oxidation-reduction; the isoprenoid side chain remains unchanged. What is the function of this chain?

3. Use of FAD Rather Than NAD⁺ in Succinate Oxidation All the dehydrogenases of glycolysis and the citric acid cycle use NAD^+ (E'° for $NAD^+/NADH$ is −0.32 V) as electron acceptor except succinate dehydrogenase, which uses covalently bound FAD (E'° for $FAD^+/FADH_2$ in this enzyme is 0.050 V). Suggest why FAD is a more appropriate electron acceptor than NAD^+ in the dehydrogenation of succinate, based on the E'° values of fumarate/succinate ($E'^\circ = 0.031$ V), $NAD^+/NADH$, and the succinate dehydrogenase $FAD/FADH_2$.

4. Degree of Reduction of Electron Carriers in the Respiratory Chain Mitochondrial conditions determine the degree of reduction of each carrier in the respiratory chain. For example, when NADH and O_2 are abundant, the steady-state degree of reduction of the carriers decreases as electrons pass from the substrate to O_2. When electron transfer is blocked, the carriers before the block become more reduced and those beyond the block become more oxidized (see Fig. 19-6). For each of the mitochondrial conditions listed, predict the state of oxidation of ubiquinone and cytochromes b, c_1, c, and $a + a_3$.

(a) Abundant NADH and O_2, but cyanide added
(b) Abundant NADH, but O_2 exhausted
(c) Abundant O_2, but NADH exhausted
(d) Abundant NADH and O_2

5. Effect of Rotenone and Antimycin A on Electron Transfer Rotenone, a toxic natural product from plants, strongly inhibits NADH dehydrogenase of insect and fish mitochondria. Antimycin A, a toxic antibiotic, strongly inhibits the oxidation of ubiquinol.

(a) Explain why rotenone ingestion is lethal to some insect and fish species.
(b) Explain why antimycin A is a poison.
(c) Given that rotenone and antimycin A are equally effective in blocking their respective sites in the electron-transfer chain, which would be a more potent poison? Explain.

6. Uncouplers of Oxidative Phosphorylation In normal mitochondria, the rate of electron transfer is tightly coupled to the demand for ATP. When the rate of ATP use is relatively low, the rate of electron transfer is low; when demand for ATP increases, the electron-transfer rate increases. Under these conditions of tight coupling, the number of ATP molecules produced per atom of oxygen consumed when NADH is the electron donor—the P/O ratio—is about 2.5.

(a) Predict the effect of a relatively low and a relatively high concentration of uncoupling agent on the rate of electron transfer and the P/O ratio.
(b) Ingestion of uncouplers causes profuse sweating and an increase in body temperature. Explain this phenomenon in molecular terms. What happens to the P/O ratio in the presence of uncouplers?
(c) Physicians used to prescribe the uncoupler 2,4-dinitrophenol (DNP) as a weight-reducing drug. How could this agent, in principle, serve as a weight-reducing aid? Physicians no longer prescribe uncoupling agents, because some deaths occurred following their use. Why might the ingestion of uncouplers cause death?

7. Effects of Valinomycin on Oxidative Phosphorylation When investigators add the antibiotic valinomycin (see Fig. 11-43) to actively respiring mitochondria, several things happen: the yield of ATP decreases, the rate of O_2 consumption increases, heat is released, and the pH gradient across the inner mitochondrial membrane increases. Does valinomycin act as an uncoupler or as an inhibitor of oxidative phosphorylation? Explain the experimental observations in terms of the antibiotic's ability to transfer K^+ ions across the inner mitochondrial membrane.

8. Cellular ADP Concentration Controls ATP Formation Although ATP synthesis requires both ADP and P_i, the rate of synthesis depends mainly on the concentration of ADP, not P_i. Why?

9. Reactive Oxygen Species Describe the role played by superoxide dismutase in ameliorating the effects of reactive oxygen species.

10. How Many Protons in a Mitochondrion? Electron transfer translocates protons from the mitochondrial matrix to the external medium, establishing a pH gradient across the inner membrane (outside more acidic than inside). The tendency of protons to diffuse back into the matrix is the driving force for ATP synthesis by ATP synthase. During oxidative phosphorylation by a suspension of mitochondria in a medium of pH 7.4, the measured pH of the matrix is 7.7.

(a) Calculate $[H^+]$ in the external medium and in the matrix under these conditions.
(b) What is the outside-to-inside ratio of $[H^+]$? How much energy for ATP synthesis is available in this concentration difference. (Hint: See Eqn 11-4, p. 392.)
(c) Calculate the number of protons in a respiring liver mitochondrion, assuming its inner matrix compartment is a sphere of diameter 1.5 μm.
(d) From these data, is the pH gradient alone sufficient to generate ATP?
(e) If not, suggest how the necessary energy for synthesis of ATP arises.

11. Rate of ATP Turnover in Rat Heart Muscle Rat heart muscle operating aerobically fills more than 90% of its ATP needs by oxidative phosphorylation. Each gram of tissue consumes O_2 at the rate of 10.0 μmol/min, with glucose as the fuel source.

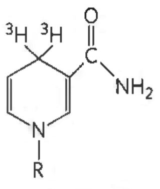
(a) Calculate the rate at which the heart muscle consumes glucose and produces ATP.
(b) For a steady-state ATP concentration of 5.0 μmol/g of heart muscle tissue, calculate the time required (in seconds) to completely turn over the cellular pool of ATP. What does this result indicate about the need for tight regulation of ATP production? (Note: Concentrations are expressed as micromoles per gram of muscle tissue because the tissue is mostly water.)

12. Rate of ATP Breakdown in Insect Flight Muscle ATP production in the flight muscle of the fly *Lucilia sericata* results almost exclusively from oxidative phosphorylation. During flight, maintaining an ATP concentration of 7.0 μmol/g of flight muscle requires 187 mL of O_2/h • g of body weight. Assuming that flight muscle makes up 20% of the fly's weight, calculate the rate at which the flight-muscle ATP pool turns over. How long would the reservoir of ATP last in the absence of oxidative phosphorylation? Assume that the glycerol 3-phosphate shuttle transfers the reducing equivalents and that O_2 is at 25 °C and 101.3 kPa (1 atm).

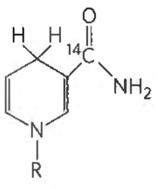
13. High Blood Alanine Level Associated with Defects in Oxidative Phosphorylation Most individuals with genetic defects in oxidative phosphorylation have relatively high concentrations of alanine in their blood. Explain this in biochemical terms.

14. Compartmentalization of Citric Acid Cycle Components Isocitrate dehydrogenase is found only in mitochondria, but malate dehydrogenase is found in both the cytosol and mitochondria. What is the role of cytosolic malate dehydrogenase?

15. Transmembrane Movement of Reducing Equivalents Under aerobic conditions, extramitochondrial NADH must undergo oxidation by the mitochondrial respiratory chain. Consider a preparation of rat hepatocytes containing mitochondria and all the cytosolic enzymes. After the introduction of [4-^{3}H]NADH, radioactivity soon appears in the mitochondrial matrix. Conversely, no radioactivity appears in the matrix after the introduction of [7-^{14}C]NADH. What do these observations reveal about the oxidation of extramitochondrial NADH by the respiratory chain?



[4-^{3}H]NADH



[7-^{14}C]NADH

16. NAD Pools and Dehydrogenase Activities Although both pyruvate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase use NAD$^+$ as their electron acceptor, the two enzymes do not compete for the same cellular NAD pool. Why?

17. The Malate–α-Ketoglutarate Transport System *n*-Butylmalonate inhibits the transport system that conveys malate and α-ketoglutarate across the inner mitochondrial membrane (see Fig. 19-31). Suppose you add *n*-butylmalonate to an aerobic suspension of kidney cells using exclusively glucose as fuel. Predict the effect of this inhibitor on **(a)** glycolysis, **(b)** oxygen consumption, **(c)** lactate formation, and **(d)** ATP synthesis.

18. Time Scales of Regulatory Events in Mitochondria Compare the likely time scales for the adjustments in respiratory rate caused by **(a)** increased [ADP] and **(b)** reduced pO_2. What accounts for the difference?

19. The Pasteur Effect When investigators add O_2 to an anaerobic suspension of cells consuming glucose at a high rate, the rate of glucose consumption declines greatly as the cells consume the O_2, and accumulation of lactate ceases. This effect, first observed by Louis Pasteur in the 1860s, is characteristic of most cells capable of both aerobic and anaerobic glucose catabolism.
(a) Why does the accumulation of lactate cease after the addition of O_2?
(b) Why does the presence of O_2 decrease the rate of glucose consumption?
(c) How does the onset of O_2 consumption slow down the rate of glucose consumption? Explain in terms of specific enzymes.

20. Respiration-Deficient Yeast Mutants and Ethanol Production Researchers can produce respiration-deficient yeast mutants (p$^-$; "petites") from wild-type parents by treatment with mutagenic agents. The mutants lack cytochrome oxidase, a deficit that markedly affects their metabolic behavior. One striking effect is that fermentation is not suppressed by O_2—that is, the mutants do not experience the Pasteur effect (see Problem 19). Some companies are very interested in using these mutants to ferment wood chips to ethanol for energy use. Why does the absence of cytochrome oxidase eliminate the Pasteur effect? Explain the advantages of using these mutants rather than wild-type yeast for large-scale ethanol production.

21. Mitochondrial Disease and Cancer Mutations in the genes that encode certain mitochondrial proteins are associated with a high incidence of some types of cancer. How might defective mitochondria lead to cancer?

22. Variable Severity of a Mitochondrial Disease Different individuals with a disease caused by the same specific defect in the mitochondrial genome may have symptoms ranging from mild to severe. Explain why.

23. Diabetes as a Consequence of Mitochondrial Defects Glucokinase is essential in the metabolism of glucose in pancreatic β cells. Humans with two defective copies of the glucokinase gene exhibit a severe, neonatal diabetes, whereas those with only one defective copy of the gene have a much milder form of the disease (maturity onset diabetes of the young, MODY2). Explain this difference in terms of the biology of the β cell.

24. Effects of Mutations in Mitochondrial Complex II Single nucleotide changes in the gene for succinate dehydrogenase (Complex II) are associated with midgut carcinoid tumors. Suggest a mechanism to explain this observation.

DATA ANALYSIS PROBLEM

25. Membrane Fluidity and Respiration Rate The mitochondrial electron transfer complexes and the F_oF_1 ATP synthase are embedded in the inner mitochondrial membrane in eukaryotes and in the inner membrane of bacteria. Electrons are shuttled between complexes in part by coenzyme Q, or ubiquinone, a factor that migrates within the membrane. Jay Keasling and coworkers explored the effect of membrane fluidity on rates of respiration in *E. coli*.

E. coli naturally adjusts its membrane lipid content to maintain membrane fluidity at different temperatures. Workers in the Keasling lab bioengineered an *E. coli* strain to allow them to control expression of the enzyme FabB, which catalyzes the limiting step in the synthesis of unsaturated fatty acids in *E. coli*.

(a) How does the content of unsaturated fatty acids affect membrane fluidity?

(b) The researchers were able to modulate the content of unsaturated fatty acids in the membrane lipid from 15% to 80%. They did not try to completely block synthesis of unsaturated fatty acids to extend the experimental range in the membrane to 0%. Why not?

(c) When the cells were grown under aerobic conditions, the researchers found that bacterial growth rate increased as the concentration of unsaturated fatty acids in the membrane increased. However, when oxygen was very limited, the unsaturated fatty acid content of the membrane had no effect on growth rate. How might you explain this observation?

(d) The researchers measured rates of respiration, finding a strong correlation between those rates and the fraction of membrane fatty acids that was unsaturated. When the unsaturated fatty acid content of the membranes was kept low, the cells accumulated pyruvate and lactate. Explain these observations.

(e) Next, they measured rates of diffusion of membrane phospholipids and ubiquinone in vesicles derived from *E. coli* membranes. The diffusion rates increased as a function of the content of unsaturated fatty acids. These measured rates were consistent with simulations carried out to model the effects of ubiquinone diffusion on respiration. What overall conclusion can be drawn from this work?

Reference

Budin, I, T. de Rond, Y. Chen, L.J.G. Chan, C.J. Petzold, and J.D. Keasling. 2018. Viscous control of cellular respiration by membrane lipid composition. *Science* 362:1186–1189.

Chapter 20

PHOTOSYNTHESIS AND CARBOHYDRATE SYNTHESIS IN PLANTS

20.1 Light Absorption *701*

20.2 Photochemical Reaction Centers *707*

20.3 Evolution of a Universal Mechanism of ATP Synthesis *716*

20.4 CO_2-Assimilation Reactions *719*

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20.6 Biosynthesis of Starch, Sucrose, and Cellulose *733*

We have now reached a turning point in our study of cellular metabolism. Thus far in Part II we have described how the major metabolic fuels—carbohydrates, fatty acids, and amino acids—are degraded through *converging catabolic* pathways that lead to the citric acid cycle and yield their electrons to the respiratory chain, driving ATP synthesis by *oxidative* phosphorylation. We now turn to *reductive*, *anabolic*, *divergent* processes fueled by energy from the sun that take place in photosynthetic organisms, and in all other organisms, driven ultimately by the photosynthetic reduction of CO_2.

As we examine this process, these principles will emerge:

P1 **The capture of solar energy by photosynthetic organisms and its conversion to the chemical energy of reduced organic compounds is the ultimate source of nearly all biological energy and organic nutrients for all of the nonphotosynthetic organisms, including humans.** It is arguably *the most important biochemical process in the biosphere.*

P2 **Photosynthetic organisms use tightly organized light-harvesting complexes to absorb sunlight and capture its energy in chemical form: a separation of positive and negative charge leading to electron flow.** The energy from an absorbed photon moves from one antenna chlorophyll to another and another until it arrives at the reaction center where it promotes the photochemical reaction that sends electrons through a series of electron carriers.

P3 **The light-driven flow of electrons through specialized protein carriers is coupled to ATP synthesis.** A strong reducing agent (NADPH) is also produced, and simultaneously, water is oxidized to O_2, which is released into the atmosphere.

P4 **Evolution yielded a universal mechanism for coupling ATP synthesis to the flow of electrons.** A proton gradient created by electron flow is used to energize the ATP-synthesizing enzyme in microorganisms, animals, and plants.

P5 **The ATP and NADPH produced in the light-dependent reactions of photosynthesis provide the energy and the reducing power to convert atmospheric CO_2 into simple organic compounds.** High concentrations of ATP and NADPH allow the chloroplast to carry out redox reactions that are thermodynamically unfavorable.

Photosynthesis encompasses two processes: the **light-dependent reactions**, in which sunlight provides the energy for the synthesis of ATP and NADPH, and the CO_2-assimilation reactions, in which ATP and NADPH are used to reduce CO_2 to form triose phosphates via a set of reactions known as the Calvin cycle (**Fig. 20-1**). We heterotrophs are alive because the enormous energy of sunlight has been captured and tamed by autotrophs by photosynthesis and made available to us as fuel, vitamins, and building blocks. How do they do it?

All vascular plants, as well as algae and cyanobacteria, carry out the same basic process of photosynthesis, but

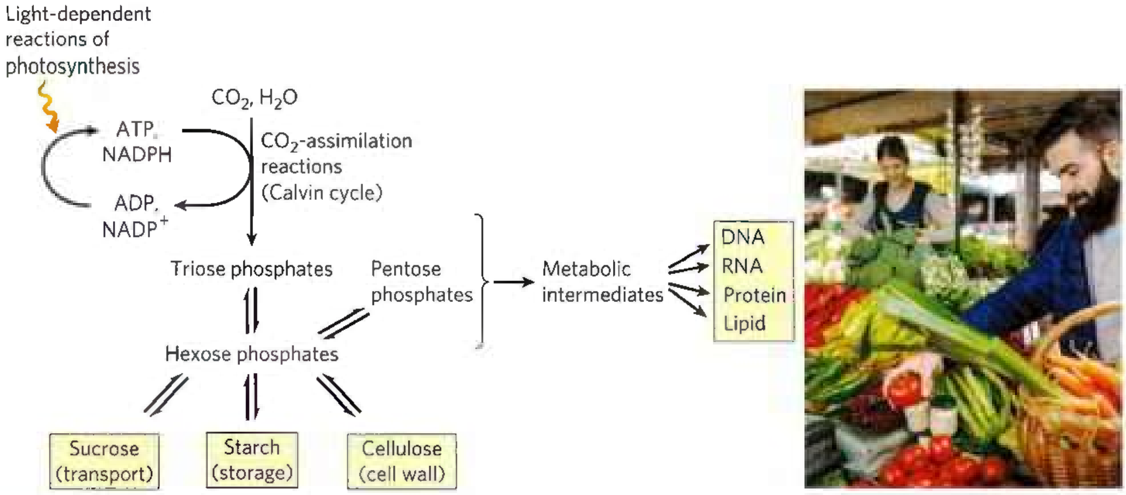


FIGURE 20-1 Assimilation of CO_2 provides all of the carbon a plant needs. The light-driven synthesis of ATP and NADPH provides energy and reducing power for the fixation of CO_2 into trioses in the Calvin cycle. All of the carbon-containing compounds of the plant cell are synthesized from this fixation of CO_2. [Emir Memedovski/Getty Images]

some are more amenable to study than others. Algae and cyanobacteria have been extensively studied because of the relative ease of culturing and manipulating them in the laboratory. Spinach is a vascular plant commonly used for studies of photosynthesis because of the ease of obtaining large amounts of material; and for genetic approaches, the small plant *Arabidopsis thaliana* is a favorite. What we say here about photosynthesis is essentially true of photosynthesis in all of these organisms.

After looking at photosynthesis, we will discuss the conversion of trioses produced in the Calvin cycle to sucrose (for sugar transport) and starch (for energy storage) (see Fig. 20-1). This conversion is accomplished by mechanisms analogous to those used by animal cells to make glycogen. We also describe the synthesis of the cellulose of plant cell walls. Finally, we consider how carbohydrate metabolism is integrated within a plant cell and throughout the plant.

Although strikingly different on the surface, the processes of photophosphorylation in the chloroplast and oxidative phosphorylation in the mitochondrion are closely similar at the molecular level, and the mechanism for ATP synthesis is virtually identical: a proton gradient drives rotary catalysis by a remarkable ATP synthase.

20.1 Light Absorption

Photophosphorylation (ATP synthesis driven by light) resembles oxidative phosphorylation in that electron flow through a series of membrane carriers is coupled to proton pumping, producing the proton motive force that powers ATP formation. The processes are compared in **Figure 20-2**. In oxidative phosphorylation, the electron donor is NADH and the ultimate electron acceptor is O_2, forming H_2O. In photophosphorylation, electrons flow in the opposite direction: H_2O is the electron *donor* and NADPH is *formed*. How is this endergonic process possible?

Water is a poor donor of electrons; its standard reduction potential is 0.816 V, compared with −0.320 V for NADH, a good electron donor. **P1** Photosynthesis requires the input of energy in the form of light to *create* a good electron donor and a good electron acceptor. **P3** Electrons flow from the electron donor through a series of membrane-bound carriers, including cytochromes, quinones, and iron-sulfur proteins, while protons are pumped across a membrane to create an electrochemical potential. Electron transfer and proton pumping are catalyzed by a membrane complex that is homologous in structure and function to Complex III of mitochondria. The electrochemical potential so produced is the driving force for ATP synthesis from ADP and P_i, catalyzed by a membrane-bound ATP synthase complex closely similar to that of mitochondria and bacteria.

Chloroplasts Are the Site of Light-Driven Electron Flow and Photosynthesis in Plants

In photosynthetic eukaryotic cells, both the light-dependent and the CO_2-assimilation reactions take place in **chloroplasts** (**Fig. 20-3**), organelles that are variable in shape and generally a few micrometers in diameter. Like mitochondria, chloroplasts are surrounded by two membranes: an outer membrane that is permeable to small molecules and ions, and an impermeable inner membrane that bears specific transporters for a variety of ions and metabolites. The space enclosed by the inner membrane is called the **stroma** in chloroplasts and is analogous to the mitochondrial matrix; it is an aqueous phase containing most of the soluble enzymes required for the CO_2-assimilation reactions. Throughout the stroma is a highly organized set of topologically continuous internal membranes, forming a single compartment or lumen. This complex membrane system forms flattened sacks called **thylakoids**. **Granal thylakoids** are disk-like pouches arranged in stacks;

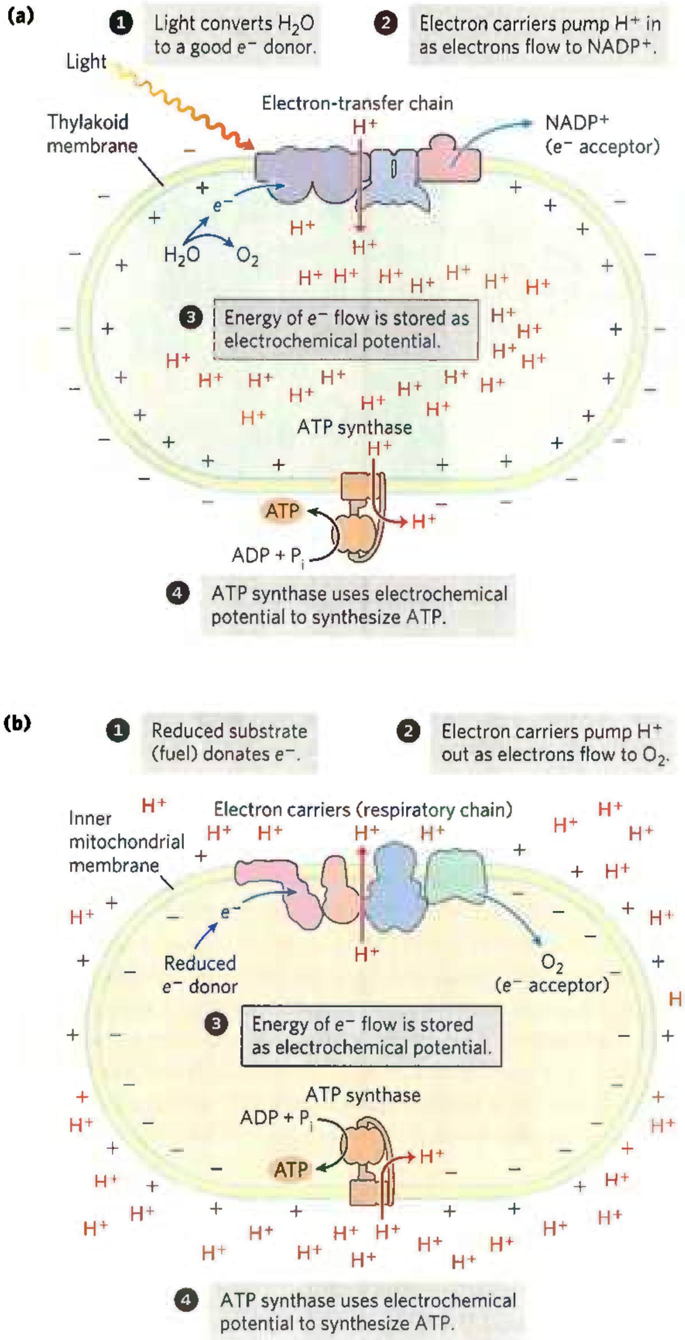

(a)
① Light converts H_2O to a good e⁻ donor.
② Electron carriers pump H^+ in as electrons flow to $NADP^+$.
Light
Electron-transfer chain
Thylakoid membrane
$NADP^+$ (e⁻ acceptor)
e⁻
H_2O
O_2
H^+
③ Energy of e⁻ flow is stored as electrochemical potential.
ATP synthase
ATP
ADP + P_i
④ ATP synthase uses electrochemical potential to synthesize ATP.
(b)
① Reduced substrate (fuel) donates e⁻.
② Electron carriers pump H^+ out as electrons flow to O_2.
Inner mitochondrial membrane
Electron carriers (respiratory chain)
Reduced e⁻ donor
O_2 (e⁻ acceptor)
③ Energy of e⁻ flow is stored as electrochemical potential.
ATP synthase
ADP + P_i
ATP
④ ATP synthase uses electrochemical potential to synthesize ATP.

FIGURE 20-2 The chemiosmotic mechanism for ATP synthesis in chloroplasts and mitochondria. (a) Movement of electrons through a chain of membrane-bound carriers in the chloroplast membrane is driven by the energy of photons absorbed by the green pigment chlorophyll. Electron flow leads to the movement of protons and positive charge across the membrane, creating an electrochemical potential. This electrochemical potential drives ATP synthesis by the membrane-bound ATP synthase, which is fundamentally similar in structure and mechanism to (b) the mitochondrial machinery for oxidative phosphorylation of mitochondria. In mitochondria, the force that moves electrons through the complexes is a large difference in the reduction potentials of electron donor and acceptor. In both systems, the energy made available by electron transfer is captured as a transmembrane proton gradient, which drives ATP synthesis by an ATP synthase.

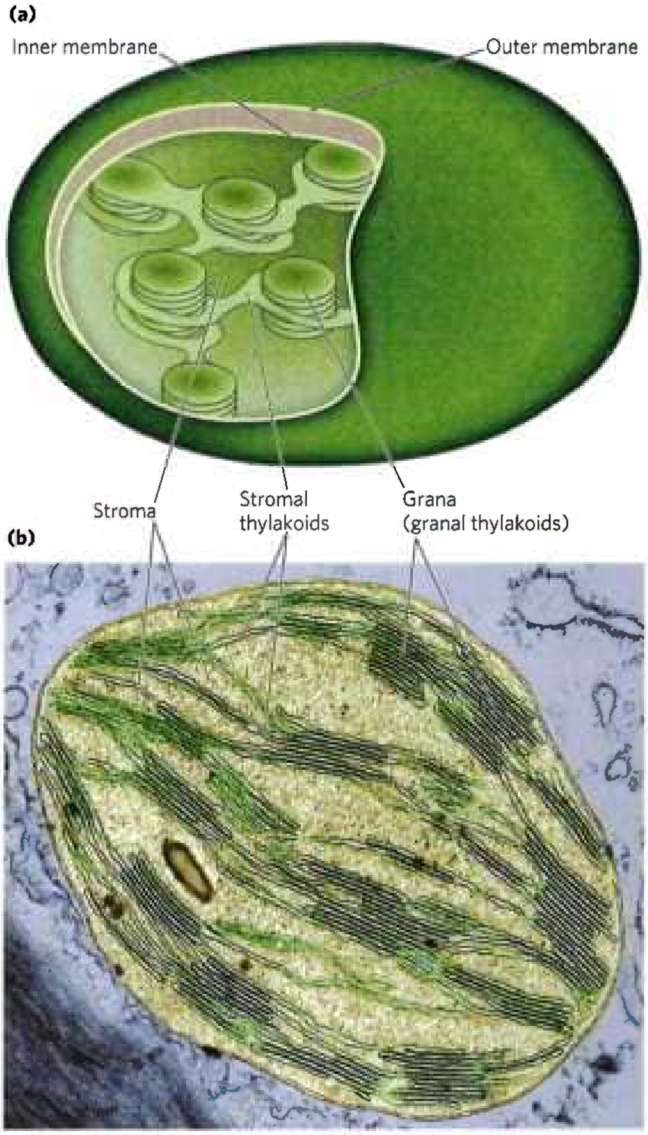

(a)
Inner membrane
Outer membrane
Stroma
Stromal thylakoids
Grana (granal thylakoids)
(b)

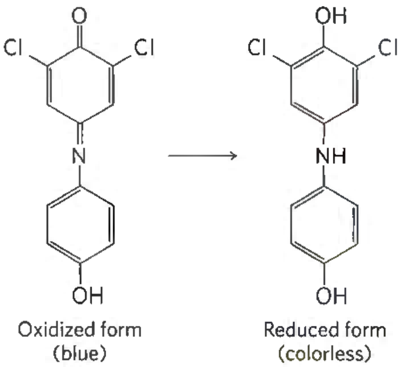
FIGURE 20-3 Chloroplast structure. (a) Schematic diagram. (b) Colorized electron micrograph at high magnification, showing the highly organized thylakoid membrane system. [(b) Biophoto Associates/Science Source]

they are connected by **stromal thylakoids**, which are flatter and spiral around a stack of grana. The thylakoid membranes provide a large area for the machinery of photophosphorylation—the photosynthetic pigments and enzyme complexes that carry out the light-dependent reactions and ATP synthesis. Traffic across these membranes is also mediated by specific transporters.

In 1937, Robert Hill found that when leaf extracts containing chloroplasts were illuminated, they (1) evolved O_2 and (2) reduced a nonbiological electron acceptor added to the medium, according to the Hill reaction

$$2H_2O + 2A \xrightarrow{\text{light}} 2AH_2 + O_2$$

where A is an artificial electron acceptor, or Hill reagent. One Hill reagent, the dye 2,6-dichlorophenolindophenol, is blue when oxidized (A) and colorless when reduced (AH_2), making the reaction easy to follow.



2,6-Dichlorophenolindophenol

When a leaf extract supplemented with the dye was illuminated, the blue dye became colorless and O_2 was evolved. In the dark, no O_2 evolution or dye reduction took place. **P2** This was the first evidence that absorbed light energy causes electrons to flow from some electron donor (now known to be H_2O) to an electron acceptor. Moreover, Hill found that CO_2 was neither required nor reduced to a stable form under these conditions; O_2 production could be dissociated from CO_2 reduction. Several years later, Severo Ochoa showed that $NADP^+$ is the biological electron acceptor in chloroplasts, according to the equation

$$2H_2O + 2NADP^+ \xrightarrow{\text{light}} 2NADPH + 2H^+ + O_2$$

To understand this photochemical process, we must first consider the more general topic of the effects of light absorption on molecular structure.

Visible light is electromagnetic radiation of wavelengths 400 to 700 nm, a small part of the electromagnetic spectrum (**Fig. 20-4**), ranging from violet to red. The energy of a single **photon** (a quantum of light) is greater at the violet end of the spectrum than at the red end; shorter wavelength (and higher frequency) corresponds to higher energy. The energy, E, in a single photon of visible light is given by the Planck equation:

$$E = h\nu = hc/\lambda$$

where h is Planck's constant (6.626×10^{-34} J • s), ν is the frequency of the light in cycles/s, c is the speed of light (3.00×10^8 m/s), and λ is the wavelength of the light in meters. The energy of a photon of visible light ranges from 150 kJ/einstein for red light to ~300 kJ/einstein for violet light.

WORKED EXAMPLE 20-1 *Energy of a Photon*

The light used by vascular plants for photosynthesis has a wavelength of about 700 nm. Calculate the energy in a "mole" of photons (an einstein) of light of this wavelength, and compare this with the energy needed to synthesize a mole of ATP.

SOLUTION: The energy in a single photon is given by the Planck equation. At a wavelength of 700×10^{-9} m, the energy of a photon is

$$\begin{aligned} E &= hc/\lambda \\ &= \frac{[(6.626 \times 10^{-34}\ \text{J} \cdot \text{s})(3.00 \times 10^8\ \text{m/s})]}{(7.00 \times 10^{-7}\ \text{m})} \\ &= 2.84 \times 10^{-19}\ \text{J} \end{aligned}$$

An einstein of light is Avogadro's number of photons (6.022×10^{23}); thus the energy of one einstein of photons at 700 nm is given by

$$\begin{aligned} (2.84 \times 10^{-19}\ \text{J/photon})(6.022 \times 10^{23}\ \text{photons/einstein}) \\ = 17.1 \times 10^4\ \text{J/einstein} \\ = 171\ \text{kJ/einstein} \end{aligned}$$

So, a "mole" of photons of red light has about five times the energy needed to produce a mole of ATP from ADP and P_i (30.5 kJ/mol).

When a photon is absorbed, an electron in the absorbing molecule (chromophore) is lifted to a higher energy level. This is an all-or-nothing event: to be absorbed, the photon must contain a quantity of energy, called a **quantum**, that exactly matches the energy of the electronic transition. A molecule that has absorbed a photon is in an **excited state**, which is generally unstable. An electron lifted into a

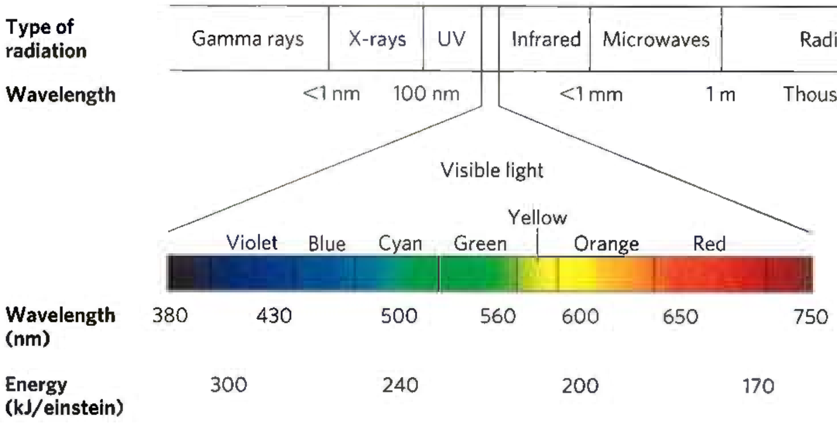
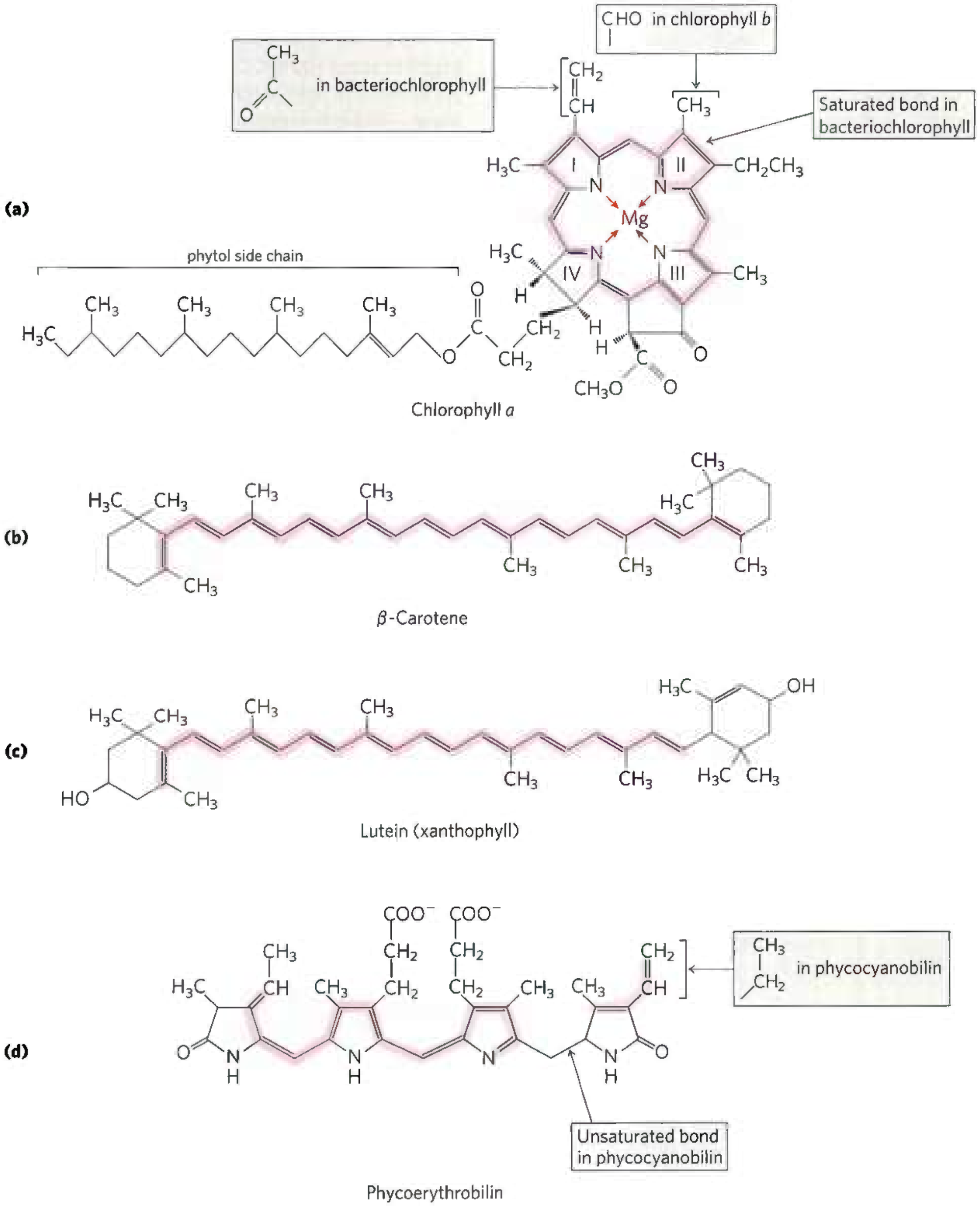


FIGURE 20-4 Electromagnetic radiation. The spectrum of electromagnetic radiation, and the energy of photons in the visible range. One einstein is 6.022×10^{23} photons.

higher-energy orbital usually returns rapidly to its lower-energy orbital; that is, the excited molecule decays to the stable **ground state**, giving up the absorbed quantum as light or heat or using it to do chemical work. Light emission accompanying decay of excited molecules, **fluorescence**, is always at a longer wavelength (lower energy) than that of the absorbed light (see Box 12-1). An alternative mode of decay, central to photosynthesis, involves direct transfer of excitation energy from an excited molecule to a neighboring molecule. Just as the photon is a quantum of light energy, so the **exciton** is a quantum of energy passed from an excited molecule to another molecule in a process called **exciton transfer**.

Chlorophylls Absorb Light Energy for Photosynthesis

The most important light-absorbing pigments in the thylakoid membranes are the **chlorophylls**, green pigments with polycyclic, planar structures resembling the protoporphyrin of hemoglobin, except that Mg^{2+}, not Fe^{2+}, occupies the central position (**Fig. 20-5a**; compare to Fig. 5-1).



Chlorophyll *a*

β-Carotene

Lutein (xanthophyll)

Phycoerythrobilin

FIGURE 20-5 Primary and secondary photopigments. (a) Chlorophylls *a* and *b* and bacteriochlorophyll are the primary gatherers of light energy. (b) β-Carotene (a carotenoid) and (c) lutein (a xanthophyll) are accessory pigments in plants. (d) Phycoerythrobilin and phycocyanobilin (phycobilins) are accessory pigments in cyanobacteria and red algae. The conjugated bond systems in these molecules (alternating single and double bonds, shaded) have delocalized electrons that are easily excited by photons with the wavelengths of visible light.

The four inward-oriented nitrogen atoms of chlorophyll are coordinated with the Mg^{2+}. All chlorophylls have a long phytol side chain, esterified to a carboxyl-group substituent in ring IV, and chlorophylls also have a fifth five-membered ring not present in heme.

The heterocyclic five-ring system that surrounds the Mg^{2+} has an extended polyene structure, with alternating single and double bonds. Such polyenes characteristically show strong absorption in the visible region of the spectrum (**Fig. 20-6**); the chlorophylls have unusually high molar extinction coefficients (see Box 3-1) and are therefore particularly well-suited for absorbing visible light during photosynthesis.

Chloroplasts always contain both chlorophyll *a* and chlorophyll *b* (Fig. 20-5a). Although both are green, their absorption spectra are sufficiently different (Fig. 20-6) that they complement each other's range of light absorption in the visible region. Most plants contain about twice as much chlorophyll *a* as chlorophyll *b*. The chlorophyll in cyanobacteria differs only slightly from those of plants.

In addition to chlorophylls, thylakoid membranes of plants contain secondary light-absorbing pigments, or **accessory pigments**, called carotenoids. **Carotenoids** may be yellow, red, or purple. The two most prominent in plant leaves are **β-carotene**, a red-orange isoprenoid, and the yellow carotenoid lutein (Fig. 20-5b, c). Cyanobacteria and red algae use the accessory pigments phycocyanobilin and phycoerythrobilin (Fig. 20-5d). Accessory pigments absorb light at wavelengths not absorbed by the chlorophylls (Fig. 20-6) and thus are supplementary light receptors. They also protect downstream components from a highly reactive form of oxygen (singlet oxygen) that is formed when intense light exceeds the system's capacity to accept electrons.

Experimental determination of the effectiveness of light of different colors in promoting photosynthesis yields an **action spectrum** (**Fig. 20-7**), often useful in identifying the pigment primarily responsible for a biological effect of light. By capturing light in a region of the spectrum not used by other organisms, a photosynthetic organism can claim a unique ecological niche.

Chlorophylls Funnel Absorbed Energy to Reaction Centers by Exciton Transfer

The light-absorbing pigments of thylakoid or bacterial membranes are arranged in functional arrays called **photosystems**. In spinach chloroplasts, for example, each photosystem contains about 200 chlorophyll and 50 carotenoid molecules. All the pigment molecules in a photosystem can absorb photons, but **P2** only one pair of chlorophyll molecules associated with the **photochemical reaction center** is specialized to transduce light into chemical energy. The other pigment molecules in a photosystem serve as **antenna molecules**. They absorb light energy and transmit it rapidly and efficiently to the reaction center (**Fig. 20-8**). Some chlorophylls are part of a core complex around the reaction center. Others form **light-harvesting complexes (LHCs)** around the periphery of the core complex. Chlorophyll and other pigments are always associated with specific binding proteins, which fix the chromophores in relation to each other, to other protein complexes, and to the membrane. For example, each monomer of the trimeric light-harvesting complex LHCII (**Fig. 20-9**) contains seven molecules of chlorophyll *a*, five of chlorophyll *b*, and two of lutein.

The chlorophyll molecules in light-harvesting complexes and other chlorophyll-binding proteins have light-absorption properties that are subtly different from those of free chlorophyll. When isolated chlorophyll molecules are excited by light, the absorbed energy is quickly released as fluorescence and heat; but when chlorophyll

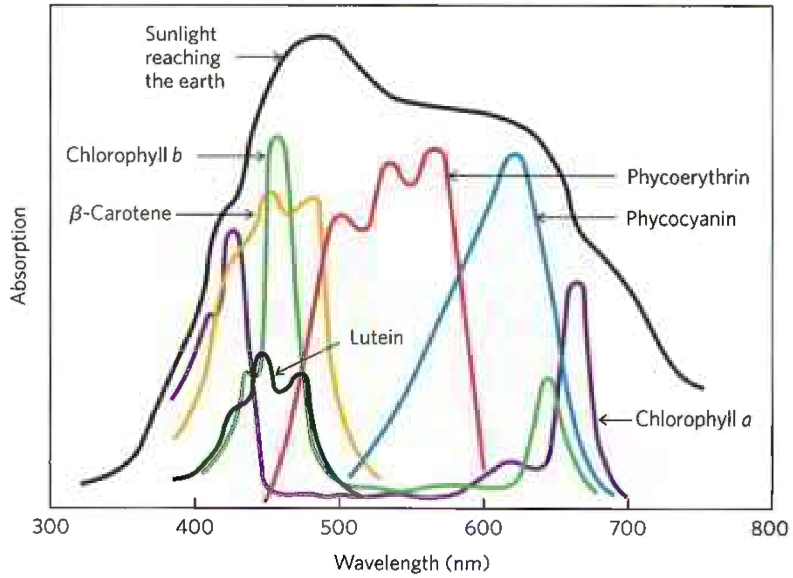


FIGURE 20-6 Absorption of visible light by photopigments. Plants are green because their pigments absorb light from the red and blue regions of the spectrum, leaving primarily green light to be reflected. Compare the absorption spectra of the pigments with the spectrum of sunlight reaching the earth's surface; the combination of chlorophylls (*a* and *b*) and accessory pigments enables plants to harvest most of the energy available in sunlight. The relative amounts of chlorophylls and accessory pigments are characteristic of a particular plant species. Variation in the proportions of these pigments is responsible for the range of colors of photosynthetic organisms, from the deep blue-green of spruce needles, to the greener green of maple leaves, to the red, brown, or purple color of some species of multicellular algae and the leaves of some foliage plants favored by gardeners.

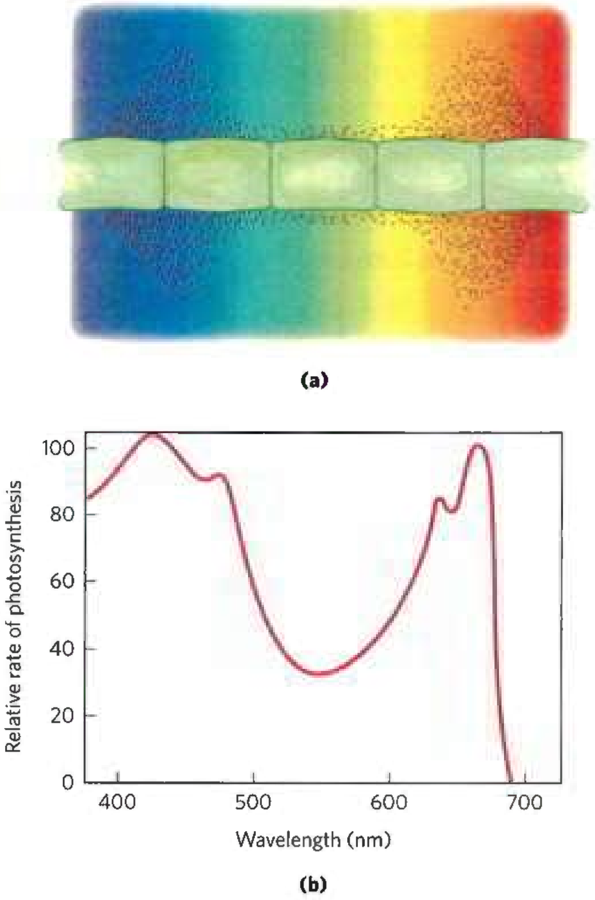


FIGURE 20-7 Two ways to determine the action spectrum for photosynthesis. (a) Results of a classic experiment performed by T. W. Engelmann in 1882 to determine the wavelength of light that is most effective in supporting photosynthesis. Engelmann placed cells of a filamentous photosynthetic alga on a microscope slide and illuminated them with light from a prism, so that one part of the algal filament received mainly blue light, another part yellow, another red. To determine which cells carried out photosynthesis most actively, Engelmann also placed on the microscope slide bacteria known to migrate toward regions of high O_2 concentration. After a period of illumination, the distribution of bacteria showed highest O_2 levels (produced by photosynthesis) in the regions illuminated with violet and red light. (b) Results of a similar experiment that used modern techniques (an oxygen electrode) for the measurement of O_2 production. An action spectrum, as shown here, describes the relative rate of photosynthesis for illumination with a constant number of photons of different wavelengths. An action spectrum is useful because, by comparison with absorption spectra (such as those in Fig. 20-6), it suggests which pigments can channel energy into photosynthesis.

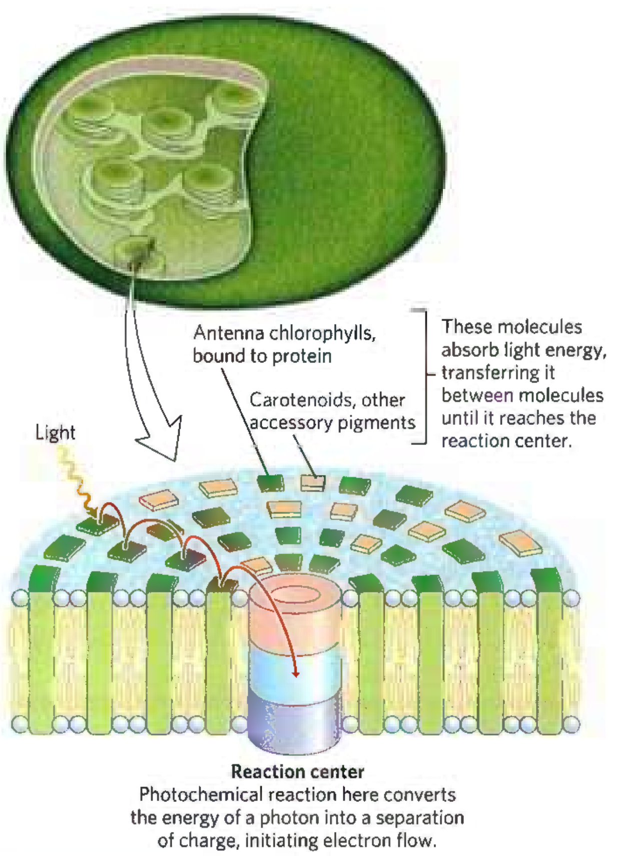

Antenna chlorophylls, bound to protein
Carotenoids, other accessory pigments
These molecules absorb light energy, transferring it between molecules until it reaches the reaction center.
Light
Reaction center
Photochemical reaction here converts the energy of a photon into a separation of charge, initiating electron flow.

FIGURE 20-8 Organization of photosystems in the thylakoid membrane. Photosystems are tightly packed in the thylakoid membrane, with several hundred antenna chlorophylls and accessory pigments surrounding each reaction center. Absorption of a photon by any of the antenna chlorophylls leads to excitation of the reaction center by exciton transfer (red arrow).

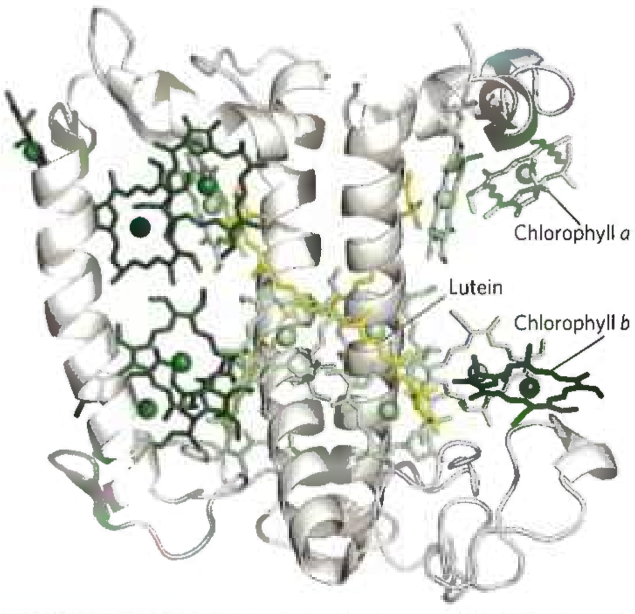

Chlorophyll *a*
Lutein
Chlorophyll *b*

FIGURE 20-9 The light-harvesting complex LHCII of the pea. The functional unit is a trimer, with 36 chlorophyll and 6 lutein molecules. Shown here is a monomer, viewed in the plane of the membrane, with its three transmembrane α-helical segments, seven chlorophyll *a* molecules (light green), five chlorophyll *b* molecules (dark green), and two molecules of lutein (yellow), which form an internal cross-brace. [Data from PDB ID 2BHW, J. Standfuss et al., *EMBO J.* 24:919, 2005.]

in intact leaves is excited by visible light (**Fig. 20-10**, step ❶), very little fluorescence is observed. Instead, the excited antenna chlorophyll transfers energy directly to a neighboring chlorophyll molecule, which becomes excited as the first molecule returns to its ground state (step ❷). This transfer of energy, exciton transfer, extends to a third, fourth, or subsequent neighbor, until one of a "special pair" of chlorophyll *a* molecules at the photochemical reaction center is excited (step ❸). The special pair of chlorophyll molecules, often designated $(Chl)_2$, are held close enough

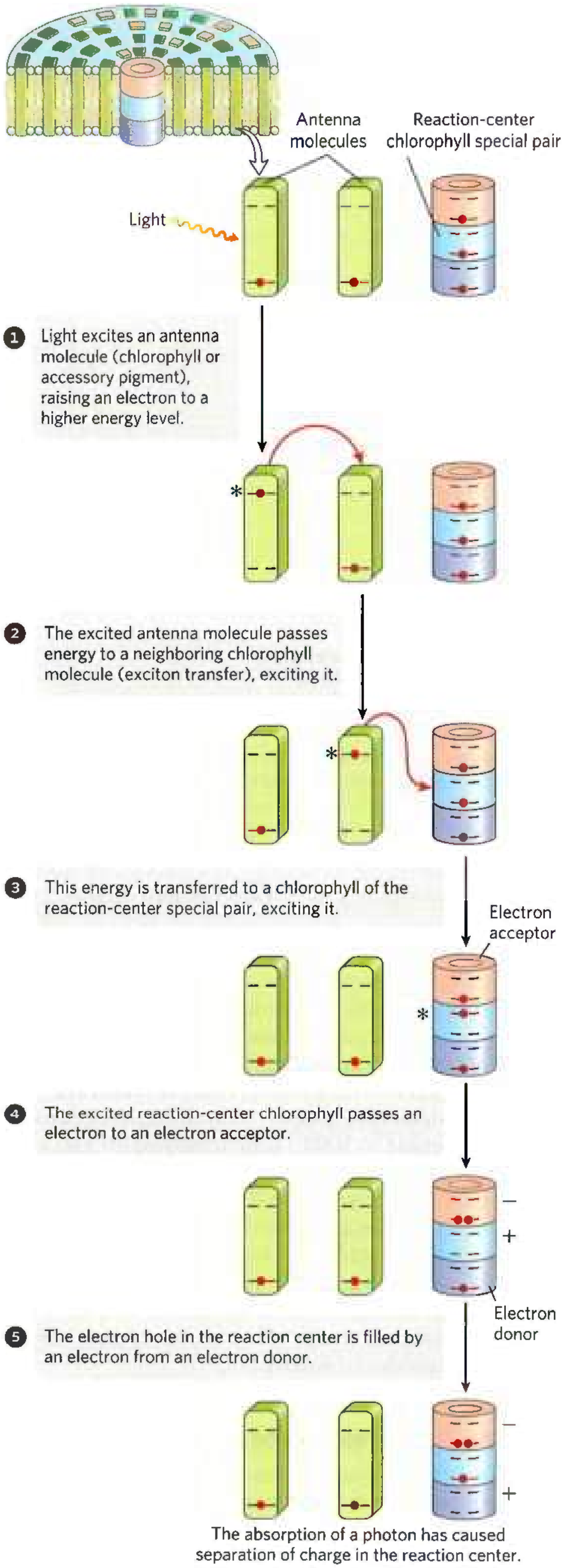

Antenna molecules
Reaction-center chlorophyll special pair
Light
① Light excites an antenna molecule (chlorophyll or accessory pigment), raising an electron to a higher energy level.
*
② The excited antenna molecule passes energy to a neighboring chlorophyll molecule (exciton transfer), exciting it.
*
③ This energy is transferred to a chlorophyll of the reaction-center special pair, exciting it.
Electron acceptor
*
④ The excited reaction-center chlorophyll passes an electron to an electron acceptor.
–
+
Electron donor
⑤ The electron hole in the reaction center is filled by an electron from an electron donor.
–
+
The absorption of a photon has caused separation of charge in the reaction center.

FIGURE 20-10 Exciton and electron transfer. This generalized scheme shows conversion of the energy of an absorbed photon into separation of charges at the reaction center. Note that step ① may repeat between successive antenna molecules until the exciton reaches the special pair of chlorophylls in the reaction center. An asterisk (*) denotes the excited state of a molecule.

to each other to share bonding orbitals, and to react as a single compound when excited. In this excited chlorophyll pair, an electron is promoted to a higher-energy orbital. This electron then passes to a nearby electron acceptor that is part of the photosynthetic electron-transfer chain, leaving the reaction-center chlorophyll pair with a missing electron (an "electron hole," denoted by + in Fig. 20-10) (step ④). The electron acceptor acquires a negative charge in this transaction. The electron lost by the reaction-center chlorophyll pair is replaced by an electron from a neighboring electron-donor molecule (step ⑤), which thereby becomes positively charged. **P1** **P2** In this way, *excitation by light causes electric charge separation and initiates an oxidation-reduction chain.*

SUMMARY 20.1 *Light Absorption*

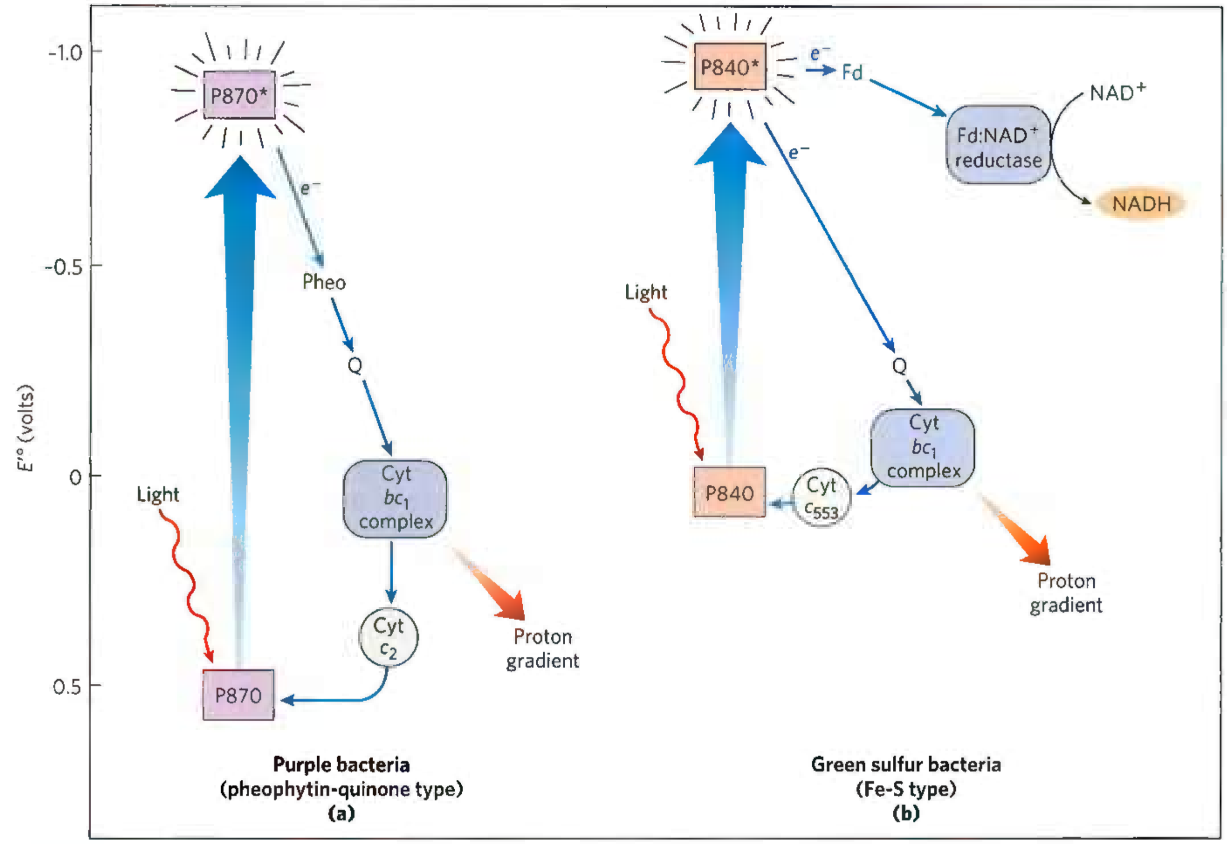
- Photosynthesis takes place in plant chloroplasts, structures enclosed in double membranes and filled with an elaborate system of thylakoid membranes containing the photosynthetic machinery.
- Chlorophyll molecules and other light-absorbing pigments are associated with proteins in light-harvesting complexes arrayed around photochemical reaction centers. The proteins are embedded in thylakoid membranes.
- The many chlorophyll molecules that surround the reaction center serve as antennas for light. When they absorb light, they pass its energy (exciton) to the reaction center. There the energy is used to create a charge separation that initiates electron flow through a series of oxidation-reduction reactions.

20.2 Photochemical Reaction Centers

Studies on a variety of bacteria that carry out photosynthesis have been helpful in understanding the mechanisms of photosynthesis in cyanobacteria, algae and vascular plants. Photosynthetic bacteria have relatively simple phototransduction machinery, with one of two general types of photosystems. Both systems send electrons through a cytochrome complex that pumps protons, producing the electrochemical gradient that drives ATP synthesis.

Photosynthetic Bacteria Have Two Types of Reaction Center

The **type II photosystem** in purple bacteria consists of three basic modules (**Fig. 20-11a**): a single P870 reaction center; a cytochrome bc_1 electron-transfer



Purple bacteria (pheophytin-quinone type)
(a)

Green sulfur bacteria (Fe-S type)
(b)

FIGURE 20-11 Functional Modules of Photosynthetic Machinery in Purple Bacteria and Green Sulfur Bacteria. The position on the vertical scale of each electron carrier reflects its standard reduction potential. (a) In purple bacteria, light energy excites an electron in the reaction center P870. The electron passes through pheophytin (Pheo), a quinone (Q), and the cytochrome bc_1 complex, then through cytochrome c_2 and thus back to the reaction center. Electron transfer through the cytochrome bc_1 complex causes proton pumping, creating an electrochemical potential that powers ATP synthesis. (b) Green sulfur bacteria have two routes for electrons driven by excitation of P840. A cyclic electron transfer route that goes through a quinone to the cytochrome bc_1 complex and back to the reaction center via cytochrome c_{553} causes proton pumping. A linear electron transfer route that goes from the reaction center through the iron-sulfur protein ferredoxin (Fd) reduces NAD^+ to NADH in a reaction catalyzed by ferredoxin:NAD^+ reductase.

complex similar to Complex III of the mitochondrial electron-transfer chain; and an ATP synthase, also similar to that of mitochondria. Illumination lifts an electron in the reaction center to its excited state (P870*), from which it passes through **pheophytin** (chlorophyll *a* lacking its central Mg^{2+}) and a quinone to the cytochrome bc_1 complex. After passing through the bc_1 complex, electrons flow through cytochrome c_2 back to the reaction center, restoring its preillumination state and completing one cycle. This light-driven **cyclic electron transfer** provides the energy for proton pumping by the cytochrome bc_1 complex. Powered by the resulting proton gradient, ATP synthase produces ATP, exactly as in mitochondria.

The **type I photosystem** in green sulfur bacteria involves the same three modules as in purple bacteria, but the process differs in several respects and includes additional enzymatic reactions (Fig. 20-11b). Excitation by light causes an electron to move from the excited reaction center to the cytochrome bc_1 complex via a quinone carrier. Electron transfer through this complex powers proton transport and creates the proton-motive force used for ATP synthesis, just as in purple bacteria and in mitochondria. However, in contrast to the cyclic electron transfer path in purple bacteria, some electrons follow a **linear electron transfer** path from the reaction center to the soluble iron-sulfur protein **ferredoxin** (see Fig. 19-5), which then passes electrons via **ferredoxin: NAD^+ reductase** to NAD^+, producing NADH. The electrons taken from the reaction center to reduce NAD^+ are replaced by the oxidation of H_2S to elemental S in the reaction that defines the green sulfur bacteria. This oxidation of H_2S by bacteria is chemically analogous to the oxidation of H_2O by oxygenic plants. Note that the path of electrons in the purple bacteria is cyclic; the path in the green sulfur bacteria can be either cyclic or linear, leading to NAD^+ and producing NADH.

In Vascular Plants, Two Reaction Centers Act in Tandem

The photosynthetic apparatus of cyanobacteria, algae, and vascular plants is more complex than the one-center bacterial systems, and it most likely evolved through the combination of two simpler bacterial photosystems.

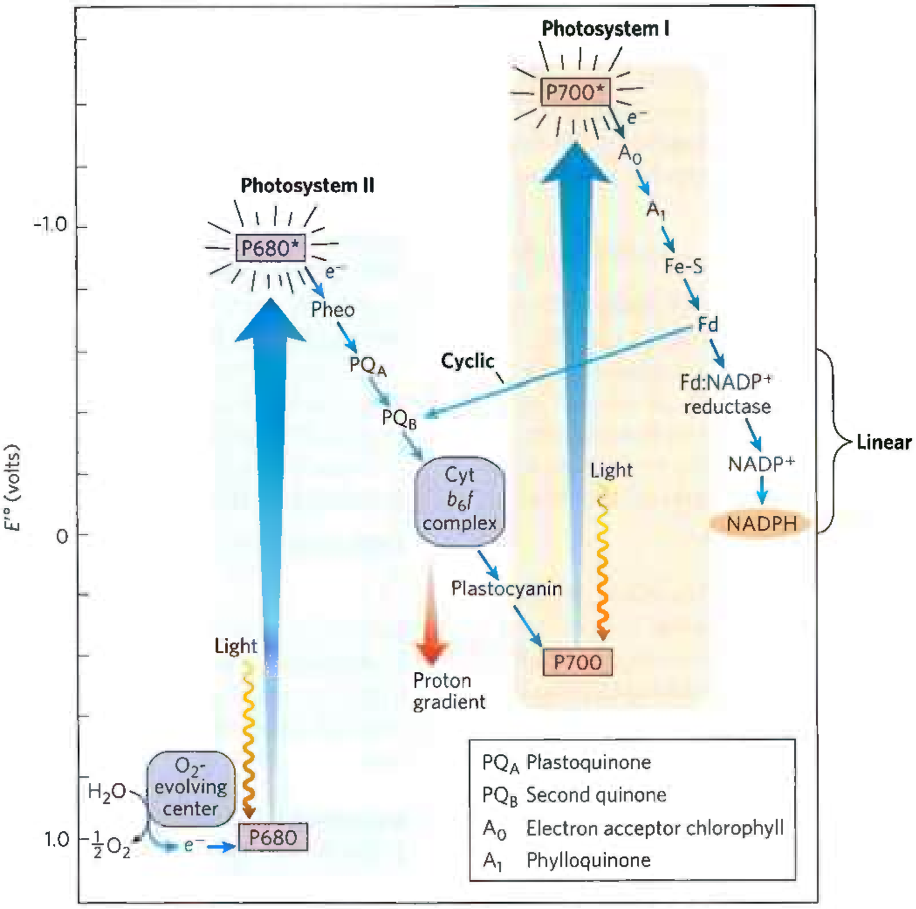


FIGURE 20-12 Integration of photosystems I and II in chloroplasts. This "Z scheme" shows the pathway of linear electron transfer from H_2O (lower left) to $NADP^+$ (far right). The position on the vertical scale of each electron carrier reflects its standard reduction potential. To raise the energy of electrons derived from H_2O to the energy level required to reduce $NADP^+$ to NADPH, each electron must be "lifted" twice (heavy arrows) by photons absorbed in PSII and PSI. One photon is required per electron in each photosystem. After excitation, the high-energy electrons flow "downhill" through the carrier chains as shown. Protons move across the thylakoid membrane during the water-splitting reaction and during electron transfer through the cytochrome b_6f complex, producing the proton gradient that is essential to ATP formation. An alternative path of electrons is cyclic electron transfer, in which electrons move from ferredoxin back to the plastoquinone and cytochrome b_6f complex, instead of reducing $NADP^+$ to NADPH. The cyclic pathway produces more ATP and less NADPH than the linear pathway.

The **Z scheme** diagram in **Figure 20-12** outlines the path of electron flow between the two photosystems and the energy relationships in the light-dependent reactions. (The Z scheme takes its name from the zigzag pattern of the pathways in the diagram.) The thylakoid membranes of chloroplasts have two different kinds of photosystems, each with its own type of photochemical reaction center and set of antenna molecules. The two systems have distinct and complementary functions. **Photosystem II (PSII)** is a pheophytin-quinone type of system (like the single photosystem of purple bacteria) containing roughly equal amounts of chlorophylls *a* and *b*. Excitation of the P680 special pair in its reaction center drives electrons through the cytochrome b_6f complex discussed below, with concomitant pumping of protons across the thylakoid membrane and ATP synthesis. **Photosystem I (PSI)** is structurally and functionally related to the photosynthetic machinery of green sulfur bacteria. It has a P700 reaction center and a high ratio of chlorophyll *a* to chlorophyll *b*. The excited P700 passes electrons through a linear chain of carriers to ferredoxin, then to $NADP^+$, producing NADPH. An alternative pathway for electrons is cyclic: instead of following the linear path that leads to $NADP^+$ reduction, electrons pass to plastoquinone (PQ) through a membrane-embedded protein complex, **cytochrome b_6f** (again, with the movement of protons into the chloroplast lumen). The thylakoid membranes of a single spinach chloroplast have many hundreds of each kind of photosystem.

These two photosystems in plants act in tandem to catalyze the light-driven movement of electrons from H_2O to $NADP^+$. The electron carriers include large, integral protein complexes (PSI, PSII, and the proton-pumping complex cytochrome b_6f); quinones that are lipid-soluble and move through the membrane between the protein complexes; and two soluble proteins, plastocyanin (analogous to cytochrome *c* in mitochondria) and ferredoxin.

To replace the electrons that move from PSII through PSI to $NADP^+$, H_2O is oxidized, producing O_2 (Fig. 20-12, bottom left). All O_2-evolving photosynthetic cells—those of plants, algae, and cyanobacteria—contain both PSI and PSII. **P3** The Z scheme thus describes the complete route by which electrons flow from H_2O to $NADP^+$, according to the equation

$$2H_2O + 2NADP^+ + 8\text{ photons} \longrightarrow O_2 + 2NADPH + 2H^+$$

For every two photons absorbed (one by each photosystem), one electron is transferred from H_2O to $NADP^+$. To form one molecule of O_2, which requires transfer of four electrons from two H_2O to two $NADP^+$, a total of eight photons must be absorbed, four by each photosystem.

Having seen the overall process, we'll now look at how the structure of the photosystems informs our understanding of the electrochemistry.

Photosystem II PSII is dimeric (**Fig. 20-13**). Each monomer is a huge complex of 19 proteins, including the accessory proteins CP47 and CP43, and the core complex of P680 reaction-center proteins D1 and D2; 2 chlorophyll-binding proteins; and associated chromophores, including carotenoids, a nonheme iron, and the critically important inorganic cofactor, Mn_4CaO_5. Of the proteins in PSII, 16 have transmembrane segments, but 3 are peripheral proteins on the lumenal side that stabilize the Mn_4CaO_5 cofactor. Surrounding PSII are additional chlorophyll-binding proteins and light-harvesting complexes. **P2** When a photon is absorbed by any of these antenna molecules, the resulting exciton moves very rapidly from one to another of the antenna chlorophylls until it reaches the reaction center and excites P680, the special pair of chlorophyll *a* molecules $(\text{Chl } a)_2$, to initiate the photochemistry.

Excitation of P680 in PSII (**Fig. 20-14**) produces P680*, an excellent electron donor that, within picoseconds, transfers an electron to pheophytin, giving it a negative charge ($^{\bullet}\text{Pheo}^-$). With the loss of its electron, P680* is transformed into a radical cation, $P680^+$. $^{\bullet}\text{Pheo}^-$ very rapidly passes its extra electron to a protein-bound **plastoquinone, PQ_A**, which in turn passes its electron to another, more loosely bound plastoquinone, PQ_B. When PQ_B has acquired two electrons in two such transfers from PQ_A and two protons from the solvent water, it is in its fully reduced quinol form, PQ_BH_2. The overall reaction initiated by light in PSII is

$$4\text{ P680} + 4H^+ + 2\text{ PQ}_B + 4\text{ photons} \longrightarrow 4\text{ P680}^+ + 2\text{ PQ}_BH_2 \qquad (20\text{-}1)$$

Eventually, the electrons in PQ_BH_2 pass through the cytochrome b_6f complex (see Fig. 20-12). The electron initially removed from P680 is replaced with an electron obtained from the oxidation of water, as described below.

Photosystem I PSI and its antenna molecules are part of a supramolecular complex composed of at least 16 proteins, including 4 chlorophyll-binding proteins arranged around the periphery of the reaction center (**Fig. 20-15**). The complex also includes 35 carotenoids of several types, three 4Fe-4S clusters, and two phylloquinones.

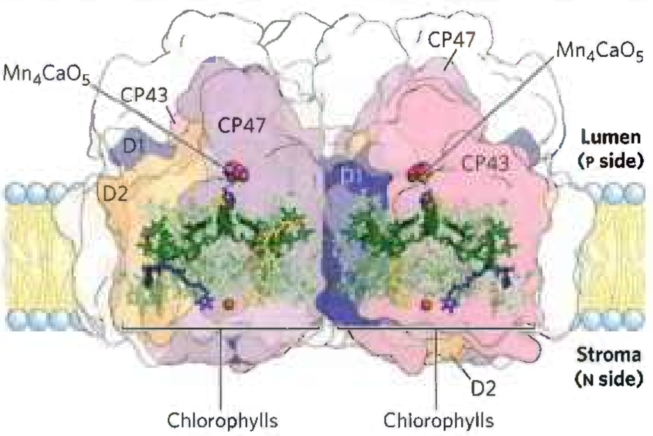


FIGURE 20-13 Structure of photosystem II of the cyanobacterium *Thermosynechococcus vulcanus*. The enormous complex, visualized by x-ray crystallography, is a dimer; each monomer has its own reaction center. Chlorophyll-binding proteins CP43 and CP47 form the core antenna, directly associated with the PSII reaction-center proteins D1 and D2. Each PSII monomer contains 35 chlorophylls, 2 pheophytins, 11 β-carotenes, 2 plastoquinones, and 1 each of *b*-type cytochrome, *c*-type cytochrome, and nonheme iron. Water is oxidized to form O_2 at the oxygen-evolving center (Mn_4CaO_5). [Data from PDB ID 3WU2, Y. Umena et al., *Nature* 473:55, 2011.]

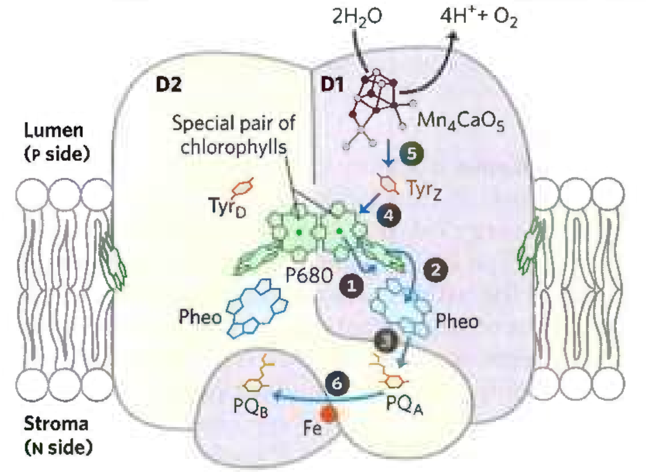


FIGURE 20-14 Electron transfer through photosystem II of the cyanobacterium *Synechococcus elongatus*. The monomeric form of the core complex shown here has two major transmembrane proteins, D1 and D2, each with its set of electron carriers. Although the two subunits are nearly symmetrical, electron transfer occurs through only one of the two branches of electron carriers: that on the right (in D1). The arrows show the path of electron transfer from the Mn_4CaO_5 ion cofactor of the oxygen-evolving complex to plastoquinone PQ_B. The photochemical events occur in the sequence indicated by the step numbers. The role of the Tyr residues and the detailed structure of the Mn_4CaO_5 cofactor are discussed below (see Fig. 20-20b).

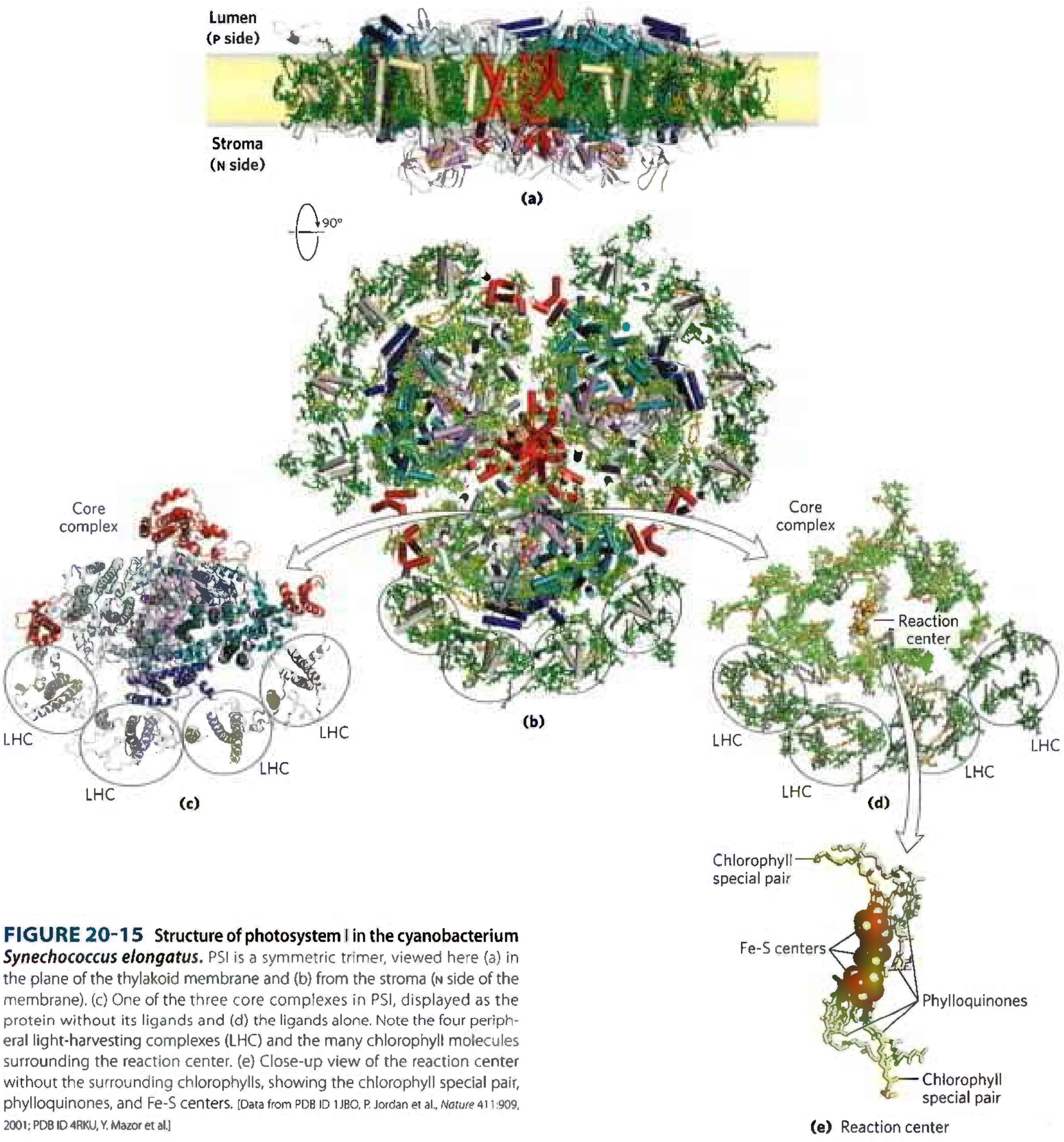

Lumen
(P side)
Stroma
(N side)
(a)
90°
Core
complex
LHC
LHC
LHC
LHC
(c)
(b)
Core
complex
Reaction
center
LHC
LHC
LHC
LHC
(d)
Chlorophyll
special pair
Fe-S centers
Phylloquinones
Chlorophyll
special pair
(e) Reaction center

FIGURE 20-15 **Structure of photosystem I in the cyanobacterium *Synechococcus elongatus*.** PSI is a symmetric trimer, viewed here (a) in the plane of the thylakoid membrane and (b) from the stroma (N side of the membrane). (c) One of the three core complexes in PSI, displayed as the protein without its ligands and (d) the ligands alone. Note the four peripheral light-harvesting complexes (LHC) and the many chlorophyll molecules surrounding the reaction center. (e) Close-up view of the reaction center without the surrounding chlorophylls, showing the chlorophyll special pair, phylloquinones, and Fe-S centers. [Data from PDB ID 1JBO, P. Jordan et al., *Nature* 411:909, 2001; PDB ID 4RKU, Y. Mazor et al.]

The photochemical events that follow the excitation of PSI at the reaction-center P700 (**Fig. 20-16**) are formally similar to those occurring in PSII. The excited reaction-center P700* loses an electron to an acceptor, designated A_0 (a chlorophyll *a* molecule, functionally homologous to the pheophytin of PSII), creating A_0^- and $P700^+$. Again, excitation results in charge separation at the photochemical reaction center. $P700^+$ is a strong oxidizing agent, which quickly acquires an electron from **plastocyanin**, a soluble Cu-containing electron-transfer protein. A_0^- is an exceptionally strong reducing agent that passes its electron through a chain of carriers that leads to $NADP^+$ (Fig. 20-12, right side). **Phylloquinone (Q_K)** accepts the electron and passes it to an iron-sulfur protein through three Fe-S centers in PSI. From here, the electron moves to ferredoxin (Fd). Recall that ferredoxin contains a 2Fe-2S center (see Fig. 19-5) that undergoes one-electron oxidation and reduction reactions.

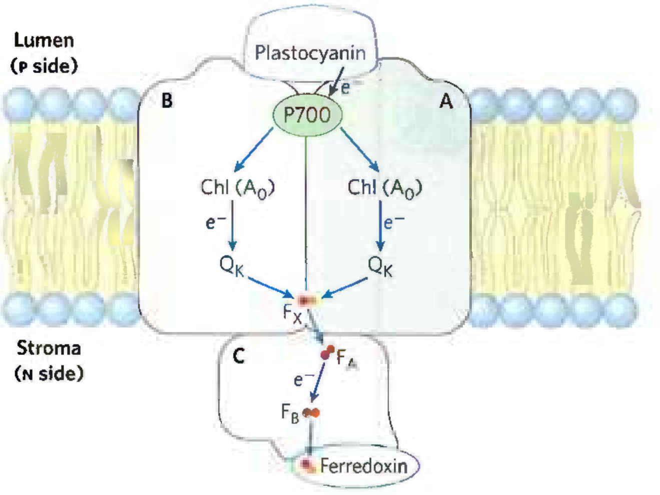


FIGURE 20-16 The path of electrons through PSI. The path of electrons (blue arrows) through PSI, viewed in the plane of the membrane. When the reaction-center P700, the special pair of chlorophylls, is excited by a photon or an exciton, its reduction potential is dramatically reduced, making it a good electron donor. P700 then passes an electron through a nearby chlorophyll (referred to as A_0) to phylloquinone (Q_K). Reduced Q_K is reoxidized as it passes two electrons, one at a time, to an Fe-S center (F_X) near the N side of the membrane. From F_X, electrons move through two more Fe-S centers (F_A and F_B) to ferredoxin in the stroma. Ferredoxin then donates electrons to $NADP^+$ (not shown), reducing it to NADPH, one of the forms in which the energy of photons is trapped in chloroplasts.

The fourth electron carrier in the chain is the flavoprotein **ferredoxin:NADP$^+$ reductase**, which transfers electrons from reduced ferredoxin (Fd_{red}) to $NADP^+$:

$$2Fd_{red} + 2H^+ + NADP^+ \longrightarrow 2Fd_{ox} + NADPH + H^+$$

The Cytochrome b_6f Complex Links Photosystems II and I, Conserving the Energy of Electron Transfer

Electrons temporarily held in plastoquinol as a result of the excitation of P680 in PSII are carried to P700 of PSI via the cytochrome b_6f complex and the soluble protein plastocyanin (see Fig. 20-12, center). With a structure and role analogous with that of Complex III in mitochondria, the cytochrome b_6f complex (**Fig. 20-17**) contains a *b*-type cytochrome with two heme groups (designated b_H and b_L), a Rieske iron-sulfur protein (M_r 20,000), and cytochrome *f* (named for the Latin *frons*, "leaf"). Electrons flow through the cytochrome b_6f complex from PQ_BH_2 to cytochrome *f*, then to plastocyanin, and finally to $P700^+$, thereby reducing it.

Like Complex III of mitochondria, cytochrome b_6f conveys electrons from a reduced quinone—a mobile, lipid-soluble carrier of two electrons (Q in mitochondria, PQ_B in chloroplasts; P for *p*lastoquinone)—to a water-soluble protein that carries one electron (cytochrome *c* in mitochondria, plastocyanin in chloroplasts) (Fig 20-17a). As in mitochondria, the function of this complex involves a Q cycle (Fig. 20-17b; see Fig. 19-11)

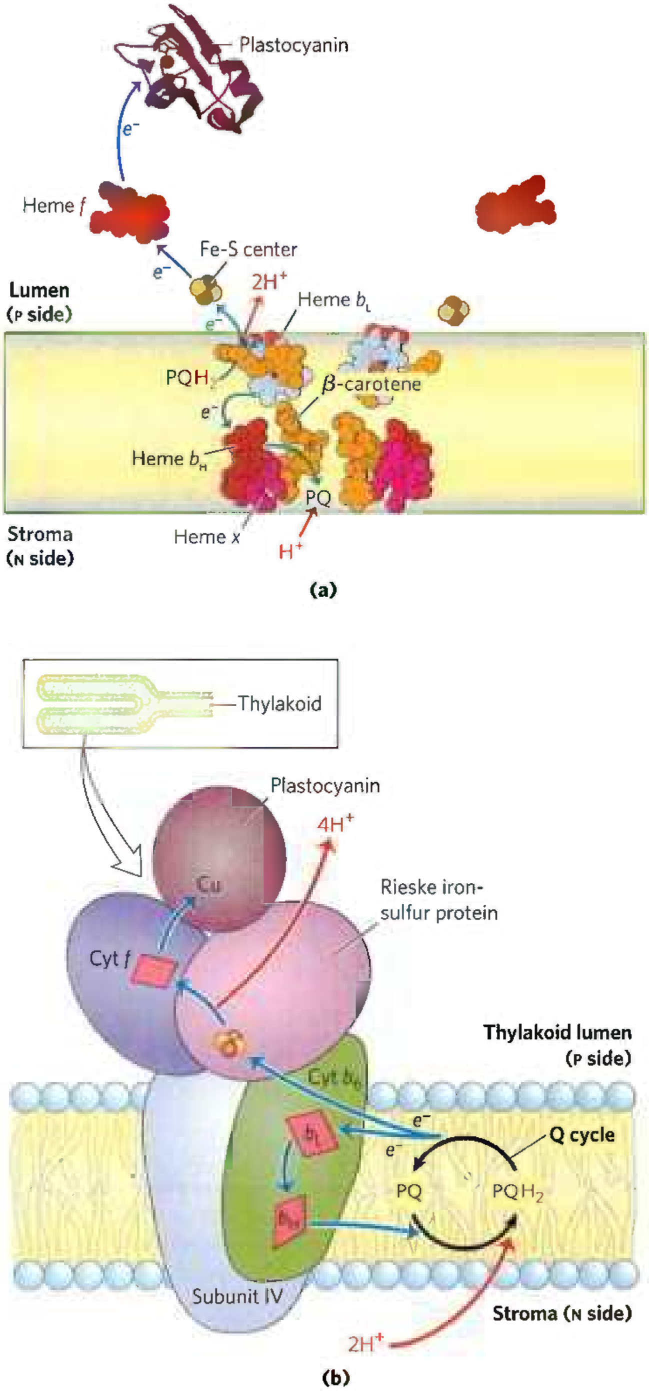


FIGURE 20-17 Electron and proton flow through the cytochrome b_6f complex. (a) In addition to the hemes of cytochrome *b* (heme b_H and b_L; also called heme b_N and b_P, respectively, because of their proximity to the N and P sides of the bilayer) and cytochrome *f* (heme *f*), there is a fourth heme (heme *x*) near heme b_H; also present is a β-carotene of unknown function. Two sites bind plastoquinone: the PQH_2 site near the P side of the bilayer, and the PQ site near the N side. The Fe-S center of the Rieske protein lies just outside the bilayer on the P side, and the heme *f* site is on a protein domain that extends well into the thylakoid lumen. The electron path is shown for just one of the monomers, but both sets of carriers in the dimer carry electrons to plastocyanin. (b) Plastoquinol (PQH_2), formed in PSII, is oxidized by the cytochrome b_6f complex in a series of steps like those of the Q cycle in Complex III of mitochondria (see Fig. 19-11). One electron from PQH_2 passes to the Fe-S center of the Rieske protein, the other to heme b_L of cytochrome b_6. The net effect is passage of electrons from PQH_2 to the soluble protein plastocyanin, which carries them to PSI. [Data from PDB ID 1VF5, G. Kurisu et al., *Science* 302:1009, 2003; PDB ID 2Q5B, Y. S. Bukhman-DeRuyter et al.]

in which electrons pass, one at a time, from PQ_BH_2 to cytochrome b_6. This cycle results in the pumping of protons across the membrane, from the stromal compartment to the thylakoid lumen. Up to four protons enter the lumen for each pair of electrons that passes through the cytochrome b_6f complex. P3 The result is production of a proton gradient across the thylakoid membrane as electrons pass from PSII to PSI. Because the volume of the flattened thylakoid lumen is small, the influx of a small number of protons has a relatively large effect on lumenal pH. The measured difference in pH between the stroma (pH 8) and the thylakoid lumen (pH 5) represents a 1,000-fold difference in proton concentration—a powerful driving force for ATP synthesis.

Cyclic Electron Transfer Allows Variation in the Ratio of ATP/NADPH Synthesized

Cyclic electron flow between PSI and cytochrome b_6f increases the production of ATP relative to NADPH. The linear path of electrons from water, through PSII, cytochrome b_6f, and PSI to $NADP^+$ produces both a proton gradient, which is used to drive ATP synthesis, and NADPH, which is used in reductive biosynthetic processes (see Fig. 20-12). Some fraction of electrons passing from P700* to ferredoxin do not continue to $NADP^+$, but cycle back through plastoquinone and the cytochrome b_6f complex to plastocyanin. Plastocyanin then donates electrons to P700. In this way, electrons are repeatedly recycled through the cytochrome b_6f complex and the reaction center of PSI, each electron propelled around the cycle by the energy of one photon. Cyclic electron flow is not accompanied by net formation of NADPH or evolution of O_2. However, it *is* accompanied by proton pumping by the cytochrome b_6f complex and by phosphorylation of ADP to ATP, referred to as **cyclic photophosphorylation**. The overall equation for cyclic electron flow and photophosphorylation is simply

$$\text{ADP} + \text{P}_i \xrightarrow{\text{light}} \text{ATP} + \text{H}_2\text{O}$$

P5 By regulating the partitioning of electrons between $NADP^+$ reduction and cyclic photophosphorylation, a plant adjusts the ratio of ATP to NADPH produced in the light-dependent reactions to match its needs for these products in the CO_2-assimilation reactions and other biosynthetic processes. As we shall see in Section 20.4, the CO_2-assimilation reactions require ATP and NADPH in the ratio 3:2. This regulation of electron-transfer pathways is part of a short-term adaptation to changes in light color (wavelength) and quantity (intensity).

State Transitions Change the Distribution of LHCII between the Two Photosystems

Photosynthetic organisms are exposed to light of highly variable intensity and wavelength in the course of a day or a season, and, although they can alter their growth patterns somewhat, they cannot uproot themselves and move to optimize their light exposure. Instead, cellular mechanisms have evolved that allow plants to accommodate changing light conditions. The energy needed to excite PSI (P700) is less (light of longer wavelength, lower energy) than the energy needed to excite PSII (P680). If PSI and PSII were physically contiguous, excitons originating in the antenna system of PSII would migrate to the reaction center of PSI, leaving PSII chronically underexcited and thus interfering with the operation of the two-center system. This imbalance in the supply of excitons is prevented by physically separating the two photosystems in the thylakoid membrane (**Fig. 20-18**). PSII is located almost exclusively in the tightly appressed membrane stacks of granal thylakoids; its associated light-harvesting complex (LHCII) mediates the tight association of adjacent membranes in the grana. PSI and the ATP synthase complex are located almost exclusively in the nonappressed membranes of the stromal thylakoids, where they have access to the contents of the stroma, including ADP and $NADP^+$. The cytochrome b_6f complex is present primarily in the granal thylakoids.

P2 The association of LHCII with PSI and PSII depends on light intensity and wavelength, which can change in the short term and lead to **state transitions** in the chloroplast. In state 1, LHCII, PSII, and PSI are poised to maximize the capture of light energy. A critical Thr residue in LHCII is unphosphorylated, and LHCII associates with PSII. Under conditions of intense or blue light, which favor absorption by PSII, that photosystem reduces plastoquinone to plastoquinol (PQH_2) faster than PSI can oxidize it. The resulting accumulation of PQH_2 activates a protein kinase that triggers the transition to state 2 by phosphorylating a Thr residue on LHCII (**Fig. 20-19**). Phosphorylation weakens the interaction of LHCII with the appressed membrane and with PSII; some LHCII dissociates and moves to the stromal thylakoids. Here it captures photons (excitons) for PSI, speeding the oxidation of PQH_2 and reversing the imbalance between electron flow in PSI and PSII. In less intense light (in the shade, with more red light), PSI oxidizes PQH_2 faster than PSII can make it, and the resulting increase in [PQ] triggers dephosphorylation of LHCII, reversing the effect of phosphorylation. The state transition in LHCII localization and the transition from cyclic to linear electron transfer are coordinately regulated: the path of electrons is primarily linear in state 1 and primarily cyclic in state 2.

When light is so intense that the combined activity of PSII and PSI cannot synthesize ATP and NADPH fast enough to keep up with the supply of photons, carotenoids in LHCII absorb the excitons and very rapidly quench the excited chlorophyll before it can create damaging reactive oxygen species (ROS). The trigger for switching from an efficient light-harvesting state to an energy-dissipating state is the lowering of pH in the lumenal space, but the detailed mechanism for this transition is not yet known.

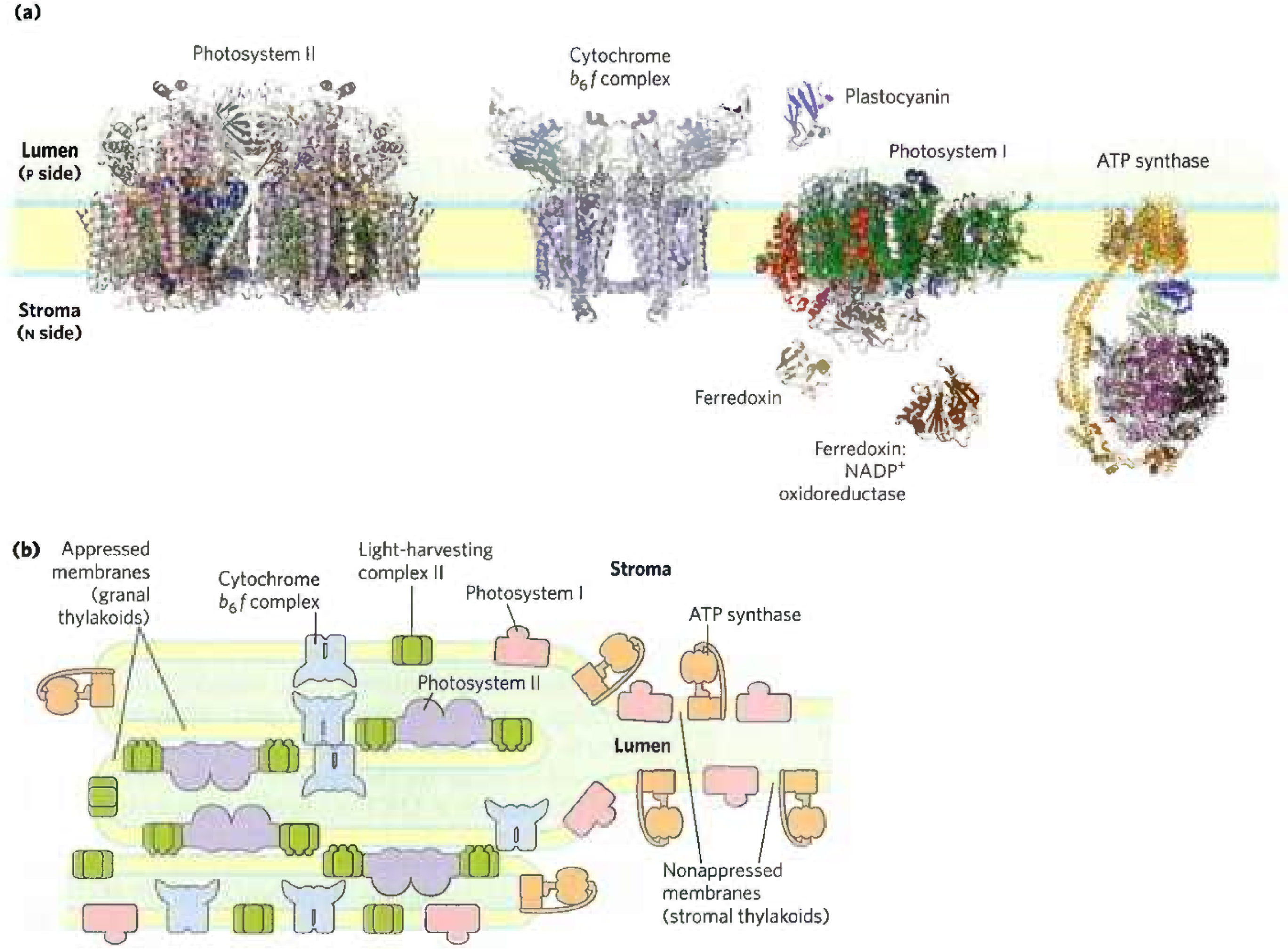

(a)
Photosystem II
Cytochrome b_6f complex
Plastocyanin
Photosystem I
ATP synthase
Lumen (P side)
Stroma (N side)
Ferredoxin
Ferredoxin: NADP+ oxidoreductase
(b) Appressed membranes (granal thylakoids)
Cytochrome b_6f complex
Light-harvesting complex II
Photosystem I
Stroma
ATP synthase
Photosystem II
Lumen
Nonappressed membranes (stromal thylakoids)

FIGURE 20-18 Localization of PSI and PSII in thylakoid membranes. (a) Structures of the complexes and soluble proteins of the photosynthetic apparatus of a vascular plant or alga, drawn to the same scale. The bovine ATP synthase is shown. (b) Light-harvesting complex LHCII and ATP synthase are located both in appressed regions of the thylakoid membrane (granal thylakoids, in which several membranes are in contact) and in nonappressed regions (stromal thylakoids), and have ready access to ADP and $NADP^+$ in the stroma. PSII is present almost exclusively in the appressed granal regions, and PSI almost exclusively in nonappressed stromal regions. LHCII is the "adhesive" that holds appressed thylakoid membranes together (see Fig. 20-19). [(a) Data from PSII: PDB ID 3WU2, Y. Umena et al., *Nature* 473:55, 2011; cyt b_6f complex: PDB ID 2E74, E. Yamashita et al., *J. Mol. Biol.* 370:39, 2007; plastocyanin: PDB ID 1AG6, Y. Xue et al., *Protein Sci.* 7:2099, 1998; PSI: PDB ID 4RKU, Y. Mazor et al.; ferredoxin: PDB ID 1A70, C. Binda et al., *Acta Crystallogr. D Biol. Crystallogr.* 54:1353, 1998; ferredoxin:NADP reductase: PDB ID 1QG0, Z. Deng et al., *Nat. Struct. Biol.* 6:847, 1999; ATP synthase: PDB ID 5ARA, A. Zhou et al., *eLife* 4:e10180, 2015.]

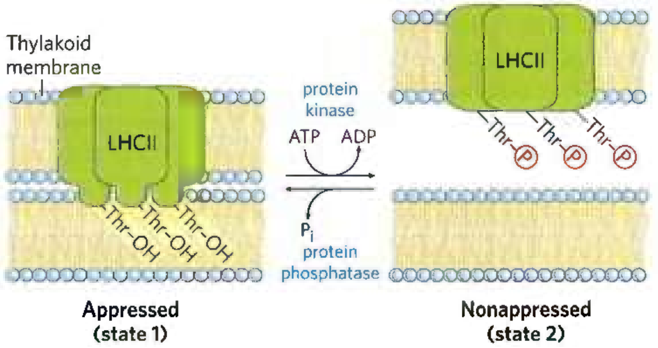
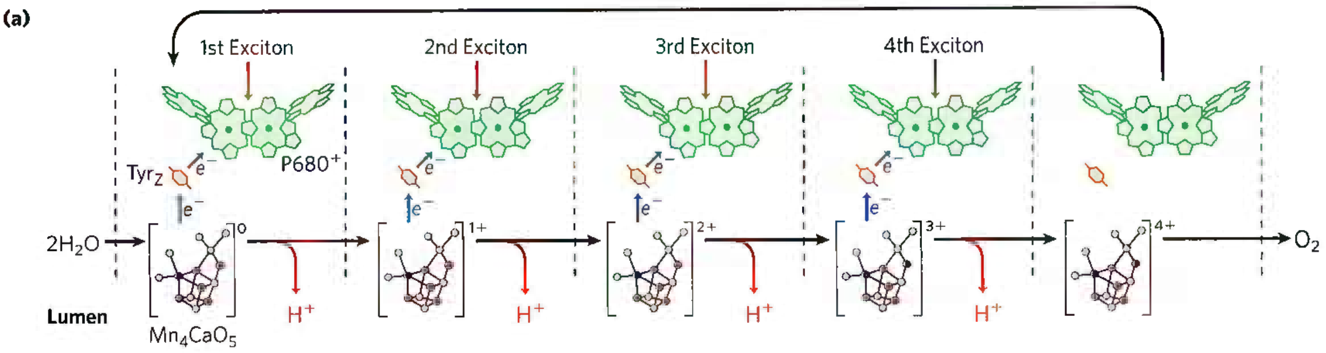

Thylakoid membrane
LHCII
Thr-OH
Thr-OH
Thr-OH
protein kinase
ATP
ADP
P_i
protein phosphatase
LHCII
Thr-P
Thr-P
Thr-P
Appressed (state 1)
Nonappressed (state 2)

FIGURE 20-19 Electron transfer in PSI and PSII is balanced through state transitions. In granal thylakoids, a hydrophobic domain of LHCII in one membrane inserts into the neighboring membrane and closely appresses the two (state 1). Accumulation of plastoquinol (not shown) stimulates a protein kinase that phosphorylates a Thr residue in the hydrophobic domain of LHCII, which reduces its affinity for the neighboring membrane and converts appressed granal thylakoids to nonappressed stromal thylakoids (state 2). A specific protein phosphatase reverses this regulatory phosphorylation when the $[PQ]/[PQH_2]$ ratio increases.

Water Is Split at the Oxygen-Evolving Center

The ultimate source of the electrons passed to NADPH in plant (oxygenic) photosynthesis is water. Having given up an electron to pheophytin, $P680^+$ (of PSII) must acquire an electron to return to its ground state in preparation for capture of another photon. In principle, the required electron might come from any number of organic or inorganic compounds. Photosynthetic bacteria use a variety of electron donors for this purpose—acetate, succinate, malate, or sulfide—depending on what is available in a particular ecological niche. About 2.5 billion years ago, evolution of primitive photosynthetic bacteria (progenitors of the modern cyanobacteria) produced a photosystem capable of taking electrons from a donor that is always available: water. Two water molecules are oxidized, yielding four electrons, four protons, and molecular oxygen:

$$2H_2O \longrightarrow 4H^+ + 4e^- + O_2$$



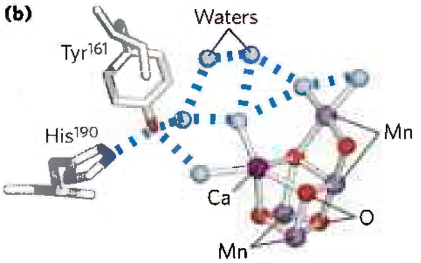


FIGURE 20-20 Water-splitting activity of the oxygen-evolving center. (a) The process that produces a four-electron oxidizing agent—a multinuclear center with four Mn ions, one Ca ion, and five oxygen atoms—in the oxygen-evolving center of PSII. The sequential absorption of four photons (excitons), each absorption causing the loss of one electron from the Mn_4CaO_5 cofactor, produces an oxidizing agent that can remove four electrons from two molecules of water, producing O_2. The electrons lost from the Mn_4CaO_5 cofactor pass one at a time to an oxidized Tyr residue in a PSII protein, then to $P680^+$. (b) The chair-shaped metallic center of the oxygen-evolving center. Tyr^{161}, known to participate in the oxidation of water, is seen hydrogen-bonded to a network of water molecules, including several directly in contact with the Mn_4CaO_5 cofactor. This is the site of one of the most important reactions in the biosphere. [(b) Data from PDB ID 3WU2, Y. Umena et al., *Nature* 473:55, 2011.]

A single photon of visible light does not have enough energy to break the bonds in water; four photons are required in this photolytic cleavage reaction.

P3 The four electrons abstracted from water do not pass directly to $P680^+$, which can accept only one electron at a time. Instead, a remarkable molecular device, the **oxygen-evolving center**, passes four electrons *one at a time* to $P680^+$ (**Fig. 20-20a**). The immediate electron donor to $P680^+$ is a Tyr residue (sometimes designated Z or Tyr_Z) in subunit D1 of the PSII reaction center. The Tyr residue loses both a proton and an electron, generating the electrically neutral Tyr free radical, •Tyr:

$$4\ P680^+ + 4\ Tyr \longrightarrow 4\ P680 + 4\ {}^\bullet Tyr \qquad (20\text{-}2)$$

The Tyr radical regains its missing electron and proton by oxidizing a cofactor of four manganese ions and one calcium ion in the oxygen-evolving center. With each single-electron transfer, the Mn_4CaO_5 cofactor becomes more oxidized; four single-electron transfers, each corresponding to the absorption of one photon, produce a charge of 4+ on the Mn_4CaO_5 cofactor (Fig. 20-20a):

$$4\ {}^\bullet Tyr + [Mn_4CaO_5]^0 \longrightarrow 4\ Tyr + [Mn_4CaO_5]^{4+} \qquad (20\text{-}3)$$

In this state, the Mn_4CaO_5 cofactor can take four electrons from a pair of water molecules, releasing four H^+ and O_2:

$$[Mn_4CaO_5]^{4+} + 2H_2O \longrightarrow [Mn_4CaO_5]^0 + 4H^+ + O_2 \qquad (20\text{-}4)$$

P4 Because the four protons produced in this reaction are released into the thylakoid lumen, the oxygen-evolving center acts as a proton pump, driven by electron transfer.

We saw in Equation 20-1 that the overall reaction initiated by light in PSII is

$$4\ P680 + 4H^+ + 2\ PQ_B + 4\ \text{photons} \longrightarrow 4\ P680^+ + 2\ PQ_BH_2$$

The sum of Equations 20-1 through 20-4 is

$$2H_2O + 2PQ_B + 4\ \text{photons} \longrightarrow O_2 + 2\ PQ_BH_2 \qquad (20\text{-}5)$$

P3 The oxygen-evolving cofactor takes the shape of a chair (Fig. 20-20b). The seat and legs of the chair are made up of three Mn ions, one Ca ion, and four O atoms; the fourth Mn and another O form the back of the chair. Four water molecules are also seen in the crystal structure, two associated with one of the Mn ions, the other two with the Ca ion. It may be one (or more) of these water molecules that undergoes oxidation to produce O_2. This metal cofactor is associated with several peripheral membrane proteins on the lumenal side of the thylakoid membrane that are believed to stabilize the cofactor. The Tyr residue designated Z, through which electrons move between water and the PSII reaction center, is connected with a network of hydrogen-bonded water molecules that includes the four associated with the Mn_4CaO_5 cofactor. The detailed mechanism of water oxidation by the Mn_4CaO_5 cofactor is not known but is under intense investigation. The reaction is central to life on Earth and may involve novel bioinorganic chemistry. Determination of the structure of the polymetallic center has inspired several reasonable and testable hypotheses. Stay tuned.

SUMMARY 20.2 *Photochemical Reaction Centers*

- Bacteria have a single photochemical reaction center. Purple bacteria have a type II photosystem where electrons from an excited special pair of chlorophyll molecules (P870*) flow through pheophytin, quinones, and a proton-pumping cytochrome complex, back to the special pair of chlorophylls. Green sulfur bacteria have a type I photosystem that can send electrons through a similar cyclic path or through a linear path that reduces NAD^+ to NADH.

- In cyanobacteria, algae, and plants, two different reaction centers are arranged in tandem. In the reaction center of PSII, when the special pair of chlorophylls (P680) is excited by light, it passes electrons to plastoquinone, and the electrons lost from P680 are replaced by electrons from H_2O. PSI passes electrons from the excited special pair (P700*) in its reaction center through a series of carriers to ferredoxin, which then reduces $NADP^+$ to NADPH.
- Electron flow from either photosystem through the cytochrome b_6f complex drives protons across the thylakoid membrane, creating a proton-motive force that provides the energy for ATP synthesis by an ATP synthase.
- Linear electron transfer through the photosystems produces NADPH and ATP. Cyclic electron transfer produces only ATP and allows variability in the proportions of NADPH and ATP formed.
- The distribution of PSI and PSII between the granal and stromal thylakoids can change and is indirectly controlled by light intensity, optimizing the distribution of excitons between PSI and PSII for efficient energy capture.
- The oxygen-evolving center, which contains a Mn_4CaO_5 cofactor, uses energy from light to split water, producing O_2. For each O_2 formed at the oxygen-evolving center, four protons are pumped into the thylakoid lumen, contributing to the proton motive force.

20.3 Evolution of a Universal Mechanism for ATP Synthesis

The combined activities of the two plant photosystems move electrons from water to $NADP^+$, conserving some of the energy of absorbed light as NADPH (Fig. 20-12). Simultaneously, protons are pumped across the thylakoid membrane and energy is conserved as an electrochemical potential. We turn now to the process by which this proton gradient drives the synthesis of ATP, the other energy-conserving product of the light-dependent reactions.

A Proton Gradient Couples Electron Flow and Phosphorylation

P4 Although the energy source and electron carriers in photophosphorylation in chloroplasts differ from those of oxidative phosphorylation in mitochondria, they use essentially the same mechanism to capture the energy of the proton gradient. Electron-transferring molecules in the chain of carriers connecting PSII and PSI are oriented asymmetrically in the thylakoid membrane, so photoinduced electron flow results in the net movement of protons across the membrane, from the stromal side to the thylakoid lumen (**Fig. 20-21**).

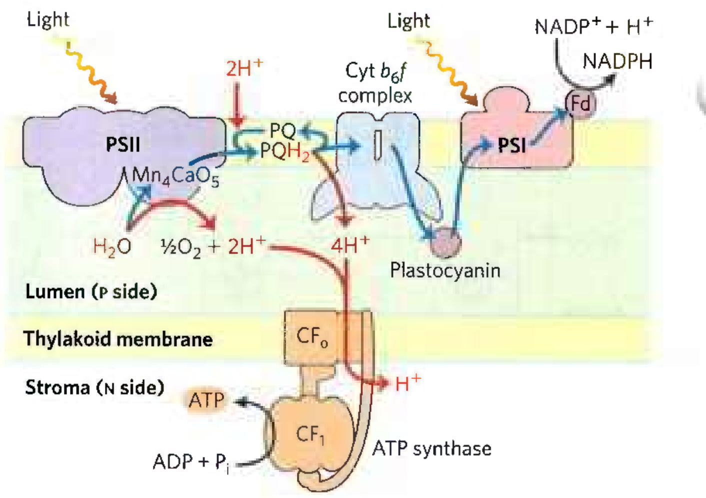


FIGURE 20-21 Proton and electron circuits during photophosphorylation. In the linear electron pathway (blue arrows), electrons move from H_2O through PSII, through the intermediate chain of carriers of the cytochrome b_6f complex, through PSI, and finally to $NADP^+$. In the cyclic pathway, electrons move from PSI back to plastoquinone and cytochrome b_6f. Protons (red arrows) are pumped into the thylakoid lumen by the flow of electrons through cytochrome b_6f, and they reenter the stroma through proton channels formed by CF_o of ATP synthase. The CF_1 subunit catalyzes synthesis of ATP.

The Approximate Stoichiometry of Photophosphorylation Has Been Established

As electrons move from water to $NADP^+$ in chloroplasts, about 12 protons move from the stroma into the thylakoid lumen per 4 electrons passed (that is, per O_2 formed). Of these protons, 4 are moved by the oxygen-evolving center, and up to 8 are moved by the cytochrome b_6f complex. The measurable result is a 1,000-fold difference in H^+ concentration across the thylakoid membrane ($\Delta pH = 3$). Recall that the energy stored in a proton gradient (the electrochemical potential) has two components: a proton concentration difference (ΔpH) and an electrical potential ($\Delta\psi$) due to charge separation. In chloroplasts, ΔpH is the dominant component; counterion movement apparently dissipates most of the electrical potential. In illuminated chloroplasts, the energy stored in the proton gradient per mole of protons is

$$\Delta G = 2.3RT\ \Delta pH + ZF\ \Delta\psi = -17\ \text{kJ/mol}$$

so the movement of 12 mol of protons across the thylakoid membrane represents conservation of about 200 kJ of energy—enough energy to drive the synthesis of several moles of ATP ($\Delta G'^\circ = 30.5$ kJ/mol). Experimental measurements yield values of about 3 ATP per O_2 produced.

At least 8 photons must be absorbed to drive 4 electrons from 2 H_2O to 2 NADPH (one photon per electron at each reaction center). The energy in 8 photons of visible light is more than enough for the synthesis of three molecules of ATP.

ATP synthesis is not the only energy-conserving reaction of photosynthesis in plants; the NADPH formed in the final electron transfer is also energetically rich. The overall equation for this linear photophosphorylation is

$$2H_2O + 8\text{ photons} + 2NADP^+ + {\sim}3ADP^+ + {\sim}3P_i \longrightarrow O_2 + {\sim}3ATP + 2NADPH \qquad (20\text{-}6)$$

The ATP Synthase Structure and Mechanism Are Nearly Universal

The enzyme responsible for ATP synthesis in chloroplasts is a large complex with two functional components, CF_o and CF_1 (*C* denoting its location in chloroplasts). CF_o is a transmembrane proton pore composed of several integral membrane proteins and is homologous to mitochondrial F_o. CF_1 is a peripheral membrane protein complex very similar in subunit composition, structure, and function to mitochondrial F_1.

Electron microscopy of sectioned chloroplasts shows ATP synthase complexes as projections on the *outside* (stromal, or N) surface of thylakoid membranes; these complexes correspond to the ATP synthase complexes that project on the *inside* (matrix, or N) surface of the inner mitochondrial membrane. Thus, the relationship between the orientation of the ATP synthase and the direction of proton pumping is the same in chloroplasts and mitochondria. In both cases, the F_1 portion of ATP synthase is located on the more alkaline (N) side of the membrane through which protons flow down their concentration gradient; the direction of proton flow relative to F_1 is the same in both cases: P to N (**Fig. 20-22**).

P4 The mechanism of chloroplast ATP synthase is essentially identical to that of its mitochondrial analog; ADP and P_i readily condense to form ATP on the enzyme surface, and the release of this enzyme-bound ATP requires a proton-motive force. Rotational catalysis sequentially engages each of the three β subunits of the ATP synthase in ATP synthesis, ATP release, and ADP + P_i binding (see Figs. 19-26 and 19-27).

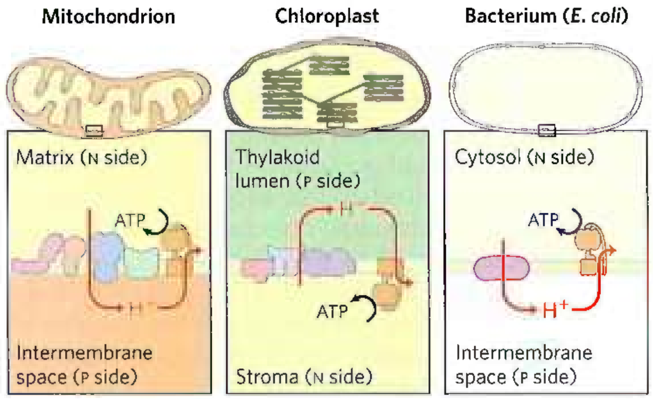


FIGURE 20-22 Orientation of ATP synthase is fixed relative to the proton gradient. Superficially, the direction of proton pumping in chloroplasts may seem to be opposite to that in mitochondria and bacteria. In mitochondria and bacteria, protons are pumped *out of* the organelle or cell, and F_1 is on the *inside* of the membrane; in chloroplasts, protons are pumped *into* the thylakoid lumen, and CF_1 is on the *outside* of the thylakoid membrane. However, exactly the same mechanism of energy conversion (from proton gradient to ATP) occurs in all three cases. ATP is synthesized in the matrix of mitochondria, the stroma of chloroplasts, and the cytosol of bacteria.

The appearance of oxygenic photosynthesis on Earth about 2.5 billion years ago was a crucial event in the evolution of the biosphere. Before that, Earth's atmosphere was composed of methane, CO_2, and N_2. The planet was essentially devoid of molecular oxygen and lacked the ozone layer that protects organisms from solar UV radiation. Oxygenic photosynthesis made available a nearly limitless supply of reducing agent (H_2O) to drive the production of organic compounds by reductive biosynthetic reactions. And mechanisms evolved that allowed organisms to use O_2 as a terminal electron acceptor in highly energetic electron transfers from organic substrates, employing the energy of oxidation to support metabolism. The complex photosynthetic apparatus of a modern vascular plant is the culmination of a series of evolutionary events, the most recent of which was the acquisition by eukaryotic cells of a cyanobacterial endosymbiont.

The chloroplasts of modern organisms share several properties with mitochondria and originated by the same mechanism that gave rise to mitochondria: endosymbiosis. Like mitochondria, chloroplasts contain their own DNA and protein-synthesizing machinery. Some of the polypeptides of chloroplast proteins are encoded by chloroplast genes and synthesized in the chloroplast; others are encoded by nuclear genes, synthesized outside the chloroplast, and imported (Chapter 27). When plant cells grow and divide, chloroplasts give rise to new chloroplasts by division, during which their DNA is replicated and divided between daughter chloroplasts. The machinery and mechanisms for light capture, electron flow, and ATP synthesis in modern cyanobacteria are similar in many respects to those in plant chloroplasts. These observations led to the now widely accepted hypothesis that the evolutionary progenitors of modern plant cells were primitive eukaryotes that engulfed photosynthetic cyanobacteria and established stable endosymbiotic relationships with them (see Fig. 1-37).

At least half of the photosynthetic activity on Earth now occurs in microorganisms—algae, other photosynthetic eukaryotes, and photosynthetic bacteria. Cyanobacteria have PSII and PSI in tandem, and the PSII has an associated oxygen-evolving activity resembling that of plants. However, the other groups of photosynthetic bacteria have single reaction centers and do not split H_2O or produce O_2. Many are obligate anaerobes and cannot tolerate O_2; they must use some compound other than H_2O as an electron donor. Some photosynthetic bacteria use inorganic compounds as electron (and hydrogen) donors. For example, green sulfur bacteria use hydrogen sulfide:

$$2H_2S + CO_2 \xrightarrow{\text{light}} (CH_2O) + H_2O + 2S$$

These bacteria, instead of producing molecular O_2, form elemental sulfur as the oxidation product of H_2S.

(They further oxidize the S to SO_4^{2-}.) Other photosynthetic bacteria use organic compounds such as lactate as electron donors:

$$2\ \text{Lactate} + CO_2 \xrightarrow{\text{light}} (CH_2O) + H_2O + 2\ \text{pyruvate}$$

The fundamental similarity of photosynthesis in plants and bacteria, despite the differences in the electron donors they employ, becomes more obvious when the equation of photosynthesis is written in the more general form

$$2H_2D + CO_2 \xrightarrow{\text{light}} (CH_2O) + H_2O + 2D$$

in which H_2D is an electron (and hydrogen) donor and D is its oxidized form. H_2D may be water, hydrogen sulfide, lactate, or some other organic compound, depending on the species. Most likely, the bacteria that first developed photosynthetic ability used H_2S as their electron source.

Modern cyanobacteria can synthesize ATP by oxidative phosphorylation or by photophosphorylation, although they have neither mitochondria nor chloroplasts. The enzymatic machinery for both processes is in a highly convoluted plasma membrane (**Fig. 20-23**). Three protein components function in both processes, giving evidence that the processes have a common evolutionary origin (**Fig. 20-24**). First, the proton-pumping cytochrome b_6f complex carries electrons from plastoquinone to cytochrome c_6 in photosynthesis, and also carries electrons from ubiquinone to cytochrome c_6 in oxidative phosphorylation—the role played by cytochrome bc_1 in mitochondria. Second, cytochrome c_6, homologous to mitochondrial cytochrome c, carries electrons from Complex III to Complex IV in cyanobacteria; it can also carry electrons from the cytochrome b_6f complex to PSI—a role performed in plants by plastocyanin. **P4** We therefore see the functional homology between the cyanobacterial cytochrome b_6f complex and the mitochondrial cytochrome bc_1 complex, and between cyanobacterial cytochrome c_6 and plant plastocyanin. The third conserved component is the ATP synthase, which functions in oxidative phosphorylation and photophosphorylation in cyanobacteria, and in the mitochondria and chloroplasts of photosynthetic eukaryotes. The structure and remarkable mechanism of this enzyme have been strongly conserved throughout evolution.

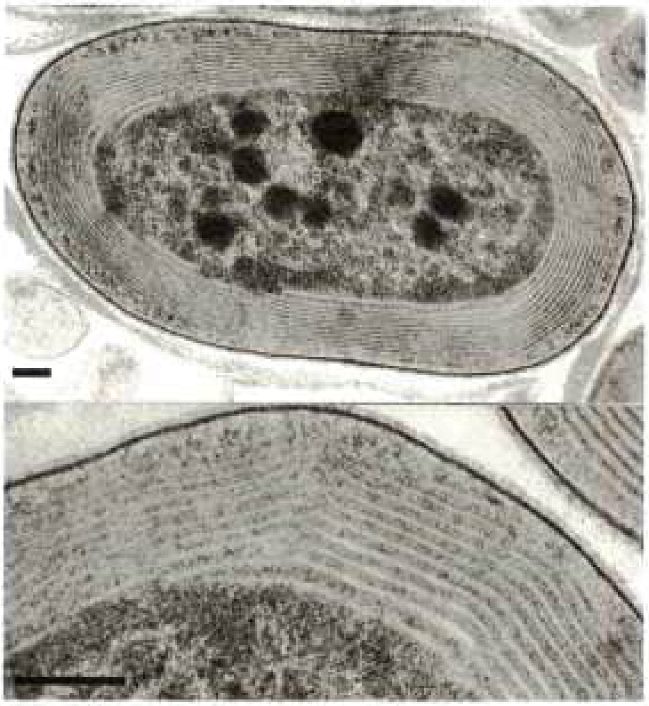


FIGURE 20-23 The photosynthetic membranes of a cyanobacterium. In these thin sections of a cyanobacterium, viewed with a transmission electron microscope, the multiple layers of the internal membranes are seen to fill half the total volume of the cell. The extensive membrane system serves the same role as the thylakoid membranes of vascular plants, providing a large surface area containing all of the photosynthetic machinery. (Bar = 100 nm.) [S. R. Miller et al. Discovery of a free-living chlorophyll *d*-producing cyanobacterium with a hybrid proteobacterial/cyanobacterial small-subunit rRNA gene. *Proc. Natl. Acad. Sci. USA* 102:850, 2005, Fig. 2. © 2005 National Academy of Sciences.]

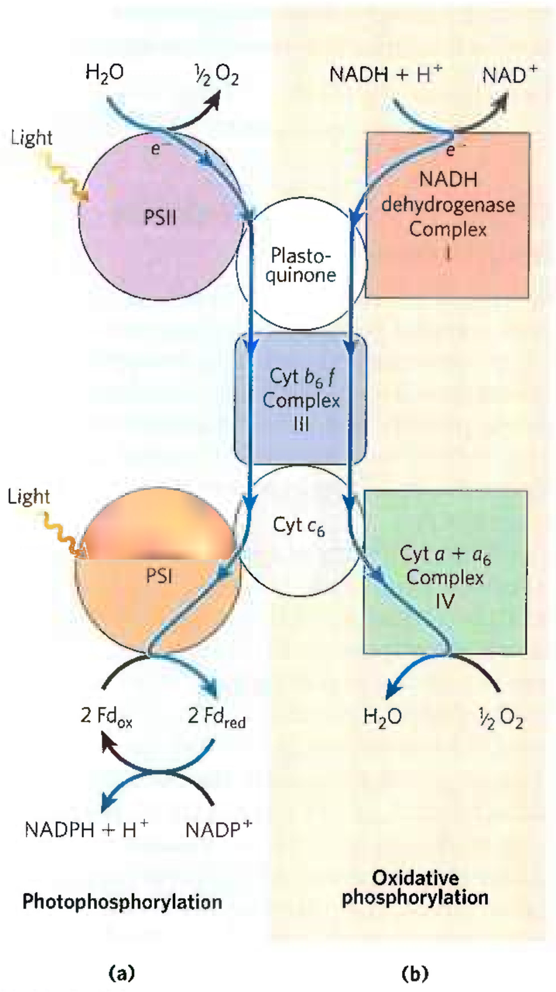


FIGURE 20-24 Dual roles of cytochrome b_6f and cytochrome c_6 in cyanobacteria reflect evolutionary origins. Cyanobacteria use cytochrome b_6f, cytochrome c_6, and plastoquinone for both oxidative phosphorylation and photophosphorylation. (a) In photophosphorylation, electrons flow (blue arrows) from water to $NADP^+$. (b) In oxidative phosphorylation, electrons flow from NADH to O_2. Both processes are accompanied by proton movement across the membrane, accomplished by a Q cycle.

SUMMARY 20.3 *Evolution of a Universal Mechanism for ATP Synthesis*

- In plants, both the water-splitting reaction and electron flow through the cytochrome b_6f complex are accompanied by proton pumping across the thylakoid membrane. The proton-motive force thus created drives ATP synthesis by a CF_oCF_1 complex similar to the mitochondrial F_oF_1 complex in both structure and catalytic mechanism.
- Direct measurements show that eight photons drive the production of one O_2 from oxidation of two H_2O, making three ATP molecules.
- About 2.5 billion years ago, cyanobacteria appeared on Earth. They had acquired two photosystems—one of the type now found in purple bacteria, the other of the type found in green sulfur bacteria—that operated in tandem, and a water-splitting activity that released oxygen into the atmosphere.
- Many photosynthetic microorganisms obtain electrons for photosynthesis not from water but from donors such as H_2S, forming an oxidized product such as elemental sulfur (not oxygen).
- Chloroplasts, like mitochondria, evolved from bacteria living as endosymbionts in early eukaryotic cells. The ATP synthases of bacteria, cyanobacteria, mitochondria, and chloroplasts share a common evolutionary precursor and a common enzymatic mechanism.

20.4 CO_2-Assimilation Reactions

Photosynthetic organisms use the ATP and NADPH produced in the light-dependent reactions of photosynthesis to synthesize all of the thousands of components that make up the organism. P5 Plants (and other autotrophs) can reduce atmospheric CO_2 to trioses, then use the trioses as precursors for the synthesis of sucrose and starch, lipids and proteins, and the many other organic components of plant cells (**Fig. 20-25**). Lacking these synthetic capacities, humans and other animals are ultimately dependent on photosynthetic organisms to provide the reduced fuels and organic precursors essential to life.

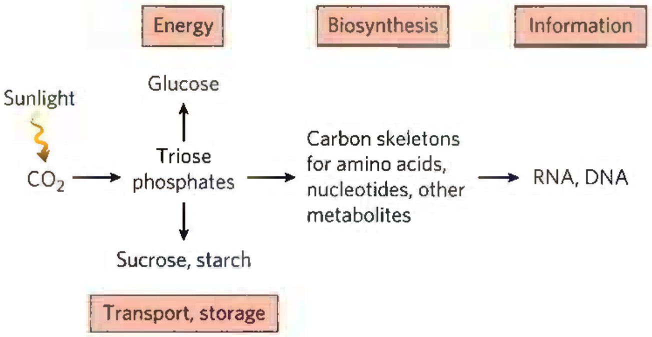


FIGURE 20-25 **Products of photosynthesis.**

Green plants contain in their chloroplasts the enzymatic machinery that catalyzes the conversion of CO_2 to simple (reduced) organic compounds, a process called **CO_2 assimilation**. This process has also been called **CO_2 fixation**, but we reserve this term for the specific reaction in which CO_2 is incorporated (fixed) into a three-carbon organic compound, the triose phosphate 3-phosphoglycerate. This simple product of photosynthesis is the precursor of more-complex biomolecules, including sugars, polysaccharides, and the metabolites derived from them, all of which are synthesized by metabolic pathways similar to those of animal tissues. Carbon dioxide is assimilated via a cyclic pathway, its key intermediates constantly regenerated. The pathway was elucidated in the early 1950s by Melvin Calvin, Andrew Benson, and James A. Bassham and is often called the **Calvin cycle** or, more descriptively, the **reductive pentose phosphate pathway**. It is essentially the reversal of a central pathway of glucose oxidation, the pentose phosphate pathway, which we described in Section 14.6.

Carbohydrate metabolism is more complex in plant cells than in animal cells or in nonphotosynthetic microorganisms. P5 *In addition to* the universal pathways of glycolysis, gluconeogenesis, and the pentose phosphate pathway, plants have the unique reaction sequences for reduction of CO_2 to triose phosphates and the associated reductive pentose phosphate pathway—all of which must be coordinately regulated to ensure proper allocation of carbon to energy production and synthesis of starch and sucrose. Key enzymes are regulated, as we shall see, by (1) reduction of disulfide bonds by electrons flowing from photosystem I and (2) changes in pH and Mg^{2+} concentration that result from illumination. When we look at other aspects of plant carbohydrate metabolism, we also find enzymes that are modulated by (3) conventional allosteric regulation by one or more metabolic intermediates and (4) covalent modification (phosphorylation).

Carbon Dioxide Assimilation Occurs in Three Stages

The first stage in the assimilation of CO_2 into biomolecules (**Fig. 20-26**) is the CO_2-fixation reaction: condensation of CO_2 with a five-carbon acceptor, **ribulose 1,5-bisphosphate**, to form two molecules of **3-phosphoglycerate**. In the second stage, the 3-phosphoglycerate is reduced to triose phosphates. P5 Overall, three molecules of CO_2 are fixed to three molecules of ribulose 1,5-bisphosphate to form six molecules of glyceraldehyde 3-phosphate (18 carbons). In the third stage, five of the six molecules of triose phosphate (15 carbons) are used to regenerate three molecules of ribulose 1,5-bisphosphate (15 carbons), the starting material. The sixth molecule of triose phosphate, the net product of photosynthesis, can be used to make hexoses for fuel and building materials, sucrose for transport to nonphotosynthetic tissues, or starch

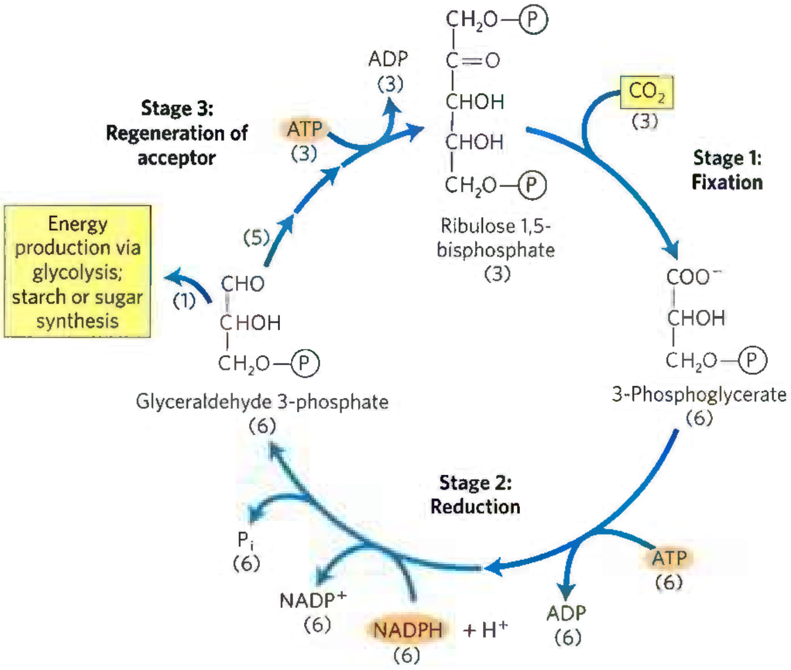


FIGURE 20-26 The three stages of CO_2 assimilation in photosynthetic organisms. Stoichiometries of three key intermediates (numbers in parentheses) reveal the fate of carbon atoms entering and leaving the photosynthetic carbon-reduction cycle (Calvin cycle). Three CO_2 are fixed for the net synthesis of one molecule of glyceraldehyde 3-phosphate.

for storage. Thus, the overall process is cyclical, with the continuous conversion of CO_2 to triose and hexose phosphates.

Fructose 6-phosphate is a key intermediate in stage 3 of CO_2 assimilation; it stands at a branch point, leading either to regeneration of ribulose 1,5-bisphosphate or to synthesis of starch. The pathway from hexose phosphate to pentose bisphosphate involves many of the same reactions used in animal cells for the conversion of pentose phosphates to hexose phosphates during the nonoxidative phase of the pentose phosphate pathway (see Fig. 14-31). In the photosynthetic assimilation of CO_2, essentially the same set of reactions operates in the reverse direction, converting hexose phosphates to pentose phosphates. This reductive pentose phosphate cycle uses the same enzymes as the oxidative pathway, and several additional enzymes that make the reductive cycle irreversible. All 13 enzymes of the pathway are in the chloroplast stroma.

Stage 1: Fixation of CO_2 into 3-Phosphoglycerate An important clue to the nature of the CO_2-assimilation mechanisms in photosynthetic organisms came in the late 1940s. Calvin and his associates illuminated a suspension of green algae in the presence of radioactive carbon dioxide ($^{14}CO_2$) for just a few seconds, then quickly killed the cells, extracted their contents, and used chromatographic methods to search for the metabolites in which the labeled carbon first appeared. The first compound that became labeled was 3-phosphoglycerate, with the ^{14}C predominantly located in the carboxyl carbon atom. These experiments strongly suggested that 3-phosphoglycerate is an early intermediate in photosynthesis.

The many plants in which this three-carbon compound is the first intermediate are called **C_3 plants**, in contrast to the C_4 plants described below. Most plant species—80% to 90%—are C_3, including most trees, wheat, oats, rice, beans, peas, and spinach. The enzyme that catalyzes incorporation of CO_2 into an organic form is **ribulose 1,5-bisphosphate carboxylase/oxygenase**, a name mercifully shortened to **rubisco**. P5 As a carboxylase, rubisco catalyzes the covalent attachment of CO_2 to the five-carbon sugar ribulose 1,5-bisphosphate and cleavage of the unstable six-carbon intermediate to form two molecules of 3-phosphoglycerate, one of which bears the carbon introduced as CO_2 in its carboxyl group (Fig. 20-26). The enzyme's oxygenase activity is discussed in Section 20.5.

There are two distinct forms of rubisco. The rubisco of vascular plants, algae, and cyanobacteria is a crucial enzyme in the production of biomass from CO_2. It has a complex form I structure (**Fig. 20-27a**), with eight identical large catalytic subunits (M_r 53,000; encoded in the chloroplast genome), and eight identical small subunits (M_r 14,000; encoded in the nuclear genome) of uncertain function. The form II rubisco of photosynthetic bacteria is simpler, having two subunits that in many respects resemble the large subunits of the plant enzyme (Fig. 20-27b). The plant enzyme has an exceptionally low turnover number; only three molecules of CO_2 are fixed per second per molecule of rubisco at 25 °C. To achieve high rates of CO_2 fixation, plants therefore need large amounts of this enzyme. Rubisco is present at about 250 mg/mL in the chloroplast stroma, corresponding to an extraordinarily high concentration of active sites (~4 mM). In fact, rubisco makes up almost 50% of soluble protein in chloroplasts and is probably one of the most abundant enzymes in the biosphere.

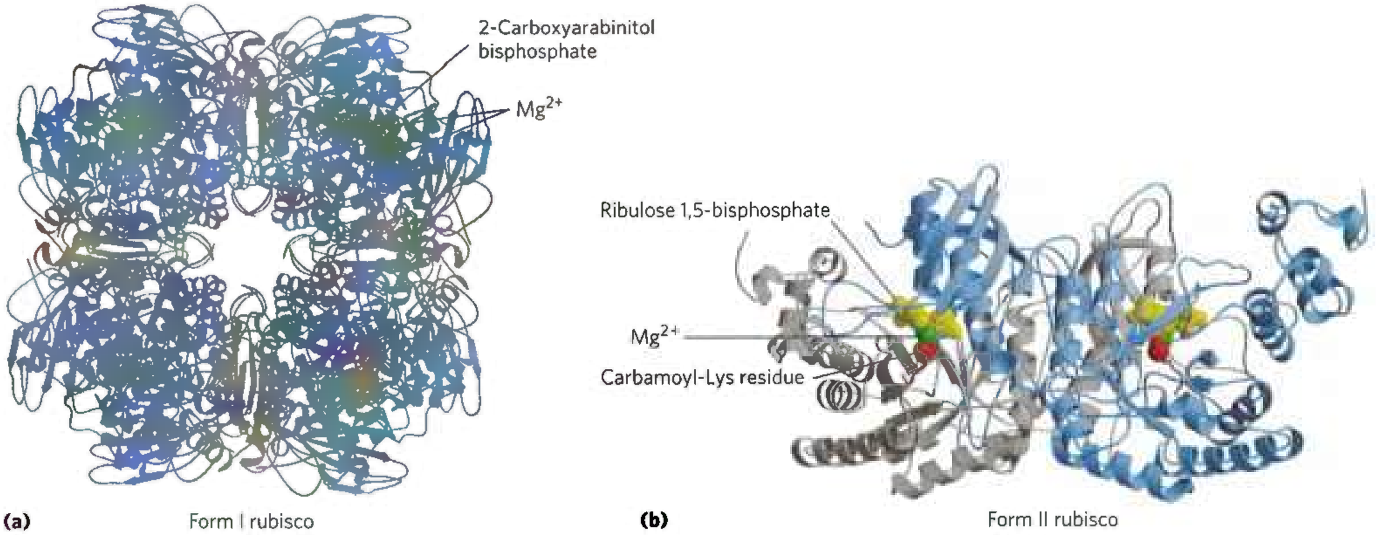


FIGURE 20-27 Structure of ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco). (a) A ribbon model of form I rubisco from spinach. The enzyme has eight large (blue) and eight small (gray) subunits, tightly packed into a structure of $M_r > 500{,}000$. A transition-state analog, 2-carboxyarabinitol bisphosphate (yellow), is shown bound to each of the eight substrate-binding sites. Mg^{2+} in the active site is shown in green. (b) Ribbon model of form II rubisco from the bacterium *Rhodospirillum rubrum*. The identical subunits are in gray and blue. [Data from (a) PDB ID 8RUC, I. Andersson, *J. Mol. Biol.* 259:160, 1996; (b) PDB ID 9RUB, T. Lundqvist and G. Schneider, *J. Biol. Chem.* 266:12,604, 1991.]

Central to the proposed mechanism for plant rubisco is a carbamoylated Lys side chain with a bound Mg^{2+} ion. The Mg^{2+} ion brings together and orients the reactants at the active site (**Fig. 20-28**), setting up for a nucleophilic attack by the five-carbon enediolate reaction intermediate formed on the enzyme (**Fig. 20-29**). The resulting six-carbon intermediate breaks down to yield two molecules of 3-phosphoglycerate.

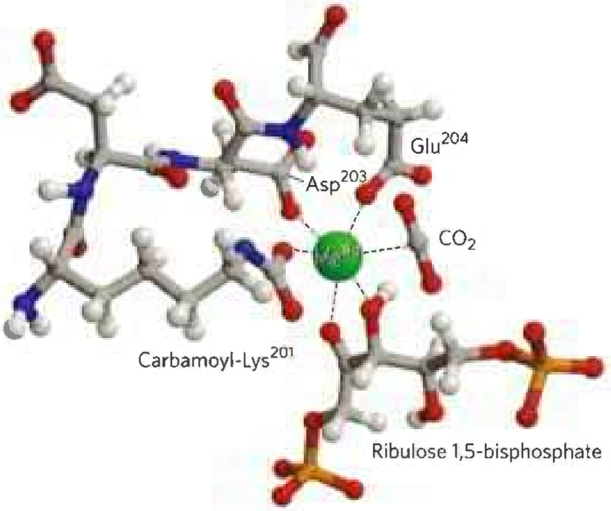
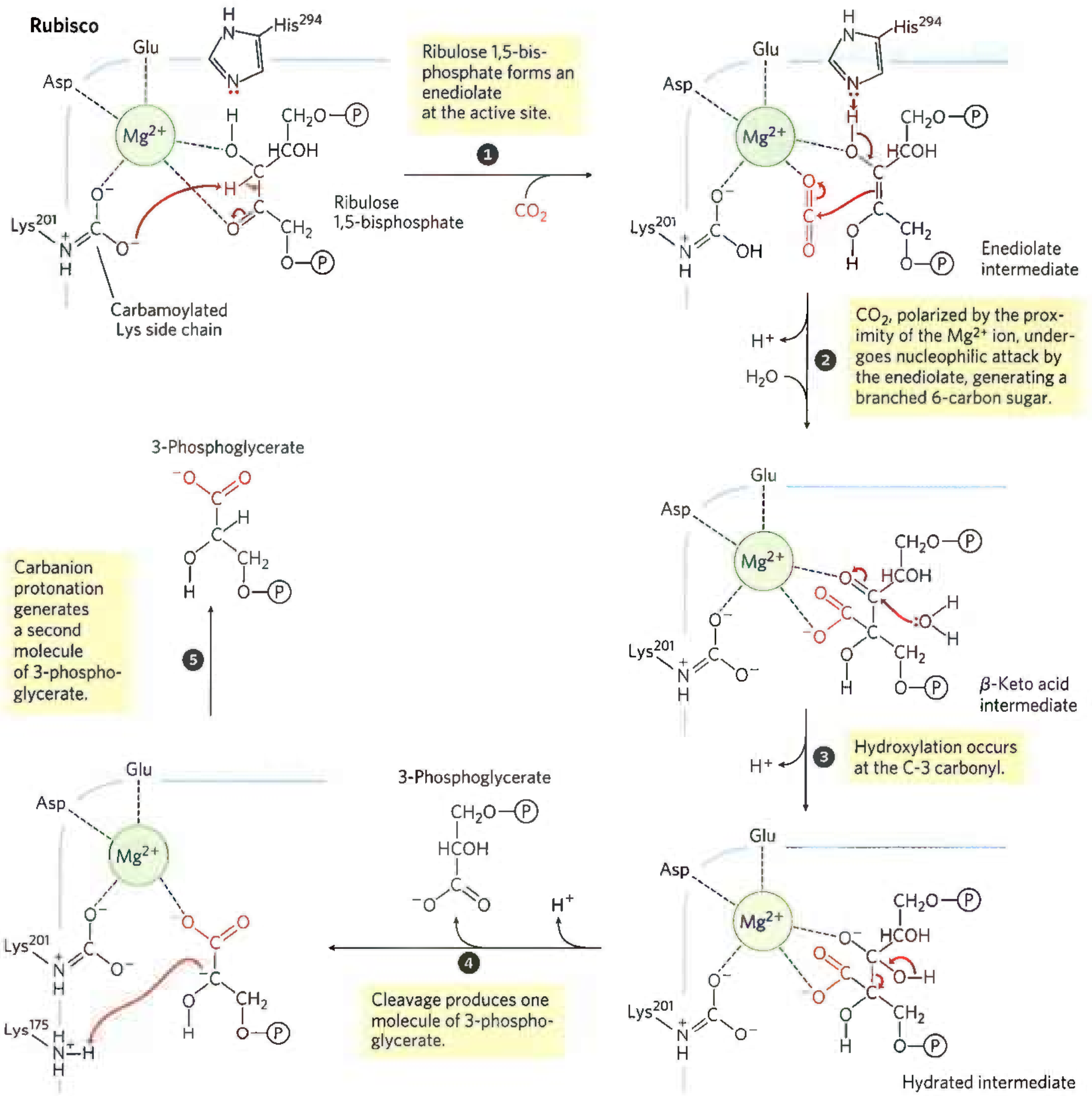


FIGURE 20-28 Central role of Mg^{2+} in the active site of rubisco. Mg^{2+} is coordinated in a roughly octahedral complex with six oxygen atoms: one oxygen in the carbamate on Lys^{201}; two in the carboxyl groups of Glu^{204} and Asp^{203}; two at C-2 and C-3 of the substrate, ribulose 1,5-bisphosphate; and one in the other substrate, CO_2. A water molecule occupies the CO_2-binding site in the crystal structure. In this figure, a CO_2 molecule is modeled in its place. (Residue numbers refer to the spinach enzyme.) [Data from PDB ID 1RXO, T. C. Taylor and I. Andersson, *J. Mol. Biol.* 265:432, 1997.]

As the catalyst for the first step of photosynthetic CO_2 assimilation, rubisco is a prime target for regulation. The enzyme is inactive until carbamoylated on the ε-amino group of Lys^{201} (**Fig. 20-30**). Ribulose 1,5-bisphosphate inhibits carbamoylation by binding tightly to the active site and locking the enzyme in the "closed" conformation, in which Lys^{201} is inaccessible. **Rubisco activase** overcomes the inhibition by promoting ATP-dependent release of the ribulose 1,5-bisphosphate, exposing the Lys amino group to nonenzymatic carbamoylation by CO_2; this is followed by Mg^{2+} binding, which activates the rubisco.

Stage 2: Conversion of 3-Phosphoglycerate to Glyceraldehyde 3-Phosphate Stage 2 begins as stromal 3-phosphoglycerate kinase catalyzes the transfer of a phosphoryl group from ATP to 3-phosphoglycerate, yielding 1,3-bisphosphoglycerate. Next, NADPH donates electrons in a reduction catalyzed by the chloroplast-specific isozyme of glyceraldehyde 3-phosphate dehydrogenase, producing glyceraldehyde 3-phosphate and P_i. The high concentrations of NADPH and ATP in the chloroplast stroma allow this thermodynamically unfavorable pair of reactions to proceed in the direction of glyceraldehyde 3-phosphate formation. Triose phosphate isomerase then interconverts glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, producing the two substrates for aldolase, which condenses them into fructose 1,6-bisphosphate. Thus far, the process has employed the same enzymes we saw in glycolysis, but operating in the reverse direction.

Most of the triose phosphate and fructose 1,6-bisphosphate produced by photosynthesis is used to regenerate ribulose 1,5-bisphosphate, the essential starting material for photosynthesis. Any excess triose


Rubisco
Glu
Asp
His^{294}
Mg^{2+}
Lys^{201}
Carbamoylated Lys side chain
Ribulose 1,5-bisphosphate
①
CO_2
Enediolate intermediate
H^+
H_2O
②
β-Keto acid intermediate
H^+
③
Hydrated intermediate
3-Phosphoglycerate
H^+
④
Lys^{175}
3-Phosphoglycerate
⑤

Ribulose 1,5-bisphosphate forms an enediolate at the active site.

CO_2, polarized by the proximity of the Mg^{2+} ion, undergoes nucleophilic attack by the enediolate, generating a branched 6-carbon sugar.

Hydroxylation occurs at the C-3 carbonyl.

Cleavage produces one molecule of 3-phosphoglycerate.

Carbanion protonation generates a second molecule of 3-phosphoglycerate.

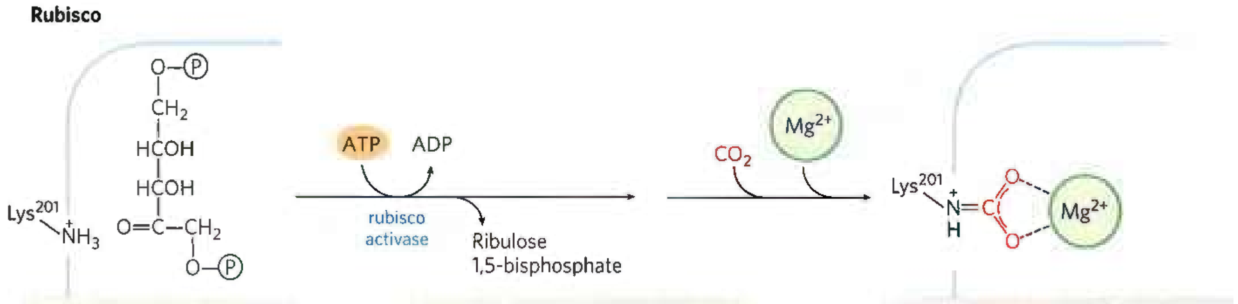
MECHANISM FIGURE 20-29 First stage of CO_2 assimilation: rubisco's carboxylase activity. The CO_2-fixation reaction is catalyzed by ribulose 1,5-bisphosphate carboxylase/oxygenase. The overall reaction accomplishes the combination of one CO_2 and one ribulose 1,5-bisphosphate to form two molecules of 3-phosphoglycerate, one of which contains the carbon atom from CO_2 (red). Additional proton transfers (not shown), involving Lys^{201}, Lys^{175}, and His^{294}, occur in several of these steps.

phosphate is either converted to starch in the chloroplast and stored for later use or immediately exported to the cytosol and converted to sucrose for transport to growing regions of the plant.

Stage 3: Regeneration of Ribulose 1,5-Bisphosphate from Triose Phosphates For the continuous flow of CO_2 into carbohydrate, ribulose 1,5-bisphosphate must be constantly regenerated. This is accomplished in a series of mostly reversible reactions (**Fig. 20-31**) that, together with stages 1 and 2, constitute the reductive pentose phosphate pathway summarized in Figure 20-26. Three exergonic reactions, shown with blue arrows in Figure 20-31, make the whole process irreversible. These are the reactions catalyzed by ② fructose 1,6-bisphosphatase, ⑤ sedoheptulose 1,7-bisphosphatase, and ⑨ ribulose 5-phosphate kinase.

Synthesis of Each Triose Phosphate from CO_2 Requires Six NADPH and Nine ATP

P5 The net result of three turns of the Calvin cycle is the conversion of three molecules of CO_2 and one molecule of phosphate to a molecule of triose phosphate. The stoichiometry of the overall path from CO_2 to triose


Rubisco
O—Ⓟ
CH₂
HCOH
HCOH
O=C—CH₂
O—Ⓟ
Lys²⁰¹
NH₃⁺
ATP
ADP
rubisco activase
Ribulose 1,5-bisphosphate
CO₂
Mg²⁺
Lys²⁰¹
N=C
H
O
O
Mg²⁺

Rubisco with unmodified Lys^{201} and bound ribulose 1,5-bisphosphate is inactive.

ATP-dependent removal of ribulose 1,5-bisphosphate uncovers ε-amino group of Lys^{201}.

ε-Amino group of Lys^{201} is carbamoylated by CO_2; Mg^{2+} binds to carbamoyl-Lys, activating rubisco.

FIGURE 20-30 Role of rubisco activase in carbamoylation of Lys^{201} of rubisco.

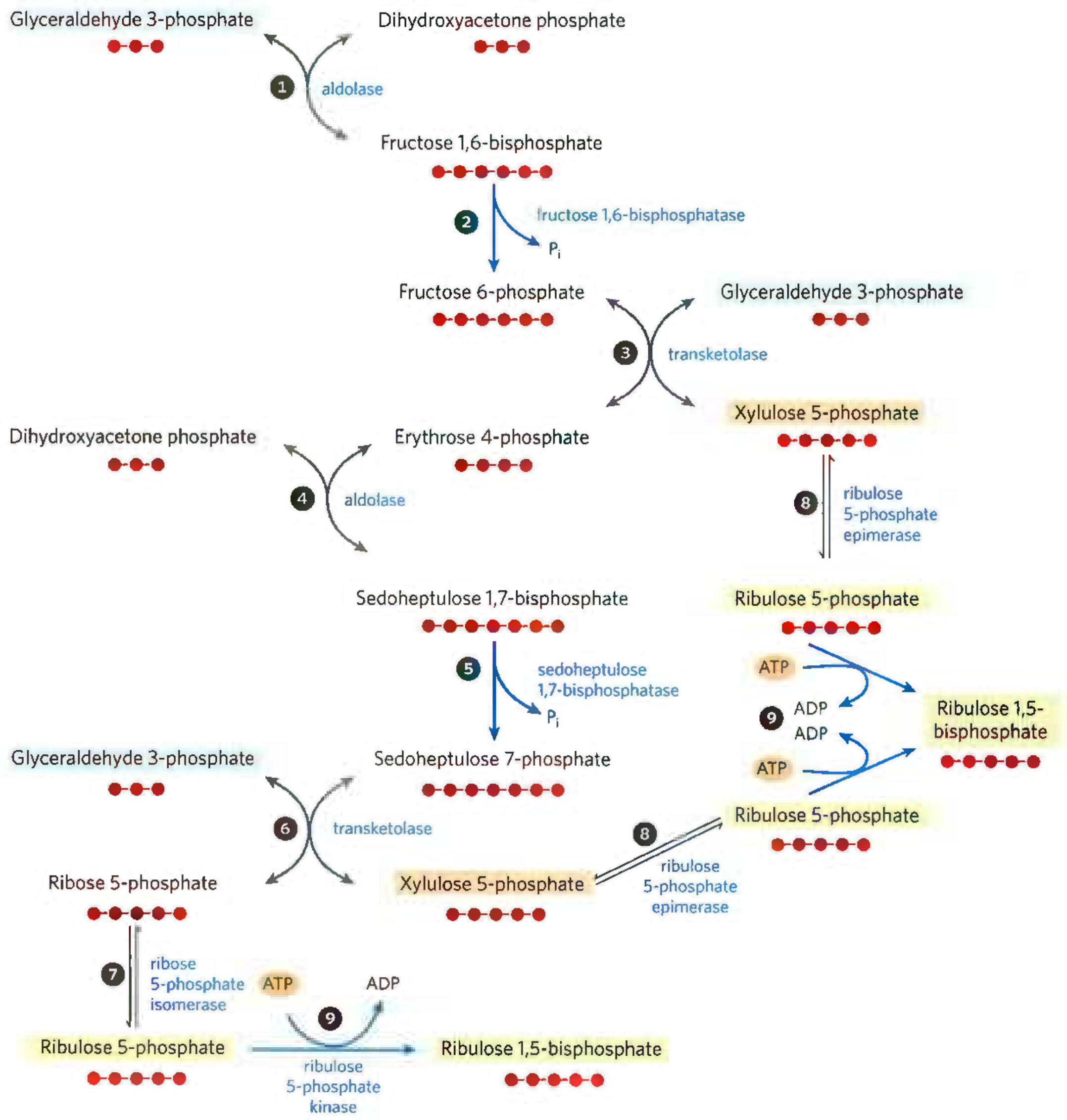

Glyceraldehyde 3-phosphate
Dihydroxyacetone phosphate
1
aldolase
Fructose 1,6-bisphosphate
2
fructose 1,6-bisphosphatase
Pᵢ
Fructose 6-phosphate
Glyceraldehyde 3-phosphate
3
transketolase
Xylulose 5-phosphate
Dihydroxyacetone phosphate
Erythrose 4-phosphate
4
aldolase
8
ribulose 5-phosphate epimerase
Sedoheptulose 1,7-bisphosphate
Ribulose 5-phosphate
5
sedoheptulose 1,7-bisphosphatase
Pᵢ
ATP
ADP
9
ADP
ATP
Ribulose 1,5-bisphosphate
Glyceraldehyde 3-phosphate
Sedoheptulose 7-phosphate
6
transketolase
Ribulose 5-phosphate
8
ribulose 5-phosphate epimerase
Ribose 5-phosphate
Xylulose 5-phosphate
7
ribose 5-phosphate isomerase
ATP
ADP
9
Ribulose 5-phosphate
ribulose 5-phosphate kinase
Ribulose 1,5-bisphosphate

FIGURE 20-31 Third stage of CO_2 assimilation. This schematic diagram shows the interconversions of triose phosphates and pentose phosphates. Red dots represent the number of carbons in each compound. Compounds that appear more than once are highlighted. The starting materials are glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Reactions catalyzed by aldolase (❶ and ❹) and transketolase (❸ and ❻) produce pentose phosphates that are converted to ribulose 1,5-bisphosphate—ribose 5-phosphate by ❼ ribose 5-phosphate isomerase and xylulose 5-phosphate by ❽ ribulose 5-phosphate epimerase. Ribulose 5-phosphate is phosphorylated ❾, regenerating ribulose 1,5-bisphosphate. The reactions with blue arrows are exergonic and make the whole process irreversible: ❷ fructose 1,6-bisphosphatase, ❺ sedoheptulose 1,7-bisphosphatase, and ❾ ribulose 5-phosphate kinase.

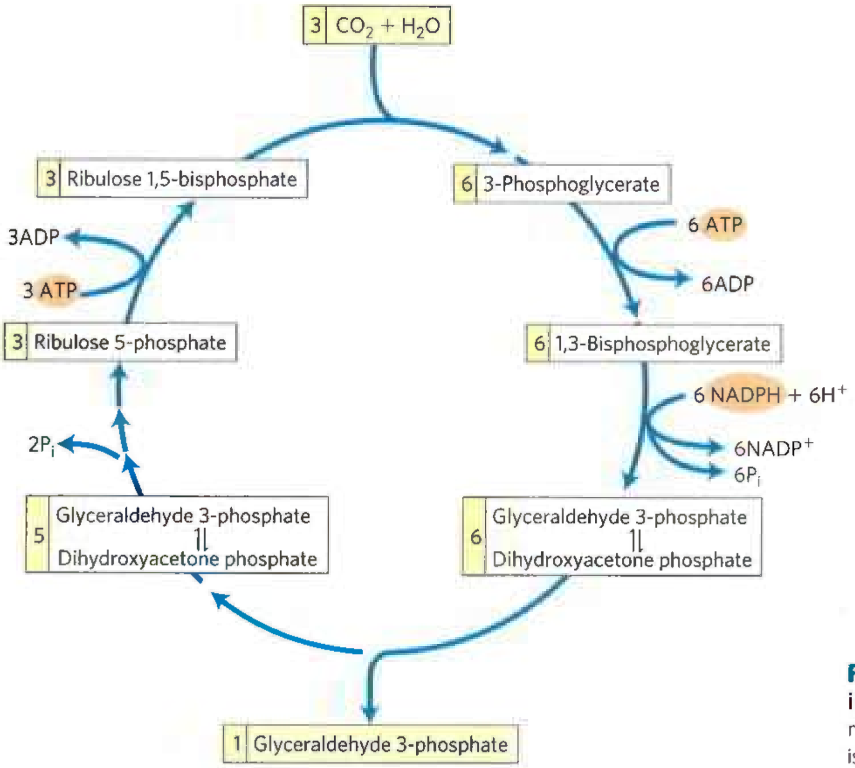

3 $CO_2 + H_2O$
6 3-Phosphoglycerate
6 ATP
6ADP
6 1,3-Bisphosphoglycerate
6 NADPH + 6H$^+$
6NADP$^+$
6P$_i$
6 Glyceraldehyde 3-phosphate ⇌ Dihydroxyacetone phosphate
1 Glyceraldehyde 3-phosphate
5 Glyceraldehyde 3-phosphate ⇌ Dihydroxyacetone phosphate
2P$_i$
3 Ribulose 5-phosphate
3 ATP
3ADP
3 Ribulose 1,5-bisphosphate

FIGURE 20-32 Stoichiometry of CO_2 assimilation in the Calvin cycle. For every three CO_2 molecules fixed, one molecule of triose phosphate (glyceraldehyde 3-phosphate) is produced and nine ATP and six NADPH are consumed.

phosphate, with regeneration of ribulose 1,5-bisphosphate, is shown in **Figure 20-32**.

One molecule of glyceraldehyde 3-phosphate is the net product of the CO_2-assimilation pathway. The other five triose phosphate molecules (15 carbons) are rearranged in steps ❶ to ❾ of Figure 20-31 to form three molecules of ribulose 1,5-bisphosphate (15 carbons). The last step in this conversion requires one ATP per ribulose 1,5-bisphosphate, or a total of three ATP. Thus, in summary, for every molecule of triose phosphate produced by photosynthetic CO_2 assimilation, six NADPH and nine ATP are required.

P5 NADPH and ATP are produced in the light-dependent reactions of photosynthesis in about the same ratio (2:3) as they are consumed in the Calvin cycle. Nine ATP molecules are converted to ADP and phosphate in the generation of a molecule of triose phosphate; eight of the phosphates are released as P_i and combined with eight ADP to regenerate ATP. The ninth phosphate is incorporated into the triose phosphate itself. To convert the ninth ADP to ATP, a molecule of P_i must be imported from the cytosol, as we shall see.

In the dark, the production of ATP and NADPH by photophosphorylation and the incorporation of CO_2 into triose phosphate (once referred to as the dark reactions) cease. The "dark reactions" of photosynthesis were so named to distinguish them from the *primary* light-driven reactions of electron transfer to NADP$^+$ and synthesis of ATP. They do not, in fact, occur at significant rates in the dark and are thus more appropriately called the CO_2-assimilation reactions. Later in this section we describe the regulatory mechanisms that turn CO_2 assimilation on in the light and turn it off in the dark.

The chloroplast stroma contains all the enzymes necessary to convert the triose phosphates produced by CO_2 assimilation (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate) to starch, which is temporarily stored in the chloroplast as insoluble granules. Aldolase condenses the triose phosphates to fructose 1,6-bisphosphate; fructose 1,6-bisphosphatase produces fructose 6-phosphate; phosphohexose isomerase yields glucose 6-phosphate; and phosphoglucomutase produces glucose 1-phosphate, the starting material for starch synthesis (see Section 20.6).

All the reactions of the Calvin cycle except those catalyzed by rubisco, sedoheptulose 1,7-bisphosphatase, and ribulose 5-phosphate kinase also take place in animal tissues. Lacking these three enzymes, animals cannot carry out significant conversion of CO_2 to glucose.

A Transport System Exports Triose Phosphates from the Chloroplast and Imports Phosphate

The inner chloroplast membrane is impermeable to most phosphorylated compounds, including fructose 6-phosphate, glucose 6-phosphate, and fructose 1,6-bisphosphate. It does, however, have a specific antiporter that catalyzes the one-for-one exchange of P_i with a triose phosphate, either dihydroxyacetone phosphate or 3-phosphoglycerate (**Fig. 20-33**). This antiporter simultaneously moves P_i into the chloroplast, where it is used in photophosphorylation, and moves triose phosphate into the cytosol, where it can be used to synthesize sucrose, the form in which the fixed carbon is transported to distant plant tissues.

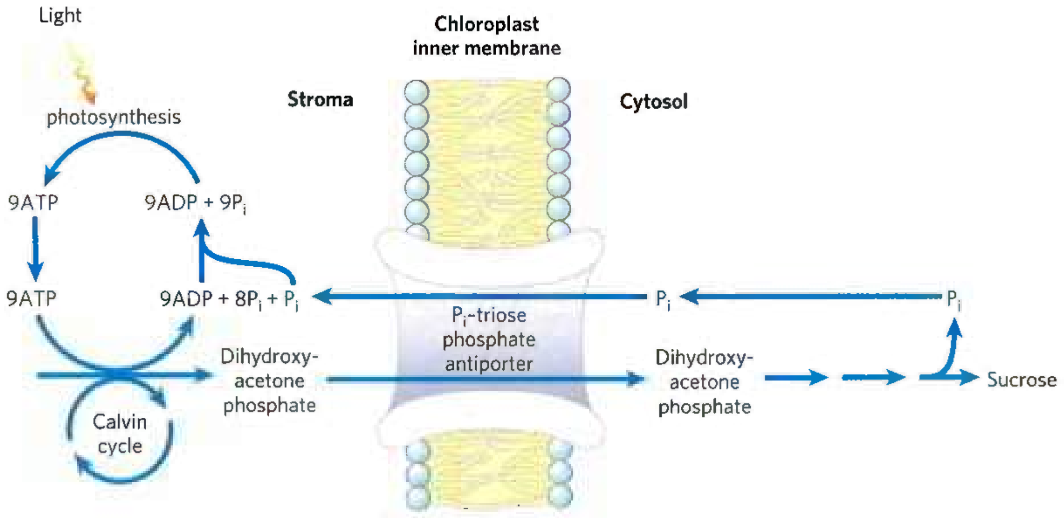

Light
photosynthesis
Stroma
Chloroplast inner membrane
Cytosol
9ATP
9ADP + 9Pᵢ
9ATP
9ADP + 8Pᵢ + Pᵢ
Pᵢ
Pᵢ
Pᵢ-triose phosphate antiporter
Dihydroxy-acetone phosphate
Dihydroxy-acetone phosphate
Sucrose
Calvin cycle

FIGURE 20-33 The P_i–triose phosphate antiport system of the chloroplast inner membrane. This transporter facilitates the exchange of cytosolic P_i for stromal dihydroxyacetone phosphate. The products of photosynthetic CO_2 assimilation are thus moved into the cytosol, where they serve as a starting point for sucrose biosynthesis, and P_i required for photophosphorylation is moved into the stroma. This same antiporter can transport 3-phosphoglycerate, and it acts indirectly in the export of ATP and reducing equivalents (see Fig. 20-34).

Sucrose synthesis in the cytosol and starch synthesis in the chloroplast are the major pathways by which the excess triose phosphate from photosynthesis is harvested. Sucrose synthesis (described later) releases four P_i molecules from the four triose phosphates required to make sucrose. For every molecule of triose phosphate removed from the chloroplast, one P_i is transported into the chloroplast, providing the ninth P_i mentioned above, to be used in regenerating ATP. If this exchange were blocked, triose phosphate synthesis would quickly deplete the available P_i in the chloroplast, slowing ATP synthesis and suppressing assimilation of CO_2 into starch.

The P_i–triose phosphate antiport system serves one additional function. ATP and reducing power are needed in the cytosol for a variety of synthetic and energy-requiring reactions. These requirements are met to an as-yet-undetermined degree by mitochondria, but a second potential source of energy is the ATP and NADPH generated in the chloroplast stroma during the light-dependent reactions. However, neither ATP nor NADPH can cross the chloroplast membrane. The P_i–triose phosphate antiport system has the indirect effect of moving ATP equivalents and reducing equivalents from the chloroplast to the cytosol (**Fig. 20-34**). Dihydroxyacetone phosphate formed in the stroma is transported to the cytosol, where it is converted by glycolytic enzymes to 3-phosphoglycerate, generating ATP and NADH. 3-Phosphoglycerate reenters the chloroplast, completing the cycle.

Four Enzymes of the Calvin Cycle Are Indirectly Activated by Light

The reductive assimilation of CO_2 requires a lot of ATP and NADPH, and their stromal concentrations increase when chloroplasts are illuminated (**Fig. 20-35**). The light-induced transport of protons across the thylakoid membrane also increases the stromal pH from about 7 to about 8, and it is accompanied by a flow of Mg^{2+} from the thylakoid compartment into the stroma, raising the $[Mg^{2+}]$ from 1 to 3 mM to 3 to 6 mM. **P5** Several stromal enzymes have evolved to take advantage of these light-induced conditions, which signal the availability of ATP and NADPH: the enzymes are more active in an alkaline environment and at high $[Mg^{2+}]$. For example, activation of rubisco by formation of carbamoyllysine is faster at alkaline pH, and high stromal $[Mg^{2+}]$ favors formation of the enzyme's active Mg^{2+} complex. Fructose 1,6-bisphosphatase requires Mg^{2+} and is very dependent on pH (**Fig. 20-36**); its activity increases more than 100-fold when pH and $[Mg^{2+}]$ rise during chloroplast illumination.

P5 Four Calvin cycle enzymes are subject to a special type of regulation by light. Ribulose 5-phosphate kinase, fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, and glyceraldehyde 3-phosphate dehydrogenase are activated by light-driven reduction of disulfide bonds between two Cys residues critical to their catalytic activities. When these Cys residues are disulfide-bonded (oxidized), the enzymes are inactive; this is the normal situation in the dark. With illumination, electrons flow from photosystem I to ferredoxin (Fig. 20-12), which passes electrons to a small, soluble, disulfide-containing protein called **thioredoxin** (**Fig. 20-37**), in a reaction catalyzed by **ferredoxin: thioredoxin reductase**. Reduced thioredoxin donates electrons for the reduction of the disulfide bonds of the light-activated enzymes, and these reductive cleavage reactions are accompanied by conformational changes that increase enzyme activities. At nightfall, the Cys residues in the four enzymes are reoxidized to their disulfide forms, the enzymes are inactivated, and ATP is not expended in CO_2 assimilation. Instead, starch

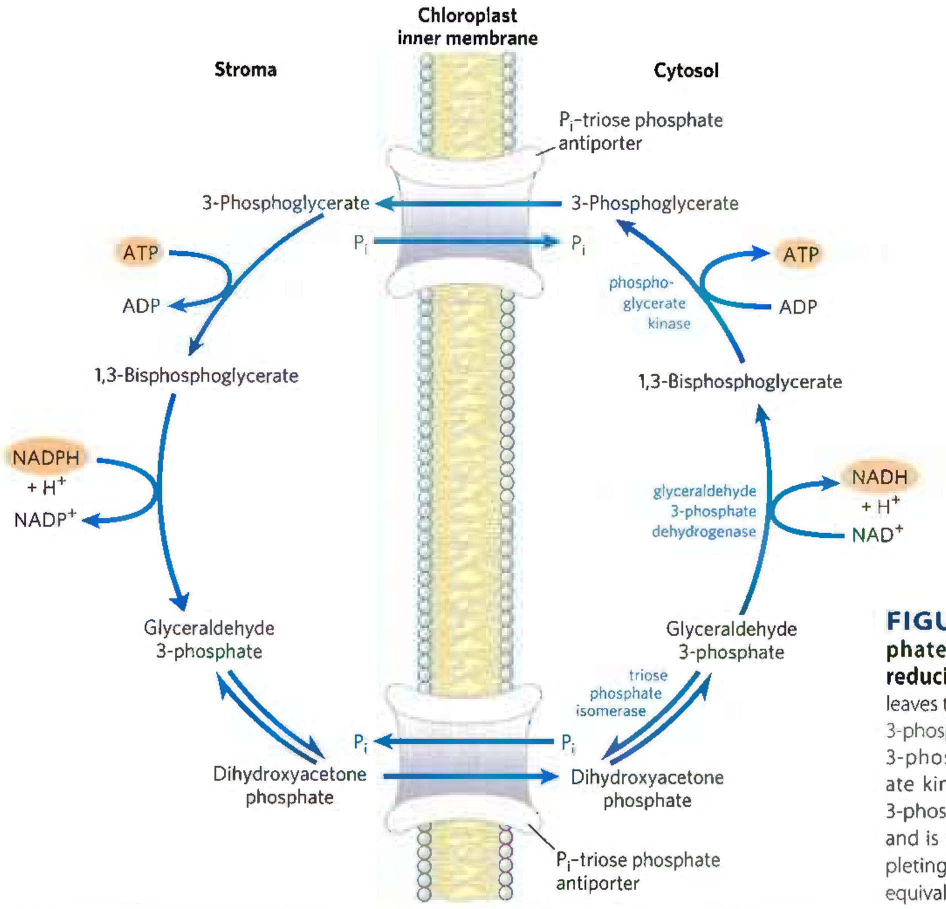

Chloroplast inner membrane
Stroma
Cytosol
P_i-triose phosphate antiporter
3-Phosphoglycerate
3-Phosphoglycerate
P_i
P_i
ATP
ADP
ATP
ADP
phosphoglycerate kinase
1,3-Bisphosphoglycerate
1,3-Bisphosphoglycerate
NADPH
+ H^+
$NADP^+$
glyceraldehyde 3-phosphate dehydrogenase
NADH
+ H^+
NAD^+
Glyceraldehyde 3-phosphate
Glyceraldehyde 3-phosphate
triose phosphate isomerase
P_i
P_i
Dihydroxyacetone phosphate
Dihydroxyacetone phosphate
P_i-triose phosphate antiporter

FIGURE 20-34 Role of the P_i–triose phosphate antiporter in the transport of ATP and reducing equivalents. Dihydroxyacetone phosphate leaves the chloroplast and is converted to glyceraldehyde 3-phosphate in the cytosol. The cytosolic glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase reactions then produce NADH, ATP, and 3-phosphoglycerate. The latter reenters the chloroplast and is reduced to dihydroxyacetone phosphate, completing a cycle that effectively moves ATP and reducing equivalents (NAD(P)H) from chloroplast to cytosol.

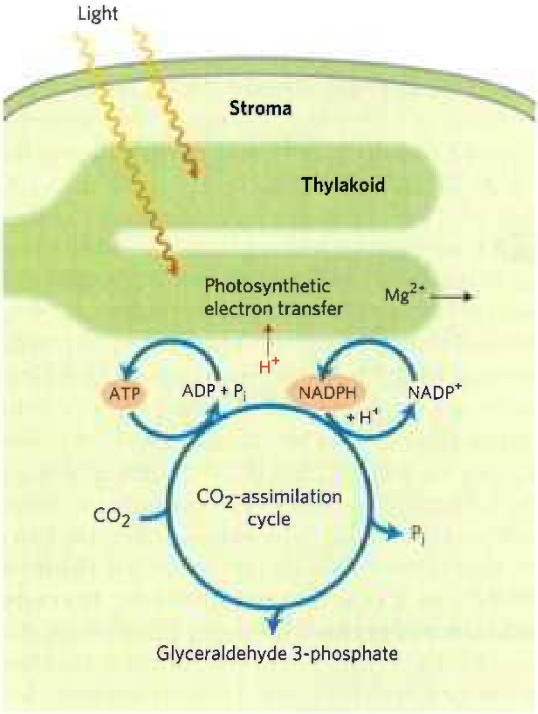

Light
Stroma
Thylakoid
Photosynthetic electron transfer
Mg^{2+}
H^+
ATP
ADP + P_i
NADPH
+ H^+
$NADP^+$
CO_2
CO_2-assimilation cycle
P_i
Glyceraldehyde 3-phosphate

FIGURE 20-35 Source of ATP and NADPH. ATP and NADPH produced by the light-dependent reactions are essential substrates for the reduction of CO_2. The photosynthetic reactions that produce ATP and NADPH are accompanied by movement of protons (red) from the stroma into the thylakoid, creating alkaline conditions in the stroma. Magnesium ions pass from the thylakoid into the stroma, increasing the stromal [Mg^{2+}].

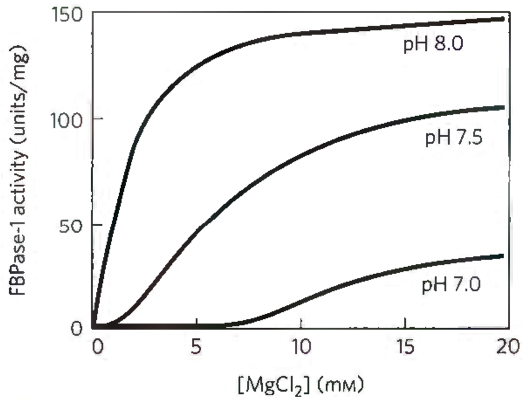


FIGURE 20-36 Activation of chloroplast fructose 1,6-bisphosphatase. Reduced fructose 1,6-bisphosphatase (FBPase-1) is activated by light and by the combination of high pH and high [Mg^{2+}] in the stroma, both of which are results of illumination. [Information from B. Halliwell, *Chloroplast Metabolism: The Structure and Function of Chloroplasts in Green Leaf Cells*, p. 97, Clarendon Press, 1984.]

synthesized and stored during the daytime is degraded to fuel glycolysis and oxidative phosphorylation at night.

Glucose 6-phosphate dehydrogenase, the first enzyme in the *oxidative* pentose phosphate pathway, is also regulated by this light-driven reduction mechanism, but in the opposite sense. During the day, when photosynthesis produces plenty of NADPH, this enzyme is not needed for NADPH production. Reduction of a critical disulfide bond by electrons from ferredoxin *inactivates* the enzyme.

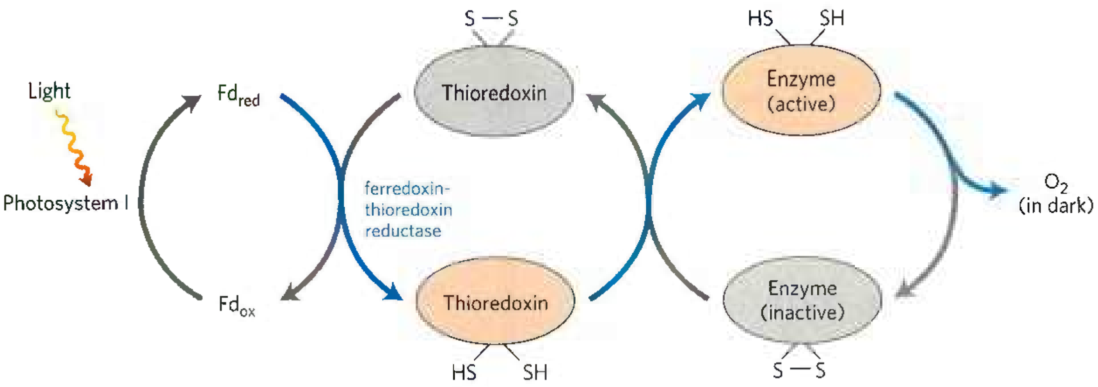


FIGURE 20-37 Light activation of several enzymes of the Calvin cycle. The light activation is mediated by thioredoxin, a small, disulfide-containing protein. In the light, thioredoxin is reduced by electrons moving from photosystem I through ferredoxin (Fd) (blue arrows), then thioredoxin reduces critical disulfide bonds in each of the enzymes sedoheptulose 1,7-bisphosphatase, fructose 1,6-bisphosphatase, ribulose 5-phosphate kinase, and glyceraldehyde 3-phosphate dehydrogenase, activating these enzymes. In the dark, the —SH groups undergo reoxidation to disulfides, inactivating the four enzymes.

SUMMARY 20.4 *Carbon-Assimilation Reactions*

- Photosynthesis in eukaryotes takes place in chloroplasts. In the CO_2-assimilating reactions (the Calvin cycle), ATP and NADPH are used to reduce CO_2 to triose phosphates. These reactions occur in three stages: the fixation reaction itself, catalyzed by rubisco; reduction of the resulting 3-phosphoglycerate to glyceraldehyde 3-phosphate; and regeneration of ribulose 1,5-bisphosphate from triose phosphates.
- Stromal enzymes rearrange the carbon skeletons of triose phosphates to generate intermediates of three, four, five, six, and seven carbons, eventually yielding pentose phosphates. The pentose phosphates are converted to ribulose 5-phosphate, which is phosphorylated to ribulose 1,5-bisphosphate to complete the Calvin cycle.
- The cost of fixing three CO_2 into one triose phosphate is nine ATP and six NADPH, which are provided by the light-dependent reactions of photosynthesis.
- An antiporter in the inner chloroplast membrane exchanges P_i in the cytosol for 3-phosphoglycerate or dihydroxyacetone phosphate molecules produced by CO_2 assimilation in the stroma. Oxidation of dihydroxyacetone phosphate in the cytosol generates ATP and NADH, thus moving ATP and reducing equivalents from the chloroplast to the cytosol.
- Four enzymes of the Calvin cycle are activated indirectly by light and are inactive in the dark, so that hexose synthesis does not compete with glycolysis—which is required to provide energy in the dark.

20.5 Photorespiration and the C_4 and CAM Pathways

As we have seen, photosynthetic cells produce O_2 (by the splitting of H_2O) during the light-driven reactions and use CO_2 during the light-independent processes, so the net gaseous change during photosynthesis is the uptake of CO_2 and release of O_2:

$$CO_2 + H_2O \longrightarrow O_2 + (CH_2O)$$

In the dark, plants also carry out **mitochondrial respiration**, the oxidation of substrates to CO_2 and the conversion of O_2 to H_2O. And there is another process in plants that, like mitochondrial respiration, consumes O_2 and produces CO_2 and, like photosynthesis, is driven by light. This process, **photorespiration**, is a costly side reaction of photosynthesis, a result of the lack of specificity of the enzyme rubisco. In this section we describe this side reaction and the strategies plants use to minimize its metabolic consequences.

Photorespiration Results from Rubisco's Oxygenase Activity

P5 Rubisco is not absolutely specific for CO_2 as a substrate. Molecular oxygen (O_2) competes with CO_2 at the active site, and about once in every three or four turnovers, rubisco catalyzes the condensation of O_2 with ribulose 1,5-bisphosphate to form 3-phosphoglycerate and **2-phosphoglycolate** (**Fig. 20-38**), a metabolically unneeded product. This is the oxygenase activity referred to in the full name of rubisco: *ribu*lose 1,5-*bis*phosphate *c*arboxylase/*o*xygenase. The reaction with O_2 results in no fixation of CO_2 and is presumably a net liability to the cell; salvaging the carbons from 2-phosphoglycolate (by the pathway outlined below) consumes significant amounts of cellular energy and releases some previously fixed CO_2.

Phosphoglycolate Is Salvaged in a Costly Set of Reactions in C_3 Plants

The **glycolate pathway** converts two molecules of 2-phosphoglycolate to a molecule of serine (three carbons) and a molecule of CO_2 (**Fig. 20-39**). In the chloroplast, a phosphatase converts 2-phosphoglycolate to glycolate, which is exported to the peroxisome. There, glycolate is oxidized by molecular oxygen, and the resulting aldehyde (glyoxylate) undergoes transamination to glycine. The hydrogen

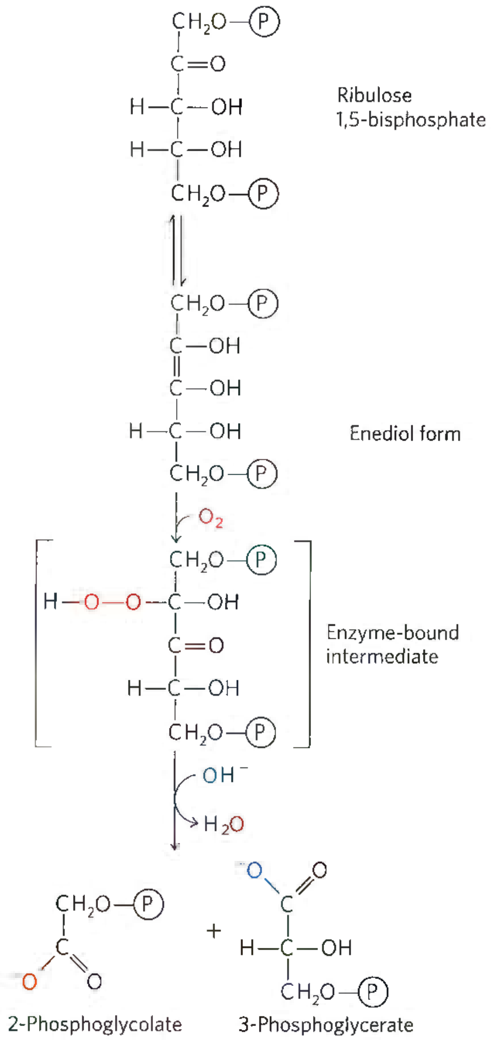

Ribulose 1,5-bisphosphate
Enediol form
O_2
Enzyme-bound intermediate
OH^-
H_2O
2-Phosphoglycolate
3-Phosphoglycerate

FIGURE 20-38 **Oxygenase activity of rubisco.** Rubisco can incorporate O_2 rather than CO_2 into ribulose 1,5-bisphosphate. The unstable intermediate thus formed splits into 2-phosphoglycolate (recycled as described in Fig. 20-39) and 3-phosphoglycerate, which can reenter the Calvin cycle.

peroxide formed as a side product of glycolate oxidation is rendered harmless by peroxidases in the peroxisome. Glycine passes from the peroxisome to the mitochondrial matrix, where it undergoes oxidative decarboxylation by the glycine decarboxylase complex, an enzyme similar in structure and mechanism to two mitochondrial complexes we have already encountered: the pyruvate dehydrogenase complex and the α-ketoglutarate dehydrogenase complex (Chapter 16). The **glycine decarboxylase complex** oxidizes glycine to CO_2 and NH_3, with the concomitant reduction of NAD^+ to NADH and transfer of the remaining carbon from glycine to the cofactor tetrahydrofolate. The one-carbon unit carried on tetrahydrofolate is then transferred to a second glycine by serine hydroxymethyltransferase, producing serine. The net reaction catalyzed by the glycine decarboxylase complex and serine hydroxymethyltransferase is

$$2\text{ Glycine} + NAD^+ + H_2O \longrightarrow \text{serine} + CO_2 + NH_3 + NADH + H^+$$

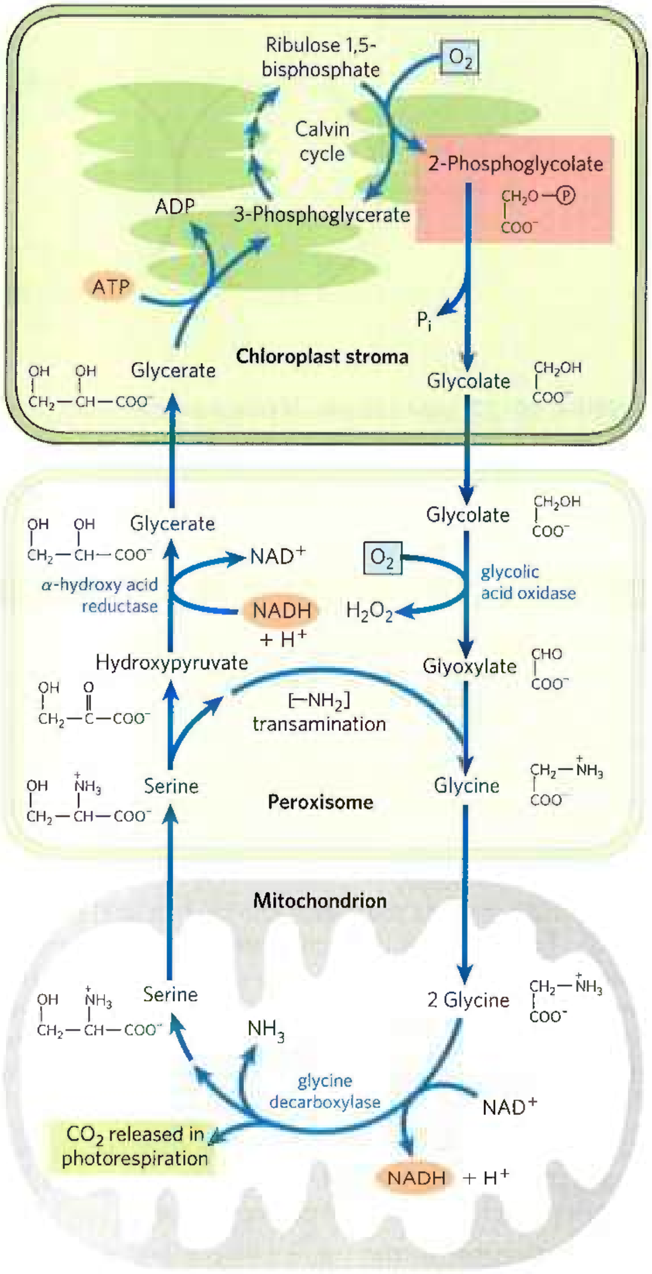

Ribulose 1,5-bisphosphate
O_2
Calvin cycle
2-Phosphoglycolate
ADP
3-Phosphoglycerate
ATP
P_i
Chloroplast stroma
Glycerate
Glycolate
Glycerate
Glycolate
NAD^+
O_2
α-hydroxy acid reductase
NADH
$+ H^+$
H_2O_2
glycolic acid oxidase
Hydroxypyruvate
Glyoxylate
[$-NH_2$] transamination
Serine
Peroxisome
Glycine
Mitochondrion
Serine
NH_3
2 Glycine
glycine decarboxylase
NAD^+
CO_2 released in photorespiration
NADH $+ H^+$

FIGURE 20-39 **Glycolate pathway.** This pathway, which salvages 2-phosphoglycolate (shaded light red) by converting it to serine and, eventually, to 3-phosphoglycerate, involves three cellular compartments. Glycolate formed by dephosphorylation of 2-phosphoglycolate in chloroplasts is oxidized to glyoxylate and transaminated to glycine in peroxisomes. In mitochondria, two glycine molecules condense to form serine and CO_2, released in photorespiration. This reaction is catalyzed by glycine decarboxylase, an enzyme present at very high levels in the mitochondria of C_3 plants. The serine is converted to hydroxypyruvate and then to glycerate in peroxisomes; glycerate reenters the chloroplasts to be phosphorylated, rejoining the Calvin cycle. Oxygen is consumed at two steps during photorespiration.

The serine is converted to hydroxypyruvate, then to glycerate, and finally to 3-phosphoglycerate, which is used to regenerate ribulose 1,5-bisphosphate, completing the long, expensive cycle (Fig. 20-39).

In bright sunlight, the carbon flux through the glycolate salvage pathway can be very high, producing about five times more CO_2 than is typically produced by all the oxidations of the citric acid cycle. To generate this large flux, mitochondria contain prodigious amounts of the glycine decarboxylase complex: the four proteins of the complex make up *half* of all the protein in the mitochondrial matrix in the leaves of pea and spinach plants. In nonphotosynthetic parts of a plant, such as potato tubers, mitochondria have very low concentrations of the glycine decarboxylase complex.

The practical effects of this inefficiency are large and costly. The average yield of soybeans and wheat in the United States is reduced by an estimated 36% and 20%, respectively, by the necessity of recycling glycolate from photorespiration.

P5 The combined activity of the rubisco oxygenase and the glycolate salvage pathway consumes O_2 and produces CO_2—hence the name photorespiration. Unlike mitochondrial respiration, photorespiration does not conserve energy and actually inhibits net biomass formation. This inefficiency has led to evolutionary adaptations in the CO_2-assimilation processes, particularly in plants that have evolved in warm climates. The apparent inefficiency of rubisco, and its effect in limiting biomass production, has inspired efforts to genetically engineer a "better" rubisco, but this goal is not, as yet, within reach (**Box 20-1**).

In C_4 Plants, CO_2 Fixation and Rubisco Activity Are Spatially Separated

In many plants that grow in the tropics (and in temperate-zone crop plants native to the tropics, such as maize, sugarcane, and sorghum) a mechanism has evolved to circumvent the problem of wasteful photorespiration. The step in which CO_2 is fixed into a three-carbon product, 3-phosphoglycerate, is preceded by several steps, one of which is temporary fixation of CO_2 into oxaloacetate, a four-carbon compound. Plants that use this process are referred to as **C_4 plants**, and the assimilation process is known as the **C_4 pathway**, by comparison to the C_3 pathway in which CO_2 is first fixed in the three-carbon compound 3-phosphoglycerate.

P5 The C_4 plants, which typically grow at high light intensity and high temperatures, have several important characteristics: high photosynthetic rates, high growth rates, low photorespiration rates, low rates of water loss, and a specialized leaf structure. Photosynthesis in the leaves of C_4 plants involves two cell types: mesophyll and bundle-sheath cells (**Fig. 20-40a**).

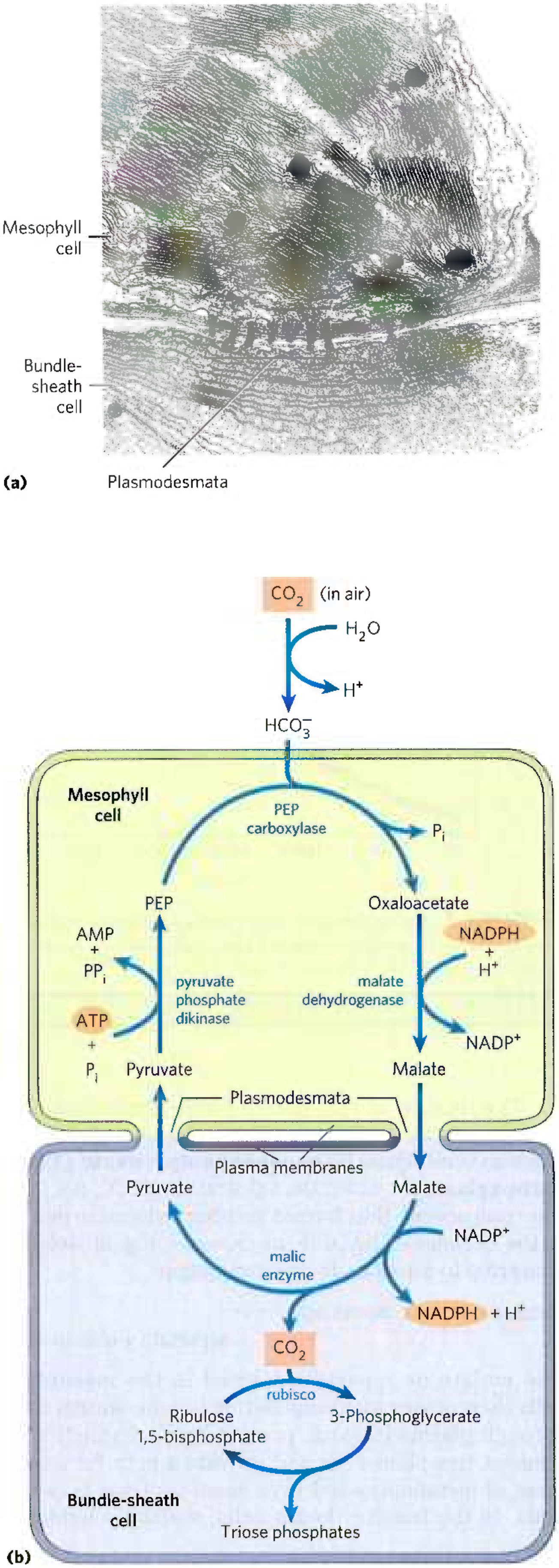

Mesophyll cell
Bundle-sheath cell
(a)
Plasmodesmata
CO_2 (in air)
H_2O
H^+
HCO_3^-
Mesophyll cell
PEP carboxylase
P_i
PEP
Oxaloacetate
AMP + PP_i
ATP + P_i
pyruvate phosphate dikinase
malate dehydrogenase
NADPH + H^+
$NADP^+$
Pyruvate
Malate
Plasmodesmata
Plasma membranes
Pyruvate
Malate
$NADP^+$
malic enzyme
NADPH + H^+
CO_2
rubisco
Ribulose 1,5-bisphosphate
3-Phosphoglycerate
Bundle-sheath cell
Triose phosphates
(b)

FIGURE 20-40 **CO_2 assimilation in C_4 plants.** The C_4 pathway, involving mesophyll cells and bundle-sheath cells, predominates in plants of tropical origin. (a) Electron micrograph showing chloroplasts of adjacent mesophyll and bundle-sheath cells. The bundle-sheath cell contains starch granules. Plasmodesmata connecting the two cells are visible. (b) The C_4 pathway of CO_2 assimilation, which occurs through a four-carbon intermediate. [(a) Dr. Ray Evert, University of Wisconsin–Madison, Department of Botany.]

BOX 20-1

Will Genetic Engineering of Photosynthetic Organisms Increase Their Efficiency?

Three pressing world problems have prompted serious attention to the possibility of engineering plants to be more efficient in converting sunlight into biomass: the greenhouse effect of increasing levels of atmospheric CO_2 on climate change, the finite supply of oil for generating energy, and the need for more and better food for the world's growing population.

The concentration of CO_2 in the earth's atmosphere has risen steadily over the past 50 years (Fig. 1), a combined effect of the use of fossil fuels for energy and the clearing and burning of tropical forests to allow use of the land for agriculture. As atmospheric CO_2 increases, the atmosphere absorbs more heat radiated from the earth's surface and reradiates more heat toward the surface of the planet (and in all other directions). Retention of heat raises the temperature at the surface of the earth; this is the greenhouse effect. One way to limit the increase in atmospheric CO_2 would be to engineer plants or microorganisms with a greater capacity for sequestering CO_2.

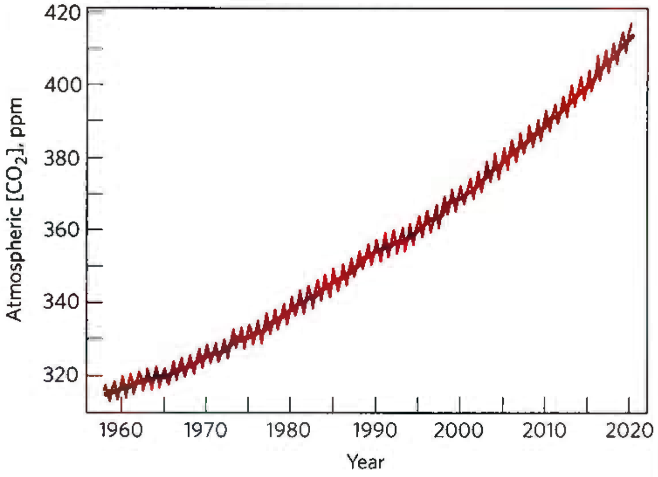


FIGURE 1 The concentration of CO_2 in the atmosphere measured at the Mauna Loa Observatory in Hawaii. [Data from the National Oceanic and Atmospheric Administration and the Scripps Institution of Oceanography CO_2 Program.]

The estimated amount of total carbon in all terrestrial systems (atmosphere, soil, biomass) is about 3,200 gigatons (GT), or 3,200 billion metric tons. The atmosphere contains another 760 GT of CO_2.

The flux of carbon through these terrestrial reservoirs (Fig. 2) is largely due to the photosynthetic activities of plants and the degradative activities of microorganisms. Plants fix some 123 GT of carbon annually, then immediately release about half of that to the atmosphere as they respire. Much of the remainder is gradually released to the atmosphere by microbial action on dead plant materials, but biomass is sequestered in woody plants and trees for decades or centuries. Anthropogenic carbon flux, the amount of CO_2 released into the atmosphere by human activities, is 9 GT per year—small compared with total biomass, but enough to tip the balance toward increased CO_2 in the atmosphere. Estimates indicate that the forests of North America sequester 0.7 GT of carbon annually, which represents about a tenth of the annual *global* production of CO_2 from fossil fuels. Clearly, preservation of forests and reforestation are effective ways to limit the flow of CO_2 back into the atmosphere.

A second approach to limiting the increase of atmospheric CO_2, while also addressing the need to replace dwindling fossil fuels, is to use renewable biomass as a source of ethanol to replace fossil fuels in internal combustion engines. This reduces the *unidirectional* movement of carbon from fossil fuels into the atmospheric pool of CO_2, replacing it with the *cyclic* flow of CO_2 from ethanol to CO_2 and back to biomass.

The fixation of CO_2 into the four-carbon oxaloacetate occurs in the cytosol of leaf mesophyll cells. The reaction is catalyzed by **phosphoenolpyruvate (PEP) carboxylase**, for which the substrate is HCO_3^-, not CO_2. The oxaloacetate thus formed is either reduced to malate at the expense of NADPH (as shown in Fig. 20-40b) or converted to aspartate by transamination:

$$\text{Oxaloacetate} + \alpha\text{-amino acid} \longrightarrow \text{L-aspartate} + \alpha\text{-keto acid}$$

The malate or aspartate formed in the mesophyll cells then passes into neighboring bundle-sheath cells through plasmodesmata, protein-lined channels that connect two plant cells and provide a path for movement of metabolites and even small proteins between cells. In the bundle-sheath cells, malate is oxidized and decarboxylated to yield pyruvate and CO_2 by the action of **malic enzyme**, reducing $NADP^+$. In plants that use aspartate as the CO_2 carrier, aspartate arriving in bundle-sheath cells is transaminated to form oxaloacetate and reduced to malate, then the CO_2 is released by malic enzyme or PEP carboxykinase. Labeling experiments show that the free CO_2 released in the bundle-sheath cells is the same CO_2 molecule originally fixed into oxaloacetate in the mesophyll cells. This CO_2 is now fixed again, this time by rubisco, in exactly the same reaction that occurs in C_3 plants: incorporation of CO_2 into C-1 of 3-phosphoglycerate.

The pyruvate formed by decarboxylation of malate in bundle-sheath cells is transferred back to the mesophyll cells, where it is converted to PEP by an unusual enzymatic reaction catalyzed by **pyruvate phosphate**

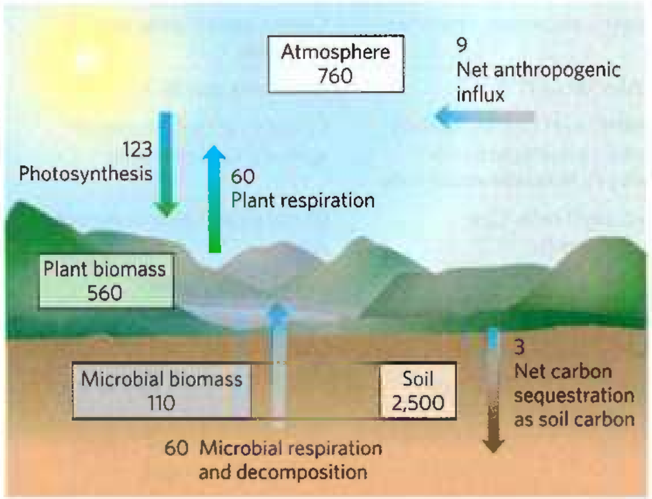


FIGURE 2 The terrestrial carbon cycle. Carbon stocks (boxed text) are shown as gigatons (GT), and fluxes (arrows) are shown in GT per year. Animal biomass is negligible here — less than 0.5 GT. [Information from C. Jansson et al., *BioScience* 60:683, 2010, Fig. 1.]

When maize, wheat, or switchgrass is fermented to ethanol for fuel, every increase in biomass production brought about by more efficient photosynthesis should result in a corresponding decrease in the use of fossil fuels.

Finally, engineering of food crops to yield more food per acre of land, or per hour of work, could improve human nutrition worldwide.

In principle, these goals might be accomplished by developing a rubisco that didn't also catalyze the wasteful reaction with O_2, or by increasing the turnover number for rubisco, or by increasing the level of rubisco or other enzymes in the pathway for CO_2 fixation. Rubisco, as we have noted, is an unusually inefficient enzyme, with a turnover number of 3 s^{-1} at 25 °C; most enzymes have turnover numbers orders of magnitude larger. It also catalyzes the wasteful reaction with oxygen, which further reduces its efficiency in fixing CO_2 and producing biomass. If rubisco could be genetically engineered to turn over faster or to be more selective for CO_2 relative to O_2, would the effect be greater photosynthetic production of biomass and thus greater sequestration of CO_2, greater production of nonfossil fuel, and improved nutrition?

The traditional view of metabolic pathways held that one step in any pathway was the slowest and therefore the limiting factor in material flow through the pathway. However, efforts to engineer cells or organisms to produce more of the "limiting" enzyme in a pathway have often given discouraging results; the organisms often show little or no change in the flux through that pathway. The Calvin cycle is an instructive case in point. Increasing the amount of rubisco in plant cells through genetic engineering has little or no effect on the rate of CO_2 conversion into carbohydrate. Similarly, changes in the levels of enzymes known to be regulated by light and therefore suspected of playing key roles in the regulation of the CO_2-assimilation pathway (fructose 1,6-bisphosphatase, 3-phosphoglycerate kinase, and glyceraldehyde 3-phosphate dehydrogenase) also produce little or no significant improvement in photosynthetic rate. This should probably not be surprising; in the living organism, pathways can be limited by more than one enzymatic step, because every change in one step results in compensating changes in other steps. **Metabolic control analysis** is the science of measuring, understanding, and eventually altering the factors that govern the overall flux through a pathway. Its application will be essential to the success of engineering plants for higher efficiency or greater yield.

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dikinase (Fig. 20-40b). This enzyme is called a dikinase because two different molecules are simultaneously phosphorylated by one molecule of ATP: pyruvate to PEP, and phosphate to pyrophosphate. The pyrophosphate is subsequently hydrolyzed to phosphate, so two high-energy phosphate groups of ATP are used in regenerating PEP. The PEP is now ready to receive another molecule of CO_2 in the mesophyll cell.

The PEP carboxylase of mesophyll cells has a high affinity for HCO_3^- (which is favored relative to CO_2 in aqueous solution) and can fix CO_2 more efficiently than can rubisco. Unlike rubisco, it does not use O_2 as an alternative substrate, so there is no competition between CO_2 and O_2. The PEP carboxylase reaction, then, serves to fix and concentrate CO_2 in the form of malate. **P5** Release of CO_2 from malate in the bundle-sheath cells yields a sufficiently high local concentration of CO_2 for rubisco to function near its maximal rate, and for suppression of the enzyme's oxygenase activity.

Once CO_2 is fixed into 3-phosphoglycerate in the bundle-sheath cells, the other reactions of the Calvin cycle take place exactly as described earlier. Thus in C_4 plants, mesophyll cells carry out CO_2 assimilation by the C_4 pathway and bundle-sheath cells synthesize starch and sucrose by the C_3 pathway.

Three enzymes of the C_4 pathway are regulated by light, becoming more active in daylight. Malate dehydrogenase is activated by the thioredoxin-dependent reduction mechanism shown in Figure 20-37; PEP carboxylase is activated by phosphorylation of a Ser residue; and pyruvate phosphate dikinase is activated by dephosphorylation.

TABLE 20-1 Comparison of C_3, C_4, and CAM Plants

	C_3 Plants	C_4 Plants	CAM Plants
Examples	Spinach, pea, rice, wheat, beans, most trees	Maize (corn), sugarcane, crabgrass	Cactus, prickly pear, orchid, pineapple
Most efficient environment	15 to 25 °C	Hot and dry; 30 to 47 °C	Extremely dry; 35 °C
Path of CO_2 fixation	C_3 photosynthesis only	Sequential C_4 and C_3 cycles spatially separated: C_4 in mesophyll cells followed by C_3 in bundle-sheath cells	C_3 and C_4 cycles, separated spatially and temporally
Cell type involved	Mesophyll cells	C_4 in mesophyll cells, C_3 in bundle-sheath cells	C_3 and C_4 in the same mesophyll cells
Light conditions	Light	Light	C_3 in light; C_4 in dark
Initial CO_2 acceptor	Ribulose 1,5-bisphosphate	Phosphoenolpyruvate	Ribulose 1,5-bisphosphate in light; phosphoenolpyruvate in dark
CO_2-fixing enzyme	Rubisco	PEP carboxylase, then rubisco	Rubisco in light; PEP carboxylase at night
First stable product of CO_2 fixation	3-Phosphoglycerate	Oxaloacetate in C_4 cycle	3-Phosphoglycerate in light; oxaloacetate in dark
Energy needed for complete reduction of one molecule of CO_2	3 ATP, 2 NADPH	5 ATP, 2 NADPH	6.5 ATP, 2 NADPH
Photorespiration	Present	Absent or suppressed	Absent or suppressed

The pathway of CO_2 assimilation has a greater energy cost in C_4 plants than in C_3 plants. For each molecule of CO_2 assimilated in the C_4 pathway, a molecule of PEP must be regenerated at the expense of two phosphoanhydride bonds in ATP. P5 Thus C_4 plants need five ATP molecules to assimilate one molecule of CO_2, whereas C_3 plants need only three (nine per triose phosphate). As the temperature increases (and the affinity of rubisco for CO_2 decreases, as noted above), a point is reached, at about 28 to 30 °C, at which the gain in efficiency from the elimination of photorespiration more than compensates for this energetic cost. C_4 plants (crabgrass, for example) outgrow most C_3 plants during the summer, as any experienced gardener can attest.

In CAM Plants, CO_2 Capture and Rubisco Action Are Temporally Separated

Succulent plants such as cactus and pineapple, which are native to very hot, very dry environments, have another variation on photosynthetic CO_2 fixation, which reduces loss of water vapor through the pores (stomata) by which CO_2 and O_2 must enter leaf tissue. Instead of separating the initial trapping of CO_2 and its fixation by rubisco across space (as do the C_4 plants), they separate these two events over time. At night, when the air is cooler and moister, the stomata open to allow entry of CO_2, which is then fixed into oxaloacetate by PEP carboxylase. The oxaloacetate is reduced to malate and stored in the vacuoles, to protect cytosolic and plastid enzymes from the low pH produced by malic acid dissociation. During the day the stomata close, preventing the water loss that would result from high daytime temperatures, and the CO_2 trapped overnight in malate is released as CO_2 by the NADP-linked malic enzyme. This CO_2 is now assimilated by the action of rubisco and the Calvin cycle enzymes. Because this method of CO_2 fixation was first discovered in stonecrops, perennial flowering plants of the family Crassulaceae, it is called crassulacean acid metabolism, and the plants are called **CAM plants**. Table 20-1 compares characteristics of C_3, C_4, and CAM plants.

SUMMARY 20.5 *Photorespiration and the C_4 and CAM Pathways*

- Rubisco is not completely specific for CO_2 as its substrate; it can also use O_2, producing 2-phosphoglycolate, which must be disposed of in an oxygen-dependent pathway. The result is increased consumption of O_2—photorespiration.
- The 2-phosphoglycolate is converted to glyoxylate, to glycine, and then to serine in a pathway that involves enzymes in the chloroplast stroma, peroxisomes, and mitochondria.
- In C_4 plants, the CO_2-assimilation pathway minimizes photorespiration: CO_2 is first fixed in mesophyll cells into a four-carbon compound, which passes into bundle-sheath cells and releases CO_2 in high concentrations. The released CO_2 is fixed by rubisco, and the remaining reactions of the Calvin cycle occur as in C_3 plants.
- In CAM plants, CO_2 is fixed into malate in the dark and stored in vacuoles until daylight, when the stomata are closed (minimizing water loss), and the stored malate serves as a source of CO_2 for rubisco.

20.6 Biosynthesis of Starch, Sucrose, and Cellulose

During active photosynthesis in bright light, a plant leaf produces more carbohydrate (as triose phosphates) than it needs for generating energy or synthesizing precursors. P5 The excess is converted to sucrose and transported to other parts of the plant, to be used as fuel or stored. In most plants, starch is the main storage form of carbohydrate, but in a few plants, such as sugar beet and sugarcane, sucrose is the primary storage form. The synthesis of sucrose and starch occurs in different cellular compartments (cytosol and plastids, respectively), and these processes are coordinated by a variety of regulatory mechanisms that respond to changes in light level and photosynthetic rate. The synthesis of sucrose and starch is important to the plant but also to humans: starch provides more than 80% of human dietary calories worldwide.

ADP-Glucose Is the Substrate for Starch Synthesis in Plant Plastids and for Glycogen Synthesis in Bacteria

Starch, like glycogen, is a high molecular weight polymer of D-glucose in ($\alpha1 \rightarrow 4$) linkage. It is synthesized in chloroplasts for temporary storage as one of the stable end products of photosynthesis, and for long-term storage it is synthesized in amyloplasts of the nonphotosynthetic parts of plants: seeds, roots, and tubers (underground stems).

The mechanism of glucose activation in starch synthesis is similar to that in glycogen synthesis, described in Chapter 15. An activated **sugar nucleotide**, in this case ADP-glucose, is formed by condensation of glucose 1-phosphate with ATP in a reaction made essentially irreversible by the presence in plastids of inorganic pyrophosphatase. **Starch synthase** then transfers glucose residues from ADP-glucose to preexisting starch molecules. The monomeric units are almost certainly added to the nonreducing end of the growing polymer, as they are in glycogen synthesis.

The amylose of starch is unbranched, but amylopectin has numerous ($\alpha1 \rightarrow 6$)-linked branches (see Fig. 7-13). Chloroplasts contain a branching enzyme, similar to the glycogen-branching enzyme that introduces the ($\alpha1 \rightarrow 6$) branches of amylopectin. Taking into account the hydrolysis by inorganic pyrophosphatase of the PP_i produced during ADP-glucose synthesis, the overall reaction for starch formation from glucose 1-phosphate is

$$\text{Starch}_n + \text{glucose 1-phosphate} + \text{ATP} \longrightarrow \text{starch}_{n+1} + \text{ADP} + 2P_i \qquad \Delta G'^{\circ} = -50 \text{ kJ/mol}$$

Starch synthesis is regulated at the level of ADP-glucose formation, as discussed below.

UDP-Glucose Is the Substrate for Sucrose Synthesis in the Cytosol of Leaf Cells

Most of the triose phosphate generated by CO_2 fixation in plants is converted to sucrose (**Fig. 20-41**) or starch.

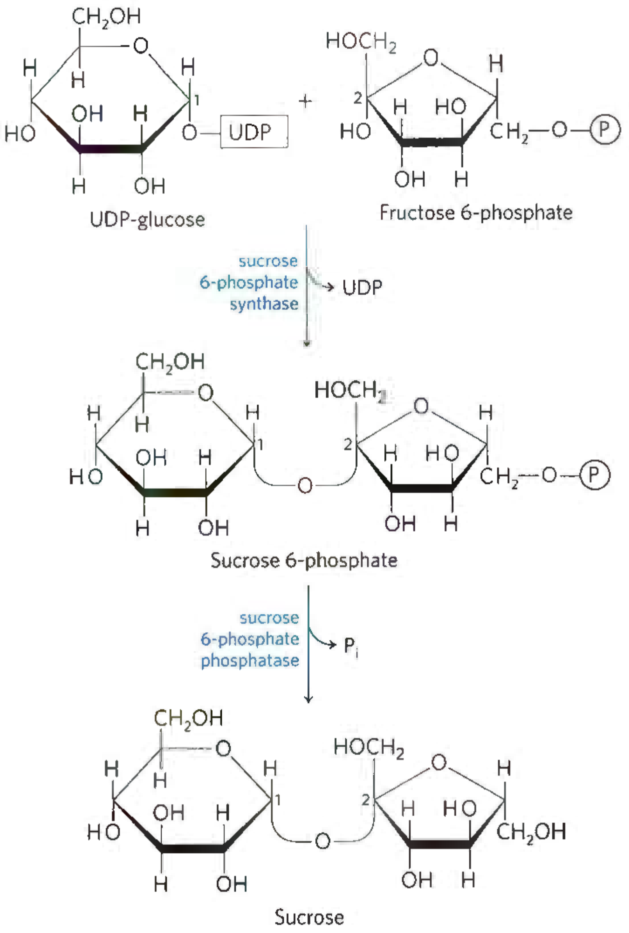


FIGURE 20-41 Sucrose synthesis. Sucrose is synthesized from UDP-glucose and fructose 6-phosphate, which are synthesized from triose phosphates in the plant cell cytosol. The sucrose 6-phosphate synthase of most plant species is allosterically regulated by glucose 6-phosphate and P_i.

In the course of evolution, sucrose may have been selected as the transport form of carbon because of its unusual linkage between the anomeric C-1 of glucose and the anomeric C-2 of fructose. This bond is not hydrolyzed by amylases or other common carbohydrate-cleaving enzymes, and the unavailability of the sucrose molecule's anomeric carbons prevents it from reacting nonenzymatically (as does glucose) with amino acids and proteins.

Sucrose is synthesized in the cytosol, beginning with dihydroxyacetone phosphate and glyceraldehyde 3-phosphate exported from the chloroplast. After condensation of two triose phosphates to form fructose 1,6-bisphosphate (catalyzed by aldolase), hydrolysis by fructose 1,6-bisphosphatase yields fructose 6-phosphate. **Sucrose 6-phosphate synthase** then catalyzes the reaction of fructose 6-phosphate with UDP-glucose to form sucrose 6-phosphate (Fig. 20-41). Finally, sucrose 6-phosphate phosphatase removes the phosphate group, making sucrose available for export to other tissues. The reaction catalyzed by sucrose 6-phosphate synthase is a low-energy process ($\Delta G'^{\circ} = -5.7$ kJ/mol), but the hydrolysis of sucrose 6-phosphate to sucrose is sufficiently exergonic ($\Delta G'^\circ = -16.5$ kJ/mol) to make the overall synthesis of sucrose thermodynamically favorable. Sucrose synthesis is regulated and closely coordinated with starch synthesis, as we shall see.

One remarkable difference between the cells of plants and animals is the absence in the plant cell cytosol of the enzyme inorganic pyrophosphatase, which catalyzes the reaction

$$PP_i + H_2O \longrightarrow 2P_i \qquad \Delta G'^\circ = -19.2 \text{ kJ/mol}$$

For many biosynthetic reactions that liberate PP_i, pyrophosphatase activity makes the process more favorable energetically, tending to make these reactions irreversible. In plants, this enzyme is present in plastids but absent from the cytosol. As a result, the cytosol of leaf cells contains a substantial concentration of PP_i—enough (~0.3 mM) to make reactions such as that catalyzed by UDP-glucose pyrophosphorylase (see Fig. 15-7) readily reversible.

Conversion of Triose Phosphates to Sucrose and Starch Is Tightly Regulated

Triose phosphates produced by the Calvin cycle in bright sunlight, as we have noted, may be stored temporarily in the chloroplast as starch, or converted to sucrose and exported to nonphotosynthetic parts of the plant, or both. P5 The balance between the two processes is tightly regulated, and both must be coordinated with the rate of CO_2 fixation. Five-sixths of the triose phosphate formed in the Calvin cycle must be recycled to ribulose 1,5-bisphosphate (Fig. 20-32); if more than one-sixth of the triose phosphate is drawn out of the cycle to make sucrose and starch, the cycle will slow or stop. However, *insufficient* conversion of triose phosphate to starch or sucrose would tie up phosphate, leaving a chloroplast deficient in P_i, which is also essential for operation of the Calvin cycle.

The flow of triose phosphates into sucrose is regulated by the activity of fructose 1,6-bisphosphatase (FBPase-1) and the enzyme that effectively reverses its action, PP_i-dependent phosphofructokinase (PP-PFK-1). These enzymes are therefore critical points for determining the fate of triose phosphates produced by photosynthesis. Both enzymes are regulated by **fructose 2,6-bisphosphate (F26BP)**, which inhibits FBPase-1 and stimulates PP-PFK-1. In vascular plants, the concentration of F26BP varies inversely with the rate of photosynthesis (Fig. 20-42). Phosphofructokinase-2, responsible for F26BP synthesis, is inhibited by dihydroxyacetone phosphate or 3-phosphoglycerate and is stimulated by fructose 6-phosphate and P_i. During active photosynthesis, dihydroxyacetone phosphate is produced and P_i is consumed, resulting in inhibition of PFK-2 and lowered concentrations of F26BP. This favors greater flux of triose phosphate into fructose 6-phosphate formation and sucrose synthesis. With this regulatory system, sucrose synthesis occurs when the level of triose phosphate produced by the Calvin cycle exceeds that needed to maintain operation of the cycle.

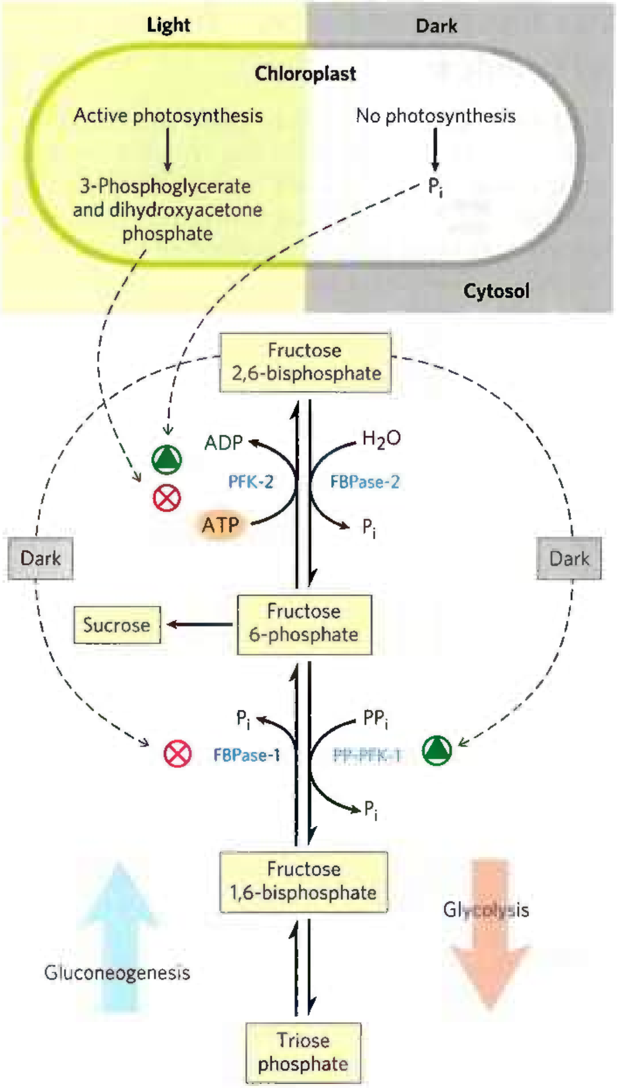


FIGURE 20-42 Fructose 2,6-bisphosphate as regulator of sucrose synthesis. The concentration of the allosteric regulator fructose 2,6-bisphosphate in plant cells is regulated by the products of photosynthetic CO_2 assimilation and by P_i. Dihydroxyacetone phosphate and 3-phosphoglycerate produced by CO_2 assimilation inhibit phosphofructokinase-2 (PFK-2), the enzyme that synthesizes the regulator; P_i stimulates PFK-2. The concentration of the regulator is therefore inversely proportional to the rate of photosynthesis. In the dark, the concentration of fructose 2,6-bisphosphate increases and stimulates the glycolytic enzyme PP_i-dependent phosphofructokinase-1 (PP-PFK-1), while inhibiting the gluconeogenic enzyme fructose 1,6-bisphosphatase (FBPase-1). When photosynthesis is active (in the light), the concentration of the regulator drops and the synthesis of fructose 6-phosphate and sucrose is favored.

Sucrose synthesis is also regulated at the level of sucrose 6-phosphate synthase, which is allosterically activated by glucose 6-phosphate and inhibited by P_i. This enzyme is further regulated by phosphorylation and dephosphorylation; a protein kinase phosphorylates the enzyme on a specific Ser residue, making it less active, and a phosphatase reverses this inactivation by removing the phosphate (Fig. 20-43). Inhibition of the kinase

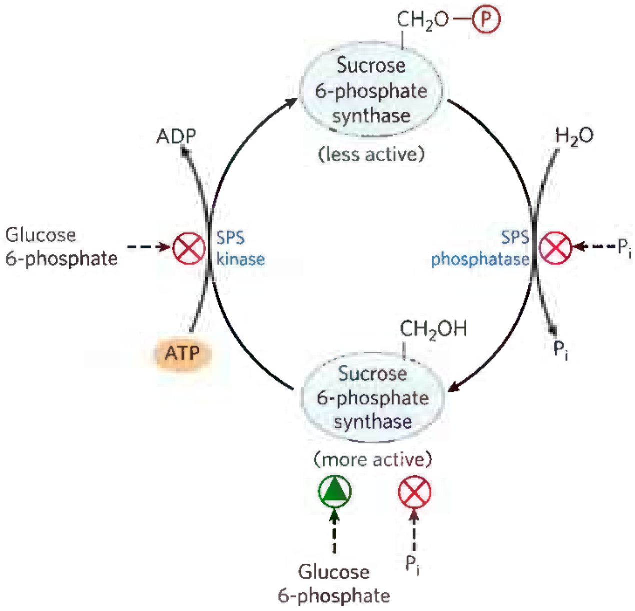


FIGURE 20-43 Regulation of sucrose phosphate synthase by phosphorylation. A protein kinase (SPS kinase) specific for sucrose phosphate synthase (SPS) phosphorylates a Ser residue in SPS, inactivating it; a specific phosphatase (SPS phosphatase) reverses this inhibition. The kinase is inhibited allosterically by glucose 6-phosphate, which also activates SPS allosterically. The phosphatase is inhibited by P_i, which also inhibits SPS directly. Thus, when the concentration of glucose 6-phosphate is high as a result of active photosynthesis, SPS is activated and produces sucrose phosphate. A high P_i concentration, which occurs when photosynthetic conversion of ADP to ATP is slow, inhibits sucrose phosphate synthesis.

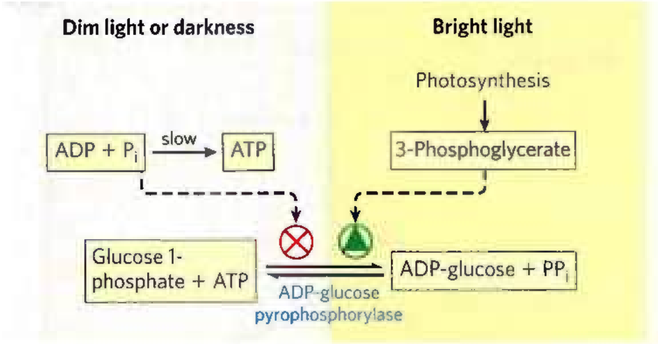


FIGURE 20-44 Regulation of ADP-glucose pyrophosphorylase by 3-phosphoglycerate and P_i. This enzyme, which produces the precursor for starch synthesis, is rate-limiting in starch production. The enzyme is stimulated allosterically by 3-phosphoglycerate (3-PGA) and inhibited by P_i; in effect, the ratio [3-PGA]/[P_i], which rises with increasing rates of photosynthesis, controls starch synthesis at this step.

by glucose 6-phosphate, and of the phosphatase by P_i, enhances the effects of these two compounds on sucrose synthesis. When hexose phosphates are abundant, sucrose 6-phosphate synthase is activated by glucose 6-phosphate; when P_i is elevated (as when photosynthesis is slow), sucrose synthesis is slowed. During active photosynthesis, triose phosphates are converted to fructose 6-phosphate, which is rapidly equilibrated with glucose 6-phosphate by phosphohexose isomerase. Because the equilibrium lies far toward glucose 6-phosphate, as soon as fructose 6-phosphate accumulates, the level of glucose 6-phosphate rises and sucrose synthesis is stimulated.

The key regulatory enzyme in starch synthesis is **ADP-glucose pyrophosphorylase** (**Fig. 20-44**); it is activated by 3-phosphoglycerate, which accumulates during active photosynthesis, and inhibited by P_i, which accumulates when light-driven condensation of ADP and P_i slows. When sucrose synthesis slows, 3-phosphoglycerate formed by CO_2 fixation accumulates, activating this enzyme and stimulating the synthesis of starch.

The Glyoxylate Cycle and Gluconeogenesis Produce Glucose in Germinating Seeds

Many plants store lipids (oils) and proteins in their seeds, to be used as sources of energy and as biosynthetic precursors during germination, before photosynthetic capacity has developed. These stored components are converted to carbohydrates by the combined action of several pathways. Glucogenic amino acids (see Table 14-4) derived from the breakdown of stored seed proteins are transaminated and oxidized to succinyl-CoA, pyruvate, oxaloacetate, fumarate, and α-ketoglutarate (Chapter 18)—all good starting materials for gluconeogenesis. Active gluconeogenesis in germinating seeds provides glucose for the synthesis of sucrose, polysaccharides, and many metabolites derived from hexoses. In plant seedlings, sucrose provides much of the chemical energy needed for initial growth.

Triacylglycerols stored in seeds also provide fuel for the germinating plants. They are hydrolyzed to free fatty acids, which undergo β oxidation to acetyl-CoA in specialized peroxisomes called **glyoxysomes** that develop during seed germination (see Fig. 17-14). The acetyl-CoA formed from seed oils enters the **glyoxylate cycle** (**Fig. 20-45**), which brings about the net conversion of acetate to succinate or other four-carbon intermediate of the citric acid cycle:

$$2\ \text{Acetyl-CoA} + \text{NAD}^+ + 2\text{H}_2\text{O} \longrightarrow \text{succinate} + 2\text{CoA} + \text{NADH} + \text{H}^+$$

In the glyoxylate cycle, acetyl-CoA condenses with oxaloacetate to form citrate, and citrate is converted to isocitrate, exactly as in the citric acid cycle. The next step, however, is not the breakdown of isocitrate by isocitrate dehydrogenase but the cleavage of isocitrate by **isocitrate lyase**, forming succinate and **glyoxylate**. The glyoxylate then condenses with a second molecule of acetyl-CoA to yield malate, in a reaction catalyzed by **malate synthase**. The malate is subsequently oxidized to oxaloacetate, which can condense with another molecule of acetyl-CoA to start another turn of the cycle. The succinate passes into the mitochondrial matrix, where it is converted by citric acid cycle enzymes to oxaloacetate. The oxaloacetate moves into the cytosol

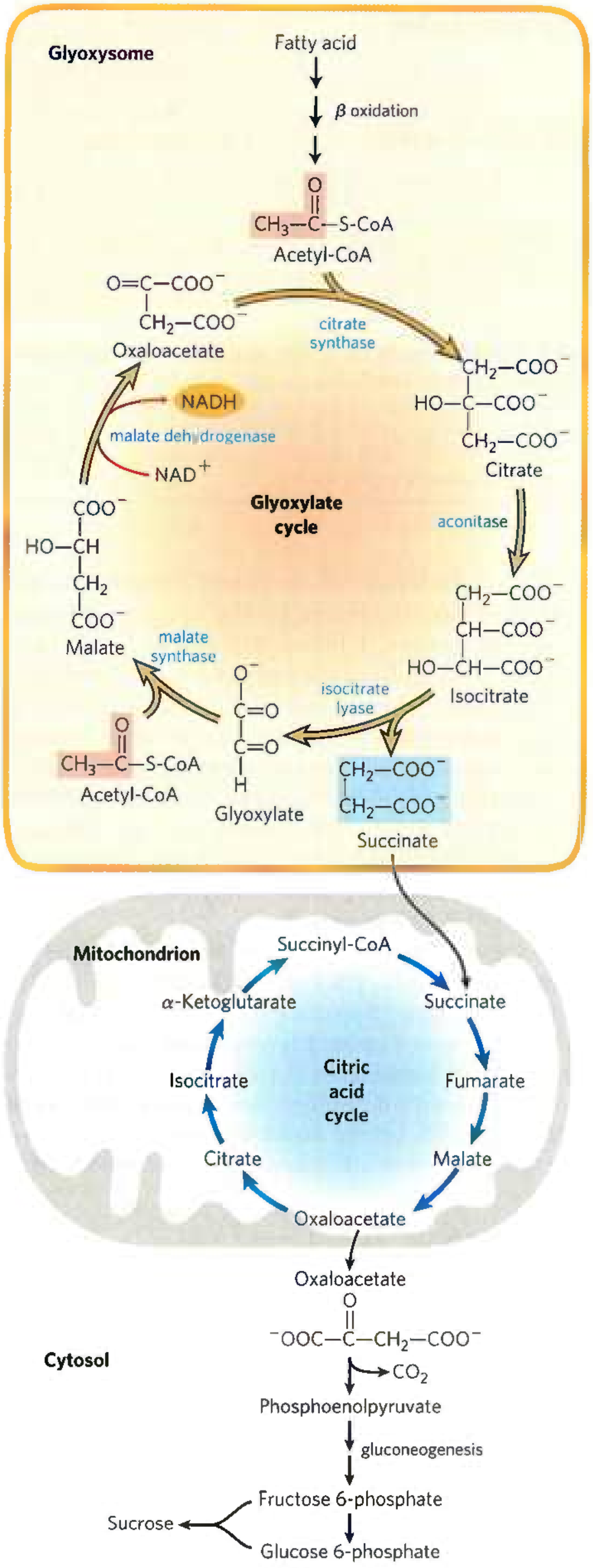

Glyoxysome
Fatty acid
β oxidation
$CH_3-\overset{O}{\overset{\|}{C}}-S\text{-}CoA$
Acetyl-CoA
$O{=}C{-}COO^-$
$CH_2{-}COO^-$
Oxaloacetate
citrate synthase
$CH_2{-}COO^-$
$HO{-}C{-}COO^-$
$CH_2{-}COO^-$
Citrate
NADH
malate dehydrogenase
NAD^+
Glyoxylate cycle
aconitase
COO^-
$HO{-}CH$
CH_2
COO^-
Malate
malate synthase
$CH_2{-}COO^-$
$CH{-}COO^-$
$HO{-}CH{-}COO^-$
Isocitrate
isocitrate lyase
O^-
$C{=}O$
$C{=}O$
H
Glyoxylate
$CH_3-\overset{O}{\overset{\|}{C}}-S\text{-}CoA$
Acetyl-CoA
$CH_2{-}COO^-$
$CH_2{-}COO^-$
Succinate
Mitochondrion
Succinyl-CoA
α-Ketoglutarate
Succinate
Isocitrate
Citric acid cycle
Fumarate
Citrate
Malate
Oxaloacetate
Oxaloacetate
$^-OOC-\overset{O}{\overset{\|}{C}}-CH_2-COO^-$
Cytosol
CO_2
Phosphoenolpyruvate
gluconeogenesis
Fructose 6-phosphate
Sucrose
Glucose 6-phosphate

FIGURE 20-45 Conversion of stored fatty acids to sucrose in germinating seeds through the glyoxylate cycle. This pathway begins in specialized peroxisomes called glyoxysomes. The citrate synthase, aconitase, and malate dehydrogenase of the glyoxylate cycle are isozymes of the citric acid cycle enzymes; isocitrate lyase and malate synthase are unique to the glyoxylate cycle. Notice that two acetyl groups enter the cycle and four carbons leave as succinate. Succinate is exported to mitochondria, where it is converted to oxaloacetate by enzymes of the citric acid cycle. Oxaloacetate enters the cytosol and serves as the starting material for gluconeogenesis and for synthesis of sucrose, the transport form of carbon in plants.

and can be converted to phosphoenolpyruvate by PEP carboxykinase, then to fructose 6-phosphate, the precursor of sucrose, by gluconeogenesis. Thus, reaction sequences carried out in three subcellular compartments (glyoxysomes, mitochondria, and cytosol) are integrated for the production of fructose 6-phosphate or sucrose from stored lipids.

Enzymes common to the citric acid and glyoxylate cycles have two isozymes, one specific to mitochondria, the other to glyoxysomes. Physical separation of the glyoxylate cycle and β-oxidation enzymes from the mitochondrial citric acid cycle enzymes prevents further oxidation of acetyl-CoA to CO_2. Each turn of the glyoxylate cycle consumes two molecules of acetyl-CoA and produces one molecule of succinate, which is then available for biosynthetic purposes. Hydrolysis of stored triacylglycerols also produces glycerol 3-phosphate, which can enter the gluconeogenic pathway, after its oxidation to dihydroxyacetone phosphate (see Fig. 14-16).

We noted in Chapter 14 that animal cells can carry out gluconeogenesis from three- and four-carbon precursors, but not from the two acetyl carbons of acetyl-CoA. Because the pyruvate dehydrogenase reaction is effectively irreversible (see Section 16.1) and animals do not have the enzymes specific to the glyoxylate cycle (isocitrate lyase and malate synthase), they have no way to convert acetyl-CoA to pyruvate or oxaloacetate. So, unlike vascular plants, animals cannot bring about the net synthesis of glucose from fatty acids.

Cellulose Is Synthesized by Supramolecular Structures in the Plasma Membrane

Cellulose is a major constituent of plant cell walls, providing strength and rigidity and preventing the swelling of the cell and rupture of the plasma membrane that might result when osmotic conditions favor water entry into the cell. Each year, worldwide, plants synthesize more than 10^{11} metric tons of cellulose, making this simple polymer one of the most abundant compounds in the biosphere. The structure of cellulose in the plant cell wall is simple: linear polymers of thousands of (β1→4)-linked D-glucose units, assembled into bundles of at least 18 chains, which co-crystalize to form microfibrils, which may in turn be assembled into larger macrofibrils. (**Fig. 20-46**).

P5 As a major component of the plant cell wall, cellulose must be synthesized from intracellular precursors but deposited and assembled *outside* the plasma membrane. The enzymatic machinery for initiation,

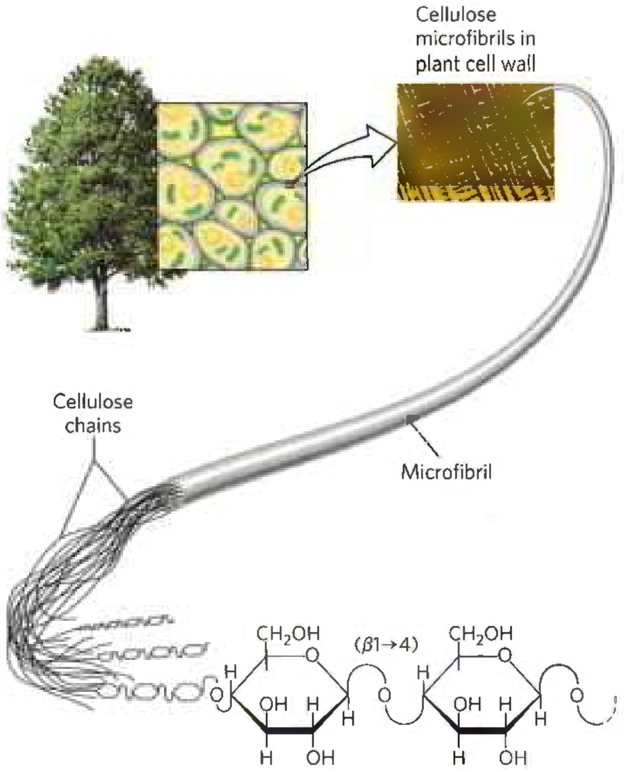

Cellulose microfibrils in plant cell wall
Cellulose chains
Microfibril
CH₂OH
(β1→4)
CH₂OH
H
O
OH H
H
OH
O
H
H
O
OH H
H
H OH
O

FIGURE 20-46 Cellulose structure. The plant cell wall is made up in part of cellulose molecules arranged side by side to form crystalline arrays—cellulose microfibrils. Several microfibrils may combine to form larger cellulose macrofibrils. The scanning electron microscope shows macrofibrils, 5 to 12 nm in diameter, laid down on the cell surface in several layers distinguishable by the different orientations of the fibrils. [Electron micrograph from Biophoto Associates/Science Source.]

elongation, and export of cellulose chains is therefore more complicated than that used to synthesize starch or glycogen (which are not exported).

The complex enzymatic machinery that assembles cellulose chains spans the plasma membrane, with one part on the cytoplasmic side positioned to bind the substrate, UDP-glucose, and elongate the chains, and another part extending to the outside, responsible for exporting the cellulose molecules to the extracellular space. Freeze-fracture electron microscopy shows a cellulose synthesis complex, or rosette, composed of six large particles arranged in a regular hexagon with a diameter of about 30 nm (**Fig. 20-47a**). Several proteins, including the catalytic subunit of **cellulose synthase**, make up this structure. The structure of the plant cellulose synthase is

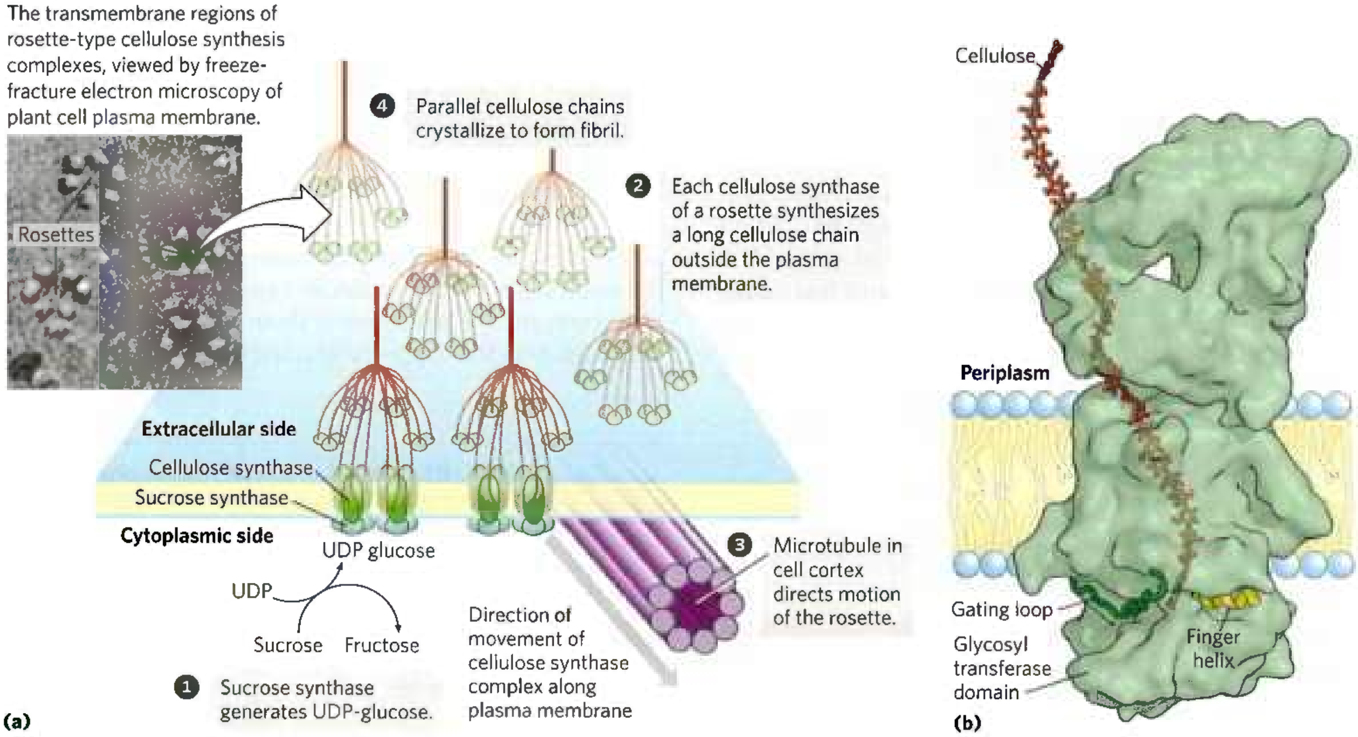

The transmembrane regions of rosette-type cellulose synthesis complexes, viewed by freeze-fracture electron microscopy of plant cell plasma membrane.
Rosettes
④ Parallel cellulose chains crystallize to form fibril.
② Each cellulose synthase of a rosette synthesizes a long cellulose chain outside the plasma membrane.
Extracellular side
Cellulose synthase
Sucrose synthase
Cytoplasmic side
UDP glucose
UDP
Sucrose
Fructose
① Sucrose synthase generates UDP-glucose.
Direction of movement of cellulose synthase complex along plasma membrane
③ Microtubule in cell cortex directs motion of the rosette.
(a)
Cellulose
Periplasm
Gating loop
Glycosyl transferase domain
Finger helix
(b)

FIGURE 20-47 A model for the synthesis of cellulose. (a) Schematic derived from a combination of genetic, electron microscopic, and biochemical studies of *Arabidopsis thaliana* and other vascular plants. (b) The structure of cellulose synthase from the bacterium *Rhodobacter sphaeroides*. The transmembrane part of the protein provides a channel through which the lengthening cellulose polymer (red) is pushed into the periplasm as the chain grows by addition of glucose units on the inside surface of the plasma membrane. Two structures of the enzyme move during the catalytic cycle. The gating loop moves into the substrate-binding site when UDP-glucose binds, then moves out to allow UDP to leave. The finger helix touches the glucose residue at the growing polymer end, then, after a new residue is added, moves so as to touch this new terminal glucose. The glycosyl transferase domain extends into the cytoplasm, where it binds its substrate UDP-glucose. [(a) Electron micrograph ©courtesy Dr. Candace H. Haigler, North Carolina State University, and Dr. Mark Grimson, Texas Tech University. (b) Data from PDB ID 5EJZ, J. L. W. Morgan et al., *Nature* 531:329, 2016. An extension of the cellulose chain was modeled in.]

similar to that of the bacterium *Rhodobacter sphaeroides*, which has been determined by x-ray crystallography (Fig. 20-47b).

In one working model of cellulose synthesis, cellulose chains are initiated by the transfer of a glucose residue from UDP-glucose to a "primer" glucose already bound to cellulose synthase on the cytoplasmic side of the plasma membrane, to form a disaccharide. As addition of further glucose residues lengthens the chain, it is extruded through a channel formed by the transmembrane helices of cellulose synthase and, on the outer surface of the plasma membrane, joins growing chains from neighboring cellulose synthase molecules to form a cellulose microfibril. Polymers of more than 6 to 8 glucose units are insoluble in water, promoting microfibril crystallization. There is no definite length for a cellulose polymer; synthesis is highly processive, and some polymers are as long as 15,000 glucose units.

The UDP-glucose used for cellulose synthesis (step ❶ in Fig. 20-47) is generated from sucrose produced during photosynthesis, in a reaction catalyzed by sucrose synthase (named for the reverse reaction):

$$\text{Sucrose} + \text{UDP} \longrightarrow \text{UDP-glucose} + \text{fructose}$$

A membrane-bound form of sucrose synthase may produce a high local concentration of UDP-glucose for cellulose synthesis.

Each of the six particles of the rosette most likely contains three cellulose synthase molecules, each synthesizing a single cellulose chain (step ❷). The large enzyme complex that catalyzes this process moves along the plasma membrane with directionality often related to the course of microtubules in the cell cortex, the cytoplasmic layer just below the membrane (step ❸). When these microtubules lie perpendicular to the axis of the plant's growth, the cellulose microfibrils are laid down similarly to promote elongation. The motion of the cellulose synthase complexes is believed to be driven by energy released in the polymerization reaction, not by a molecular motor such as kinesin.

The fundamental cellulose microfibril made by one rosette-type cellulose synthesis complex is thought to be composed of 18 chains lying side by side with the same (parallel) orientation of nonreducing and reducing ends. The 18 separate polymers coalesce on the outer surface of the cell and crystallize soon after they are polymerized (step ❹), just prior to integrating into the cell wall.

In UDP-glucose, the glucose is α-linked to the nucleotide, but in cellulose, the glucose residues are ($\beta1\rightarrow4$)-linked, so there is an inversion of configuration at the anomeric carbon (C-1) as the glycosidic bond forms. Glycosyltransferases that invert configuration are generally assumed to use a single-displacement mechanism, with nucleophilic attack by the acceptor species at the anomeric carbon of the donor sugar (in this case, UDP-glucose).

Pools of Common Intermediates Link Pathways in Different Organelles

Although we have described metabolic transformations in plant cells in terms of individual pathways, these pathways interconnect so completely that we should instead consider pools of metabolic intermediates shared among these pathways and connected by readily reversible reactions (**Fig. 20-48**). One such **metabolite pool** includes the hexose phosphates glucose 1-phosphate, glucose 6-phosphate, and fructose 6-phosphate; a second includes the 5-phosphates of the pentoses ribose, ribulose, and xylulose; a third includes the triose phosphates dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Metabolite fluxes through these pools change in magnitude and direction in response to changes in the circumstances of the plant, and they vary with tissue type. Transporters in the membranes of each organelle move specific compounds in and out, and the regulation of these transporters presumably influences the degree to which the pools mix.

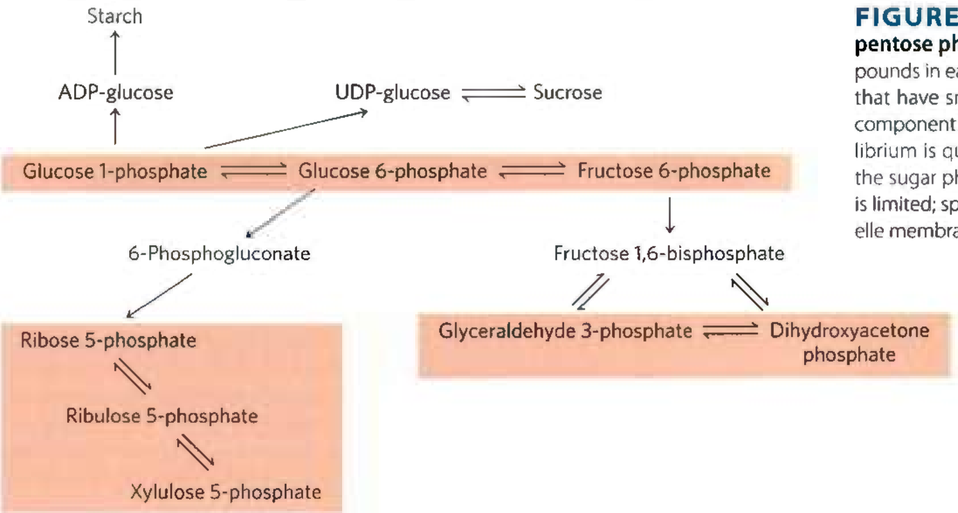


FIGURE 20-48 Pools of hexose phosphates, pentose phosphates, and triose phosphates. The compounds in each pool are readily interconvertible by reactions that have small standard free-energy changes. When one component of the pool is temporarily depleted, a new equilibrium is quickly established to replenish it. Movement of the sugar phosphates between intracellular compartments is limited; specific transporters must be present in an organelle membrane.

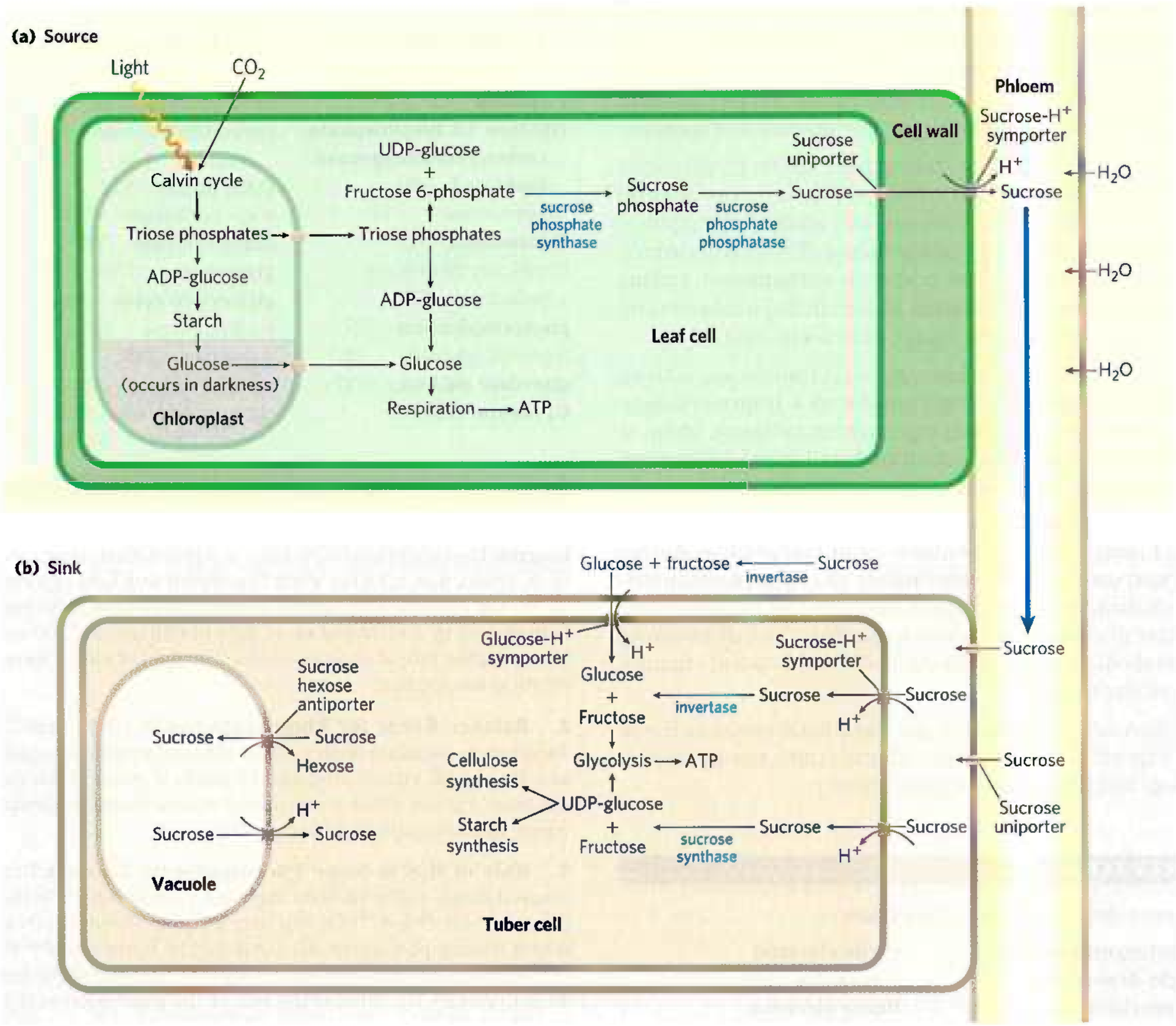

(a) Source
Light
CO_2
Calvin cycle
Triose phosphates
ADP-glucose
Starch
Glucose
(occurs in darkness)
Chloroplast
UDP-glucose
+
Fructose 6-phosphate
Triose phosphates
ADP-glucose
Glucose
Respiration
ATP
sucrose phosphate synthase
Sucrose phosphate
sucrose phosphate phosphatase
Sucrose
Sucrose uniporter
Leaf cell
Cell wall
Phloem
Sucrose-H^+ symporter
H^+
Sucrose
H_2O
H_2O
H_2O
(b) Sink
Glucose + fructose
invertase
Sucrose
Glucose-H^+ symporter
H^+
Glucose
+
Fructose
Sucrose-H^+ symporter
invertase
Sucrose
Sucrose
H^+
Sucrose
Sucrose hexose antiporter
Sucrose
Sucrose
Hexose
H^+
Sucrose
Sucrose
Vacuole
Cellulose synthesis
Starch synthesis
Glycolysis
ATP
UDP-glucose
+
Fructose
sucrose synthase
Sucrose
Sucrose
H^+
Sucrose
Sucrose uniporter
Tuber cell

FIGURE 20-49 Movement of sucrose between source and sink tissues. (a) In daylight, photosynthetic leaves (source tissue) fix CO_2 into triose phosphates via the Calvin cycle in chloroplasts. Some of the triose phosphate is used in the chloroplasts to synthesize starch; the rest is exported to the cytosol, where it can be converted via gluconeogenesis to fructose 6-phosphate and glucose 1-phosphate. Sucrose, synthesized from UDP-glucose and fructose, is exported from leaf mesophyll cells to the plant phloem; the resulting high sucrose content draws water into the phloem by osmosis. The resulting increased turgor pressure (p. 52) pushes the solution in the phloem toward sink tissues. (b) Sucrose moves from the phloem into the sink tissues, where it is converted to starch or cell wall cellulose, or is used as fuel for glycolysis, the citric acid cycle, and oxidative phosphorylation to provide ATP for these nonphotosynthetic tissues. Sugar transport across the plasma membrane and between intracellular compartments is catalyzed by several symporters and antiporters coupled to a proton gradient. [Information from Dr. Gerald Edwards, School of Biological Sciences, Washington State University.]

During daylight hours, triose phosphates produced in photosynthetic leaf tissue ("source" tissues, in which there is a net fixation of CO_2) move out of the chloroplast and into the cytosolic hexose phosphate pool, where they are converted to sucrose for transport via the plant phloem (sap) to nonphotosynthetic "sink" tissues (Fig. 20-49). In sink tissues such as roots, tubers, and bulbs, sucrose is converted to starch for storage or is used as an energy source via glycolysis. In growing plants, hexose phosphates are also withdrawn from the pool for the synthesis of cell walls. At night, starch is metabolized by glycolysis and oxidative phosphorylation to provide energy for both source and sink tissues.

SUMMARY 20.6 *Biosynthesis of Starch, Sucrose, and Cellulose*

- Starch synthase in chloroplasts and amyloplasts catalyzes the addition of single glucose residues, donated by ADP-glucose, to the growing polymer chain.
- Sucrose is synthesized in the cytosol from UDP-glucose and fructose 1-phosphate, in two steps.

- The partitioning of triose phosphates between sucrose synthesis and starch synthesis is regulated by fructose 2,6-bisphosphate (F26BP). [F26BP] varies inversely with the rate of photosynthesis, and F26BP inhibits the synthesis of fructose 6-phosphate, the precursor of sucrose.
- The glyoxylate cycle, taking place in the glyoxysomes of germinating seeds of some plants, uses several citric acid cycle enzymes and two additional enzymes: isocitrate lyase and malate synthase. The two decarboxylation steps of the citric acid cycle are bypassed, making possible the *net* formation of succinate, oxaloacetate, and other cycle intermediates from acetyl-CoA.
- Cellulose synthase has a glycosyl transferase activity in its cytoplasmic domain and forms a transmembrane channel through which the growing cellulose chain is extruded. Glucose units are transferred from UDP-glucose to the nonreducing end of the growing chain.
- The plant cell shares pools of common intermediates, including hexose-, pentose-, and triose-phosphates. Transporters in the membranes of chloroplasts, mitochondria, and amyloplasts mediate the movement of sugar phosphates between organelles. The direction of metabolite flow through the pools within a leaf changes from day to night.
- Sucrose produced in a photosynthetic (source) tissue is exported to nonphotosynthetic (sink) tissue such as roots and tubers via the plant phloem.

KEY TERMS

Terms in bold are defined in the glossary.

photosynthesis	700
light-dependent reactions	700
photophosphorylation	701
chloroplast	701
stroma	701
thylakoid	701
photon	703
excited state	703
ground state	704
exciton	704
exciton transfer	704
chlorophylls	704
accessory pigments	705
carotenoids	705
β-carotene	705
action spectrum	705
photosystem	705
photochemical reaction center	705
light-harvesting complexes (LHCs)	705
cyclic electron transfer	708
linear electron transfer	708
ferredoxin	708
Z scheme	709
photosystem II (PSII)	709
photosystem I (PSI)	709
cytochrome b_6f	709
plastoquinone (PQ_A)	710
plastocyanin	711
phylloquinone (PQ_K)	711
cyclic photophosphorylation	713
state transition	713
oxygen-evolving center	715
CO_2 assimilation	719
CO_2 fixation	719
Calvin cycle	719
reductive pentose phosphate pathway	719
ribulose 1,5-bisphosphate	719
3-phosphoglycerate	719
C_3 plants	720
ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco)	720
rubisco activase	721
thioredoxin	725
ferredoxin:thioredoxin reductase	725
photorespiration	727
2-phosphoglycolate	727
glycolate pathway	727
C_4 plants	729
C_4 pathway	729
phosphoenolpyruvate carboxylase	730
malic enzyme	730
pyruvate phosphate dikinase	730
CAM plants	732
sugar nucleotide	733
starch synthase	733
glyoxysome	735
glyoxylate cycle	735
isocitrate lyase	735
glyoxylate	735
malate synthase	735
cellulose synthase	737

PROBLEMS

1. **Photochemical Efficiency of Light at Different Wavelengths** The rate of photosynthesis in a green plant, measured by O_2 production, is higher when illuminated with light of wavelength 680 nm than with light of wavelength 700 nm. However, illumination by a combination of light of 680 nm and 700 nm gives a higher rate of photosynthesis than light of either wavelength alone. Explain.

2. **Balance Sheet for Photosynthesis** In 1804, Nicolas-Théodore de Saussure observed that the total weight of oxygen and dry organic matter produced by plants is greater than the weight of carbon dioxide consumed during photosynthesis. Where does the extra weight come from?

3. **Role of H_2S in Some Photosynthetic Bacteria** Illuminated purple sulfur bacteria carry out photosynthesis in the presence of H_2O and $^{14}CO_2$, but only if H_2S is added and O_2 is absent. During photosynthesis, measured by formation of $[^{14}C]$ carbohydrate, the bacteria convert H_2S to elemental sulfur but do not produce O_2. What is the role of the conversion of H_2S to sulfur? Why doesn't photosynthesis produce O_2 in these bacteria?

4. **Electron Transfer through Photosystems I and II** Predict how an inhibitor of electron passage through pheophytin would affect electron transfer through **(a)** photosystem II and **(b)** photosystem I. Explain your reasoning.

5. **Limited ATP Synthesis in the Dark** In a laboratory experiment, a researcher illuminates spinach chloroplasts in the absence of ADP and P_i. Then, the researcher turns the light off and adds ADP and P_i. ATP synthesis occurs for a short time in the dark. Explain this finding.

6. **Mode of Action of the Herbicide DCMU** Treating chloroplasts with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, or diuron), a potent herbicide, causes oxygen evolution and photophosphorylation to cease. Adding a Hill reagent (an external electron acceptor) restores oxygen evolution, but not photophosphorylation. How does DCMU act as a weed killer? Suggest a location for the inhibitory action of this herbicide in the scheme shown in Figure 20-12. Explain.

7. Effect of Venturicidin on Oxygen Evolution Venturicidin is a powerful inhibitor of the chloroplast ATP synthase, interacting with CF_o and blocking proton passage through the CF_oCF_1 complex. How would venturicidin affect oxygen evolution in a suspension of well-illuminated chloroplasts? Would your answer change if the experiment were done in the presence of an uncoupling reagent such as 2,4-dinitrophenol (DNP)? Explain.

8. Light Energy for a Redox Reaction Suppose you have isolated a new photosynthetic microorganism that oxidizes H_2S and passes the electrons to NAD^+. What wavelength of light would provide enough energy for H_2S to reduce NAD^+ under standard conditions? Assume 100% efficiency in the photochemical event, and use an E'° of −243 mV for H_2S and −320 mV for NAD^+. See Figure 20-4 for the energy equivalents of wavelengths of light.

9. Equilibrium Constant for Water-Splitting Reactions The coenzyme $NADP^+$ is the terminal electron acceptor in chloroplasts, according to the reaction

$$2H_2O + 2NADP^+ \longrightarrow 2NADPH + 2H^+ + O_2$$

Use information in Chapter 19 (Table 19-2) to calculate the equilibrium constant for this reaction at 25 °C. (The relationship between K'_{eq} and $\Delta G'^\circ$ is discussed on p. 468.) How can the chloroplast overcome this unfavorable equilibrium?

10. Energetics of Phototransduction During photosynthesis, pigment molecules in chloroplasts must absorb eight photons (four by each photosystem) for every O_2 molecule they produce, according to the equation

$$2H_2O + 2NADP^+ + 8\text{ photons} \longrightarrow 2NADPH + 2H^+ + O_2$$

The $\Delta G'^\circ$ for the light-independent production of O_2 is 400 kJ/mol. Assuming that these photons have a wavelength of 700 nm (red) and that the light absorption and use of light energy are 100% efficient, calculate the free-energy change for the process.

11. Electron Transfer to a Hill Reagent Isolated spinach chloroplasts evolve O_2 when illuminated in the presence of potassium ferricyanide (a Hill reagent), according to the equation

$$2H_2O + 4Fe^{3+} \longrightarrow O_2 + 4H^+ + 4Fe^{2+}$$

where Fe^{3+} represents ferricyanide and Fe^{2+} represents ferrocyanide. Does this process produce NADPH? Explain.

12. How Often Does a Chlorophyll Molecule Absorb a Photon? The amount of chlorophyll *a* (M_r 892) in a spinach leaf is about 20 $\mu g/cm^2$ of leaf surface. In noonday sunlight (average energy reaching the leaf is 5.4 $J/cm^2 \cdot min$), the leaf absorbs about 50% of the radiation. How often does a single chlorophyll molecule absorb a photon? Given that the average lifetime of an excited chlorophyll molecule in vivo is 1 ns, what fraction of the chlorophyll molecules are excited at any one time?

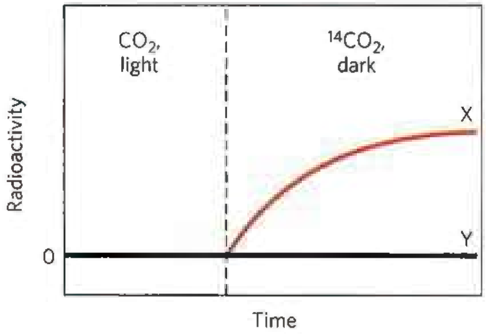
13. Effect of Monochromatic Light on Electron Flow Using a spectrophotometer, researchers can sometimes directly observe the extent of oxidation or reduction of an electron carrier during photosynthetic electron transfer. Illuminating chloroplasts with 700 nm light oxidizes cytochrome *f*, plastocyanin, and plastoquinone. Illuminating chloroplasts with 680 nm light, however, reduces these electron carriers. Explain.

14. Function of Cyclic Photophosphorylation When the $[NADPH]/[NADP^+]$ ratio in chloroplasts is high, photophosphorylation is predominantly cyclic (see Fig. 20-12). Does cyclic electron transfer evolve O_2? Does cyclic electron transfer produce NADPH? Explain. What is the main function of cyclic electron transfer?

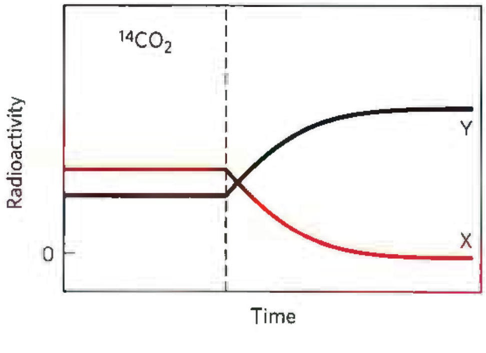
15. Phases of Photosynthesis A researcher illuminates a suspension of green algae in the absence of CO_2. He then incubates the algae with $^{14}CO_2$ in the dark and observes the conversion of $^{14}CO_2$ to $[^{14}C]$glucose for a brief time. What is the significance of this observation with regard to the CO_2-assimilation process, and how is it related to the light-dependent reactions of photosynthesis? Why does the conversion of $^{14}CO_2$ to $[^{14}C]$glucose stop after a brief time?

16. Identification of Key Intermediates in CO_2 Assimilation Calvin and his colleagues used the unicellular green alga *Chlorella* to study the CO_2-assimilation reactions of photosynthesis. They incubated $^{14}CO_2$ with illuminated suspensions of algae and followed the time course of appearance of ^{14}C in two compounds, X and Y, under two sets of conditions. Suggest the identities of X and Y, based on your understanding of the Calvin cycle.

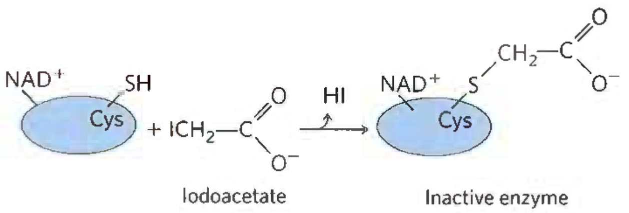
(a) They grew illuminated *Chlorella* with unlabeled CO_2, then turned off the light and added $^{14}CO_2$ (vertical dashed line in the graph below). Under these conditions, X was the first compound to become labeled with ^{14}C; Y was unlabeled.



(b) They grew illuminated *Chlorella* cells with $^{14}CO_2$. Illumination was continued until all the $^{14}CO_2$ had been taken up (vertical dashed line in the graph below). Under these conditions, X became labeled quickly but lost its radioactivity with time, whereas Y became more radioactive with time.



17. Regulation of the Calvin Cycle Iodoacetate reacts irreversibly with the free —SH groups of Cys residues in proteins. Predict which Calvin cycle enzyme(s) would be inhibited by iodoacetate, and explain why.



18. Comparison of the Reductive and Oxidative Pentose Phosphate Pathways The *reductive* pentose phosphate pathway generates several intermediates identical to those of the *oxidative* pentose phosphate pathway (Chapter 14). What role does each pathway play in cells where it is active?

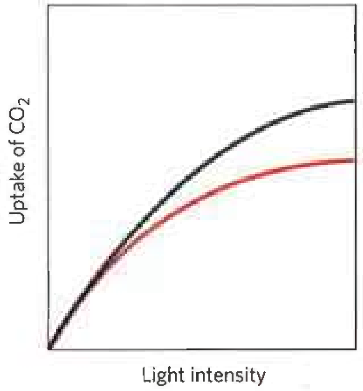
19. Photorespiration and Mitochondrial Respiration Compare the oxidative photosynthetic carbon cycle, also called *photorespiration*, with the *mitochondrial respiration* that drives ATP synthesis. Why are both processes referred to as respiration? Where in the cell do they occur, and under what circumstances? What is the path of electron flow in each?

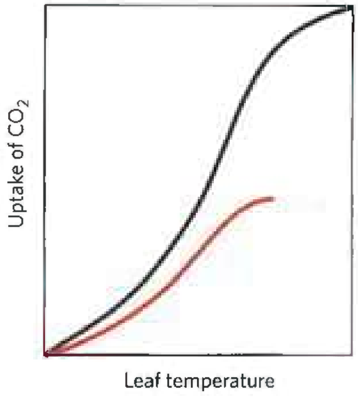
20. Pathway of CO_2 Assimilation in Maize Researchers illuminate a maize (corn) plant in the presence of $^{14}CO_2$. After about 1 second of illumination, they find more than 90% of all the radioactivity incorporated in the leaves at C-4 of malate, aspartate, and oxaloacetate. Only after 60 seconds does ^{14}C appear at C-1 of 3-phosphoglycerate. Explain.

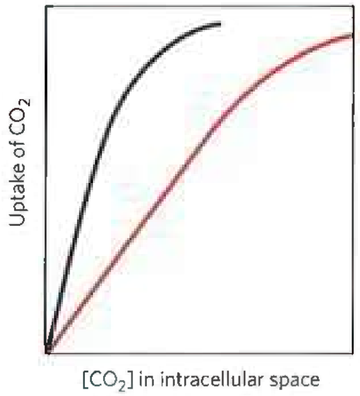
21. Identifying CAM Plants Given some $^{14}CO_2$ and all the tools typically present in a biochemistry research lab, how would you design a simple experiment to determine whether a plant is a typical C_4 plant or a CAM plant?

22. Chemistry of Malic Enzyme: Variation on a Theme Malic enzyme, found in the bundle-sheath cells of C_4 plants, carries out a reaction that has a counterpart in the citric acid cycle. What is the analogous reaction? Explain your choice.

23. Differences between C_3 and C_4 Plants The plant genus *Atriplex* includes some C_3 and some C_4 species. In the plots, the black curve represents species 1; the red curve represents species 2. From the data in the plots, identify which is a C_3 plant and which is a C_4 plant. Justify your answer in molecular terms that account for the data in all three plots.







24. Inorganic Pyrophosphatase The enzyme inorganic pyrophosphatase contributes to making many biosynthetic reactions that generate inorganic pyrophosphate essentially irreversible in cells. By keeping the concentration of PP_i very low, the enzyme "pulls" these reactions in the direction of PP_i formation. The synthesis of ADP-glucose in chloroplasts is one such reaction. However, the synthesis of UDP-glucose in the plant cytosol, which also produces PP_i, is readily reversible in vivo. How do you reconcile these two facts?

25. Regulation of Starch and Sucrose Synthesis Sucrose synthesis occurs in the cytosol and starch synthesis occurs in the chloroplast stroma, yet the two processes are intricately balanced. What factors shift the reactions in favor of **(a)** starch synthesis and **(b)** sucrose synthesis?

26. Regulation of Sucrose Synthesis In the regulation of sucrose synthesis from the triose phosphates produced during photosynthesis, 3-phosphoglycerate and P_i play critical roles (see Fig. 20-42). Explain why the concentrations of these two regulators reflect the rate of photosynthesis.

27. Sucrose and Dental Caries The most prevalent infection in humans worldwide is dental caries, which stems from the colonization and destruction of tooth enamel by a variety of acidifying microorganisms. These organisms synthesize and live within a water-insoluble network of dextrans, called dental plaque, composed of (α1→6)-linked polymers of glucose with many (α1→3) branch points. Polymerization of dextran requires dietary sucrose, and the bacterial enzyme dextran-sucrose glucosyltransferase catalyzes the reaction.

(a) Write the overall reaction for dextran polymerization.
(b) In addition to providing a substrate for the formation of dental plaque, how does dietary sucrose also provide oral bacteria with an abundant source of metabolic energy?

28. Partitioning between the Citric Acid and Glyoxylate Cycles In an organism (such as *Escherichia coli*) that has both the citric acid cycle and the glyoxylate cycle, what determines which of these pathways isocitrate will enter?

DATA ANALYSIS PROBLEM

29. Photophosphorylation: Discovery, Rejection, and Rediscovery In the 1930s and 1940s, researchers were beginning to make progress toward understanding the mechanism of photosynthesis. At the time, the role of "energy-rich phosphate bonds" (today, "ATP") in glycolysis and cellular respiration was just becoming known. There were many theories about the mechanism of photosynthesis, especially about the role of light. This problem focuses on what was then called the "primary photochemical process"—that is, on what, exactly, the energy from captured light produces in the photosynthetic cell. Interestingly, one important part of the modern model of photosynthesis was proposed early on, only to be rejected, ignored for several years, then finally revived and accepted.

In 1944, Emerson, Stauffer, and Umbreit proposed that "the function of light energy in photosynthesis is the formation of 'energy-rich' phosphate bonds" (p. 107). In their model (hereafter, the "Emerson model"), the free energy necessary to drive both CO_2 fixation *and* reduction came from these "energy-rich phosphate bonds" (i.e., ATP), produced as a result of light absorption by a chlorophyll-containing protein.

This model was explicitly rejected by Rabinowitch (1945). After summarizing Emerson and coauthors' findings, Rabinowitch stated: "Until more positive evidence is provided, we are inclined to consider as more convincing a general argument against this hypothesis, which can be derived from energy considerations. Photosynthesis is eminently a problem of energy *accumulation*. What good can be served, then, by converting light quanta (even those of red light, which amount to about 43 kcal per Einstein) into 'phosphate quanta' of only 10 kcal per mole? This appears to be a start in the wrong direction—toward *dissipation* rather than toward accumulation of energy" (p. 228). This argument, along with other evidence, led to abandonment of the Emerson model until the 1950s, when it was found to be correct—albeit in a modified form.

For each piece of information from Emerson and coauthors' article presented in (a) through (d), answer the following three questions:

1. How does this information support the Emerson model, in which light energy is used directly by chlorophyll *to make ATP*, and the ATP then provides the energy to drive CO_2 fixation and reduction?
2. How would Rabinowitch explain this information, based on his model (and most other models of the day), in which light energy is used directly by chlorophyll *to make reducing compounds*? Rabinowitch wrote: "Theoretically, there is no reason why *all* electronic energy contained in molecules excited by the absorption of light should not be available for oxidation-reduction" (p. 152). In this model, the reducing compounds are then used to fix and reduce CO_2, and the energy for these reactions comes from the large amounts of free energy released by the reduction reactions.
3. How is this information explained by our modern understanding of photosynthesis?

(a) Chlorophyll contains a Mg^{2+} ion, which is known to be an essential cofactor for many enzymes that catalyze phosphorylation and dephosphorylation reactions.
(b) A crude "chlorophyll protein" isolated from photosynthetic cells showed phosphorylating activity.
(c) The phosphorylating activity of the "chlorophyll protein" was inhibited by light.
(d) The levels of several different phosphorylated compounds in photosynthetic cells changed dramatically in response to light exposure. (Emerson and coworkers were not able to identify the specific compounds involved.)

As it turned out, the Emerson and Rabinowitch models were both partly correct and partly incorrect.
(e) Explain how the two models relate to our current model of photosynthesis.

In his rejection of the Emerson model, Rabinowitch went on to say: "The difficulty of the phosphate storage theory appears most clearly when one considers the fact that, in weak light, eight or ten quanta of light are sufficient to reduce one molecule of carbon dioxide. If each quantum should produce one molecule of high-energy phosphate, the accumulated energy would be only 80–100 kcal per Einstein—while photosynthesis requires *at least* 112 kcal per mole, and probably more, because of losses in irreversible partial reactions" (p. 228).
(f) How does Rabinowitch's value of 8 to 10 photons per molecule of CO_2 reduced compare with the value accepted today?
(g) How would you rebut Rabinowitch's argument, based on our current knowledge about photosynthesis?

References

Emerson, R.L., J.F. Stauffer, and W.W. Umbreit. 1944. Relationships between phosphorylation and photosynthesis in *Chlorella*. *Am. J. Botany* 31:107–120.

Rabinowitch, E.I. 1945. *Photosynthesis and Related Processes*, Vol. I. New York: Interscience Publishers.

Chapter 21

LIPID BIOSYNTHESIS

21.1 Biosynthesis of Fatty Acids and Eicosanoids *744*

21.2 Biosynthesis of Triacylglycerols *760*

21.3 Biosynthesis of Membrane Phospholipids *764*

21.4 Cholesterol, Steroids, and Isoprenoids: Biosynthesis, Regulation, and Transport *771*

Lipids play a variety of cellular roles, some recognized only recently. This chapter describes the biosynthetic pathways for some of the most common cellular lipids, illustrating the strategies employed in assembling these water-insoluble products from water-soluble precursors such as acetate. We first describe the biosynthesis of fatty acids, the primary components of both triacylglycerols and phospholipids, then examine the assembly of fatty acids into triacylglycerols and the simpler membrane phospholipids. Finally, we consider the synthesis of cholesterol, a component of some membranes and the precursor of steroids such as bile acids, sex hormones, and adrenocortical hormones.

Our coverage of lipid biosynthesis is organized around the following principles:

P1 **Lipids are the principal form of stored energy in most higher organisms, as well as the major constituents of membranes.**

P2 **Anabolism is not simply the reverse of catabolism.** Biosynthetic pathways typically diverge from breakdown pathways to overcome irreversible steps in catabolism.

P3 **Like other anabolic pathways, the reaction sequences in lipid biosynthesis are endergonic and reductive.** They use ATP as a source of metabolic energy and a reduced electron carrier (usually NADPH) as a reductant.

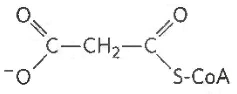
P4 **Lipid biosynthesis, like other anabolic pathways, is subject to regulation to respond to cellular and organismal requirements.** The places where catabolic and anabolic pathways diverge (Principle 2) provide opportunities to impose metabolic regulation to conserve resources and avoid futile cycles.

P5 **Like other major classes of biological molecules, lipids have a plethora of cellular functions.** Specialized lipids serve as pigments (retinal, carotene), cofactors (vitamin K), detergents (bile salts), transporters (dolichols), hormones (vitamin D derivatives, sex hormones), extracellular and intracellular messengers (eicosanoids, phosphatidylinositol derivatives), and anchors for membrane proteins (covalently attached fatty acids, prenyl groups, phosphatidylinositol). The ability to synthesize a variety of lipids is essential to all organisms.

We will focus on eukaryotes, with occasional digressions to highlight important distinctions in bacteria and plants.

21.1 Biosynthesis of Fatty Acids and Eicosanoids

P2 Even when compared to other major classes of metabolites, the division between fatty acid biosynthesis and breakdown is particularly striking. The two processes occur by different pathways, catalyzed by different sets of enzymes, and, in eukaryotes, occur in different cellular compartments. Fatty acid breakdown occurs in the mitochondria, whereas biosynthesis occurs in the cytosol. Moreover, biosynthesis requires the participation of a three-carbon intermediate, **malonyl-CoA**, that does not appear in the path of fatty acid breakdown.



Malonyl-CoA

The general pathway of fatty acid synthesis and its regulation now take center stage. We consider the biosynthesis of longer-chain fatty acids, unsaturated fatty acids, and their eicosanoid derivatives at the end of this section.

Malonyl-CoA Is Formed from Acetyl-CoA and Bicarbonate

The formation of malonyl-CoA from acetyl-CoA is an irreversible three-step process, catalyzed by **acetyl-CoA carboxylase**. In animal cells, all three steps are catalyzed in the cytoplasm by a single multifunctional polypeptide (**Fig. 21-1**). The enzyme contains a biotin prosthetic group covalently bound in amide linkage to the ε-amino group of a Lys residue in one of the domains of the enzyme molecule.

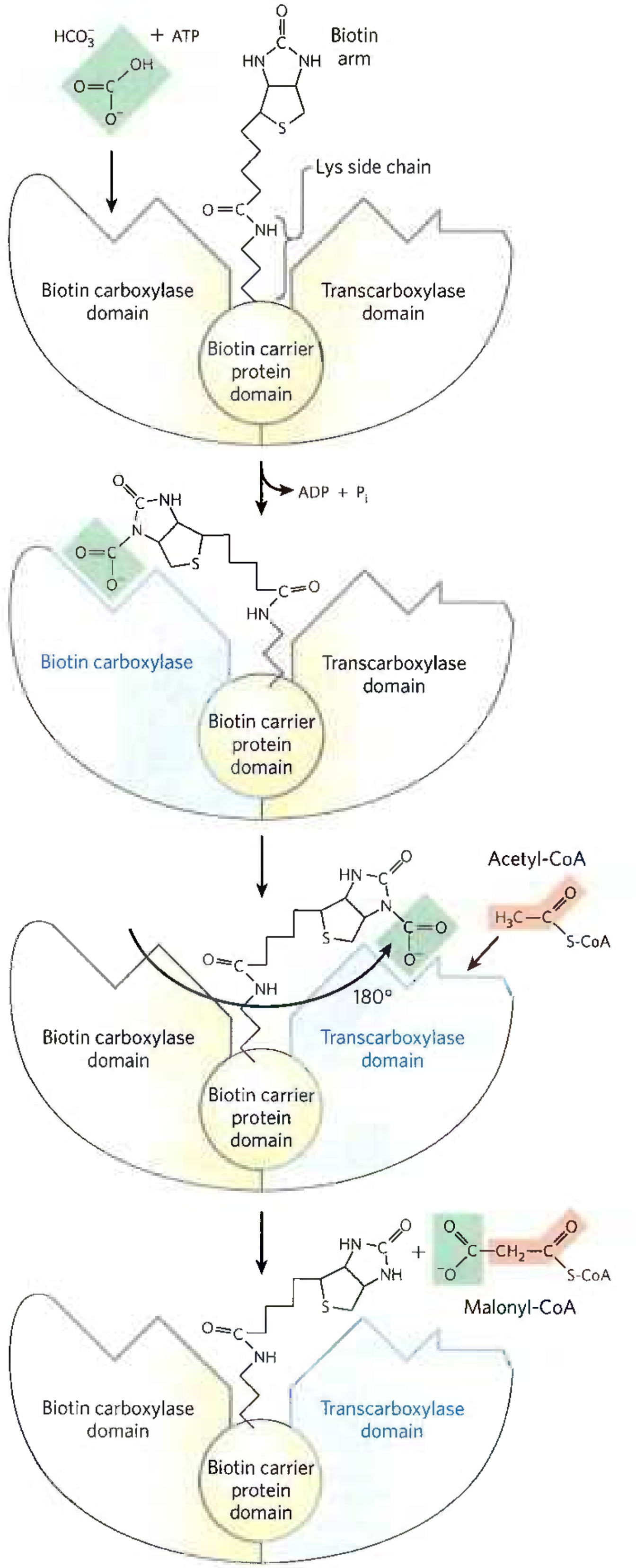

HCO_3^-
+ ATP
Biotin arm
Lys side chain
Biotin carboxylase domain
Transcarboxylase domain
Biotin carrier protein domain
ADP + P_i
Biotin carboxylase
Transcarboxylase domain
Biotin carrier protein domain
Acetyl-CoA
H_3C-C
S-CoA
180°
Biotin carboxylase domain
Transcarboxylase domain
Biotin carrier protein domain
S-CoA
Malonyl-CoA
Biotin carboxylase domain
Transcarboxylase domain
Biotin carrier protein domain

FIGURE 21-1 The acetyl-CoA carboxylase reaction. The mammalian acetyl-CoA carboxylase of the cytoplasm has three functional domains with distinct functions: biotin carrier protein; biotin carboxylase, which activates CO_2 by attaching it to a nitrogen in the biotin ring in an ATP-dependent reaction; and transcarboxylase, which transfers activated CO_2 (shaded green) from biotin to acetyl-CoA, producing malonyl-CoA. Part of the biotin carrier protein domain and the long, flexible biotin arm rotate to carry the activated CO_2 from the biotin carboxylase active site to the transcarboxylase active site. The active domain in each step is shaded in blue.

The reaction catalyzed by this enzyme is very similar to other biotin-dependent carboxylation reactions, such as those catalyzed by pyruvate carboxylase (see Fig. 16-16) and propionyl-CoA carboxylase (see Fig. 17-12). A carboxyl group, derived from bicarbonate (HCO_3^-), is first transferred to biotin in an ATP-dependent reaction. In a second step, the carboxyl group is carried by the biotin to a different active site, where the CO_2 is transferred to acetyl-CoA in the third and final step to yield malonyl-CoA. As we will see, this carboxylation has the same function as the carboxylation of pyruvate by pyruvate carboxylase—it renders the next step in the reaction sequence much more favorable thermodynamically.

The bacterial version of acetyl-CoA carboxylase is similar but has three separate polypeptide subunits (including a separate biotin carrier protein) that catalyze the three steps. Plant cells contain both types of acetyl-CoA carboxylase.

Fatty Acid Synthesis Proceeds in a Repeating Reaction Sequence

The long carbon chains of fatty acids are assembled in the cytosol in a repeating four-step sequence (**Fig. 21-2**), catalyzed by a system collectively referred to as **fatty acid synthase**. A saturated acyl group produced by each four-step series of reactions becomes the substrate for subsequent condensation with an activated malonyl group. With each passage through the cycle, the fatty acyl chain is extended by two carbons.

In the four-step pathway, a condensation reaction is followed by a reduction-dehydration-reduction sequence to convert the C-3 carbonyl to a methylene. The latter three steps are the chemical reverse of the oxidation-hydration-oxidation sequence in the β oxidation of fatty acids (Fig. 17-8a). **P2** However, both the electron-carrying cofactor and the activating groups in the reductive anabolic sequence differ from those in the oxidative catabolic process. Recall that in β oxidation, NAD^+ and FAD serve as electron acceptors and the activating group is the thiol (—SH) group of coenzyme A. By contrast, the reducing agent in the synthetic sequence is NADPH and the activating groups are two different enzyme-bound —SH groups, as described below.

In mammals, this synthetic system is called fatty acid synthase I (FAS I). There are seven active sites to catalyze the four-step cycle plus the charging steps described below. The active sites for different reactions lie in separate domains

(Fig. 21-3a), all within a single multifunctional polypeptide chain (M_r 240,000). Two of these multifunctional polypeptides function as a homodimer (M_r 480,000; Fig. 21-3b). The two subunits seem to function independently. When all the active sites in one subunit are inactivated by mutation, fatty acid synthesis is only moderately reduced.

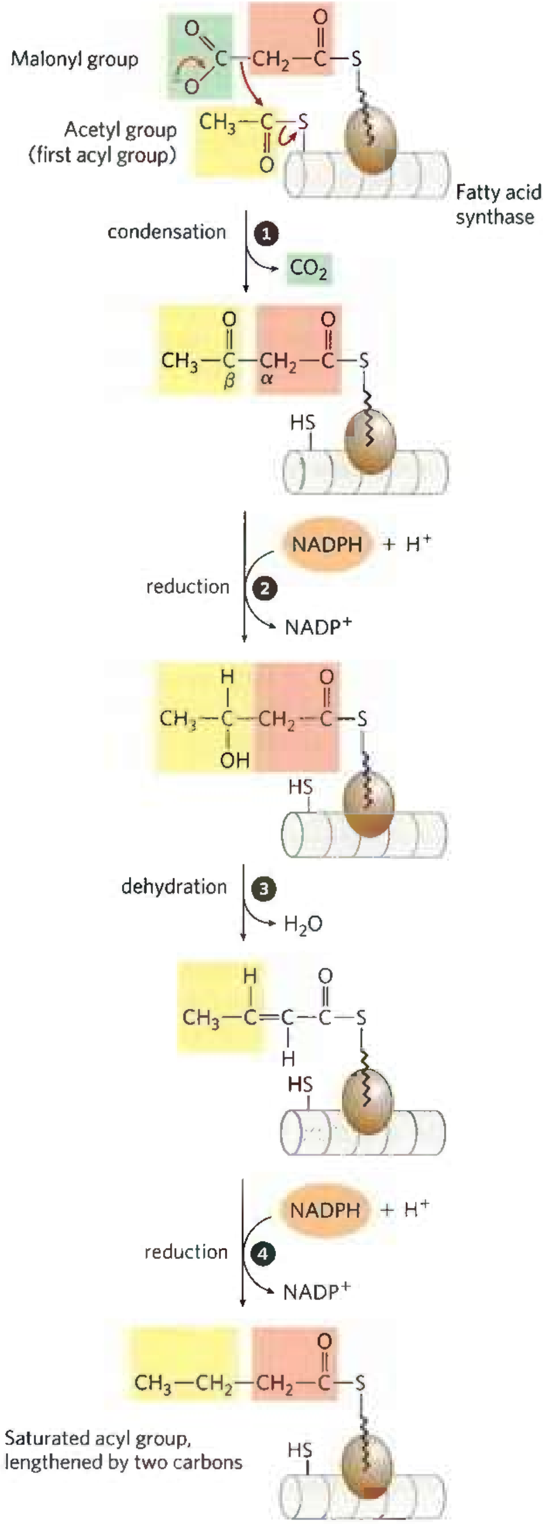

Malonyl group
Acetyl group (first acyl group)
Fatty acid synthase
condensation
1
CO_2
β
α
HS
reduction
2
NADPH + H^+
$NADP^+$
dehydration
3
H_2O
reduction
4
NADPH + H^+
$NADP^+$
Saturated acyl group, lengthened by two carbons

FIGURE 21-2 Addition of two carbons to a growing fatty acyl chain: a four-step sequence. Each malonyl group and acetyl (or longer acyl) group is activated by a thioester that links it to fatty acid synthase, a multienzyme system. ① Condensation of an activated acyl group (an acetyl group from acetyl-CoA is the first acyl group) and two carbons derived from malonyl-CoA, with elimination of CO_2 from the malonyl group, extends the acyl chain by two carbons. The mechanism of the first step of this reaction is given to illustrate the role of decarboxylation in facilitating condensation. The β-keto product of the condensation is then reduced in three more steps nearly identical to the reactions of β oxidation, but in the reverse sequence: ② the β-keto group is reduced to an alcohol, ③ elimination of H_2O creates a double bond, and ④ the double bond is reduced to form the corresponding saturated fatty acyl group.

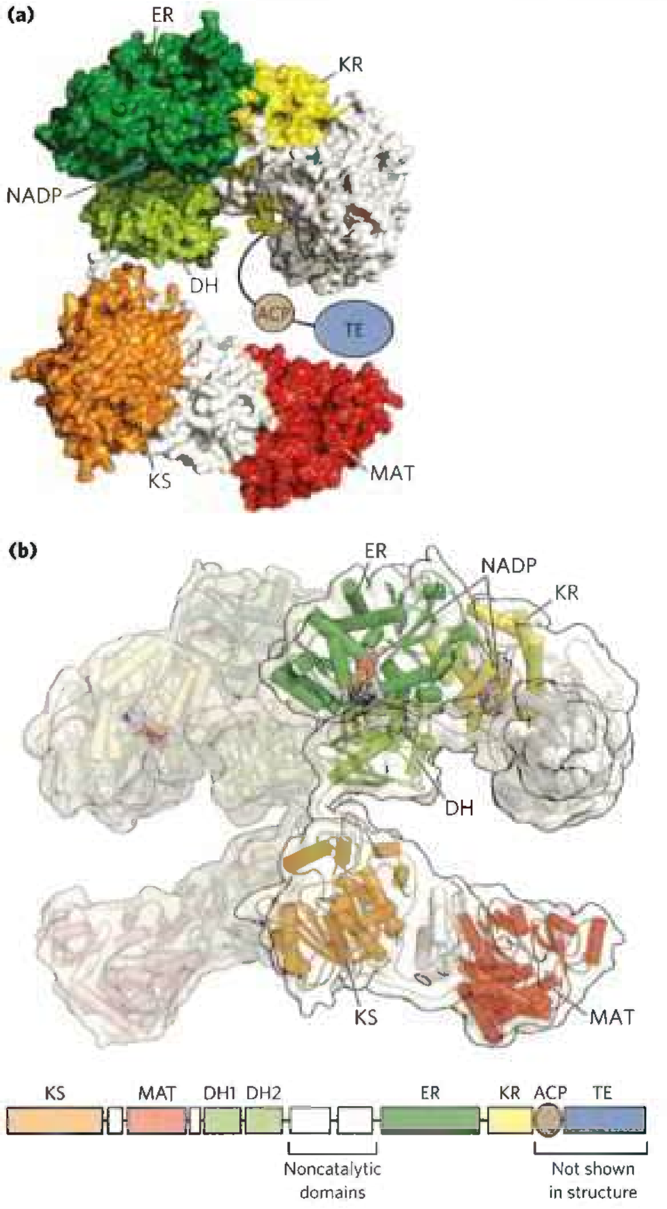

(a)
ER
KR
NADP
DH
ACP
TE
KS
MAT
(b)
ER
NADP
KR
DH
KS
MAT
KS
MAT
DH1
DH2
ER
KR
ACP
TE
Noncatalytic domains
Not shown in structure

FIGURE 21-3 The structure of a fatty acid synthase type I system. Shown here is the structure of a single (monomeric) polypeptide chain of the mammalian (porcine) enzyme system. (a) All of the active sites in the mammalian system are located in different domains within a single large polypeptide chain. The different enzymatic activities are β-ketoacyl-ACP synthase (KS), malonyl/acetyl-CoA–ACP transferase (MAT), β-hydroxyacyl-ACP dehydratase (DH), enoyl-ACP reductase (ER), and β-ketoacyl-ACP reductase (KR). ACP is the acyl carrier protein. The seventh domain is a thioesterase (TE) that releases the palmitate product from ACP when synthesis is completed. The ACP and TE domains are disordered in the crystal and are therefore not shown in the structure. (b) The native dimeric structure is shown, with one polypeptide transparent to show how the two independently operating subunits come together. The linear arrangement of the domains in the polypeptide is shown below the structure. [Data from PDB ID 2CF2, T. Maier et al., *Science* 311:1258, 2006.]

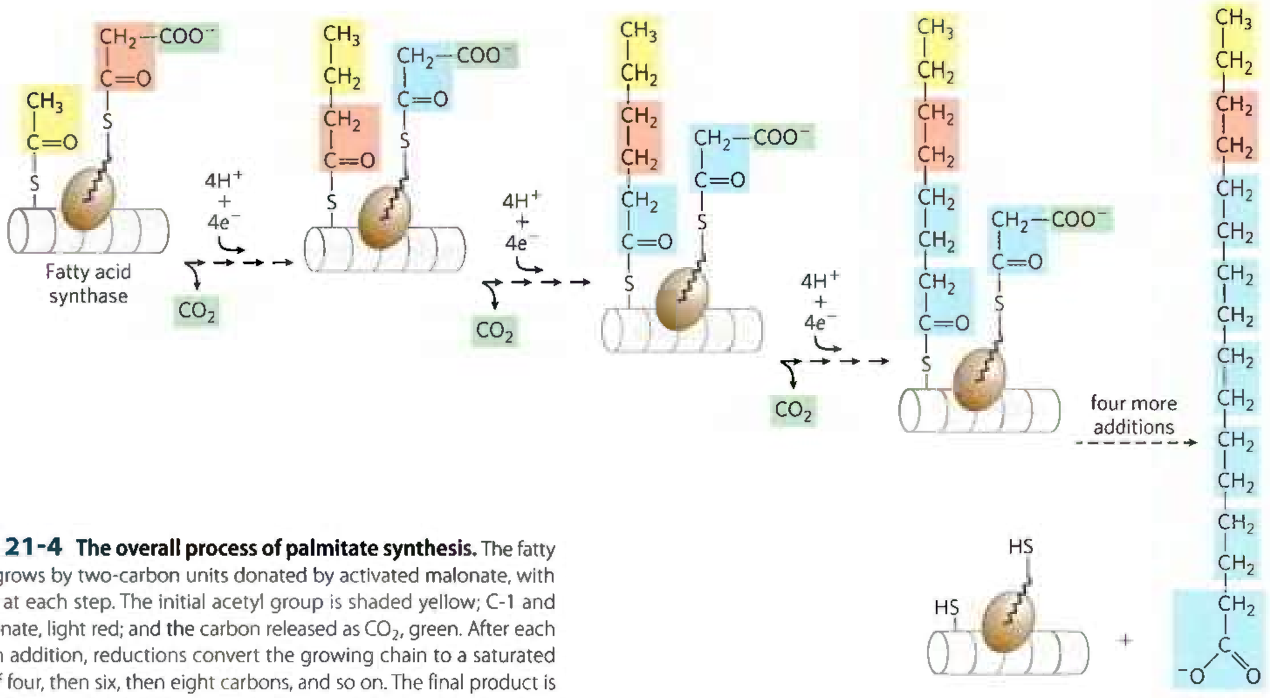


FIGURE 21-4 The overall process of palmitate synthesis. The fatty acyl chain grows by two-carbon units donated by activated malonate, with loss of CO_2 at each step. The initial acetyl group is shaded yellow; C-1 and C-2 of malonate, light red; and the carbon released as CO_2, green. After each two-carbon addition, reductions convert the growing chain to a saturated fatty acid of four, then six, then eight carbons, and so on. The final product is palmitate (16:0).

With FAS I systems, fatty acid synthesis leads to a single product. As it goes through the cycle, the acyl group is covalently linked to **acyl carrier protein (ACP)**, which shuttles it from one active site to another in sequence. Acyl carrier protein is yet another contiguous part of the single FAS I polypeptide. No intermediates are released. When the chain length reaches 16 carbons, that product (palmitate, 16:0; see Table 10-1) leaves the cycle. Carbons C-16 and C-15 of the palmitate are derived from the methyl and carboxyl carbon atoms, respectively, of an acetyl-CoA used directly to prime the system at the outset (**Fig. 21-4**); the rest of the carbon atoms in the chain are derived from acetyl-CoA via malonyl-CoA.

A somewhat different FAS I is found in yeast and other fungi, and is made up of two multifunctional polypeptides that form a complex with an architecture distinct from that of the vertebrate systems. Three of the seven required active sites are found on the α subunit and four on the β subunit. A different system, called FAS II, is found in plants and most bacteria. FAS II is a dissociated system; each step in the synthesis is catalyzed by a separate enzyme. Unlike FAS I, FAS II generates a variety of products, including saturated fatty acids of several lengths, as well as unsaturated, branched, and hydroxy fatty acids. An FAS II system is also found in vertebrate mitochondria, yet another indication of the bacterial origins of mitochondria in evolution.

The Mammalian Fatty Acid Synthase Has Multiple Active Sites

The multiple domains of mammalian FAS I function as distinct but linked enzymes. The active site for each enzyme is found in a separate domain within the larger polypeptide. Throughout the process of fatty acid synthesis, the intermediates remain covalently attached as thioesters to one of two thiol groups. One point of attachment is the —SH group of a Cys residue in one of the synthase domains (β-ketoacyl-ACP synthase; KS); the other is the —SH group of acyl carrier protein, a separate domain of the same polypeptide. Hydrolysis of thioesters is highly exergonic, and the energy released helps to make two steps (❶ and ❺ in Fig. 21-6) in fatty acid synthesis thermodynamically favorable.

Acyl carrier protein is the shuttle that holds the system together, containing the prosthetic group **4′-phosphopantetheine**, also found in coenzyme A (**Fig. 21-5**). The 4′-phosphopantetheine serves as a flexible arm, tethering the growing fatty acyl chain to the surface of the fatty acid synthase complex while carrying the reaction intermediates from one enzyme active site to the next. The same prosthetic group is used in FAS II systems.

Fatty Acid Synthase Receives the Acetyl and Malonyl Groups

Before the condensation reactions that build up the fatty acid chain can begin, the two thiol groups on the enzyme complex must be charged with the correct acyl groups (**Fig. 21-6a**). First, the acetyl group of acetyl-CoA is transferred to ACP in a reaction catalyzed by the **malonyl/acetyl-CoA–ACP transferase** (MAT) domain of the multifunctional polypeptide. The acetyl group is then transferred to the Cys —SH group of the **β-ketoacyl-ACP synthase** (KS). The second reaction, transfer of the malonyl group from malonyl-CoA to the —SH group of ACP, is also catalyzed by malonyl/acetyl-CoA–ACP transferase. In the charged synthase

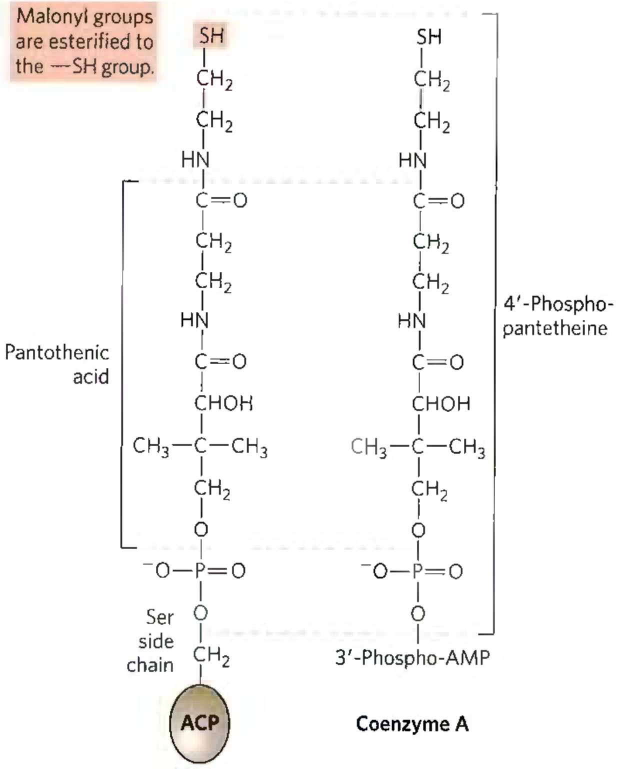


FIGURE 21-5 Acyl carrier protein (ACP). The prosthetic group is 4′-phosphopantetheine, which is covalently attached to the hydroxyl group of a Ser residue in ACP. Phosphopantetheine, also found in the coenzyme A molecule, contains the B vitamin pantothenic acid. Its —SH group is the site of entry of malonyl groups during fatty acid synthesis. Coenzyme A, shown here for comparison, serves a similar chemical purpose in general metabolism.

complex, the acetyl and malonyl groups are activated for the chain-lengthening process. We now consider the first four steps of this process in some detail; all step numbers refer to Figure 21-6.

Step ① Condensation The first reaction in the formation of a fatty acid chain is a formal Claisen condensation (see reaction class in Fig. 13-4) of the activated acetyl and malonyl groups to form **acetoacetyl-ACP**, an acetoacetyl group bound to ACP through the phosphopantetheine —SH group; simultaneously, a molecule of CO_2 is produced. In this reaction, catalyzed by β-ketoacyl-ACP synthase, the acetyl group is transferred from the Cys —SH group of the enzyme to the malonyl group on the —SH of ACP, becoming the methyl-terminal two-carbon unit of the new acetoacetyl group.

The carbon atom of the CO_2 formed in this reaction is the same carbon originally introduced into malonyl-CoA from HCO_3^- in the acetyl-CoA carboxylase reaction (Fig. 21-1). Thus, CO_2 is only transiently in covalent linkage during fatty acid biosynthesis; it is removed as each two-carbon unit is added.

Why do cells go to the trouble of adding CO_2 to make a malonyl group from an acetyl group, only to lose the CO_2 during the formation of acetoacetate? **P3** The use of activated malonyl groups rather than acetyl groups makes the condensation reactions thermodynamically favorable. The methylene carbon (C-2) of the malonyl group, sandwiched between carbonyl and carboxyl carbons, forms a good nucleophile. In the condensation step, decarboxylation of the malonyl group facilitates nucleophilic attack of the methylene carbon on the thioester linking the acetyl group to β-ketoacyl-ACP synthase, displacing the enzyme's —SH group. Coupling the condensation to the decarboxylation of the malonyl group renders the overall process highly exergonic. A similar carboxylation-decarboxylation sequence facilitates the formation of phosphoenolpyruvate from pyruvate in gluconeogenesis (see Figs. 14-17 and 14-18).

P2 **P3** By using activated malonyl groups in the synthesis of fatty acids and activated acetate in their degradation, the cell makes both processes energetically favorable, although one is effectively the reversal of the other. The extra energy required to make fatty acid synthesis favorable is provided by the ATP used to synthesize malonyl-CoA from acetyl-CoA and HCO_3^- (Fig. 21-1).

Step ② Reduction of the Carbonyl Group The acetoacetyl-ACP formed in the condensation step now undergoes reduction of the carbonyl group at C-3 to form D-β-hydroxybutyryl-ACP. This reaction is catalyzed by **β-ketoacyl-ACP reductase** (KR), and the electron donor is NADPH. Notice that the D-β-hydroxybutyryl group does not have the same stereoisomeric form as the L-β-hydroxyacyl intermediate in fatty acid oxidation (see Fig. 17-8).

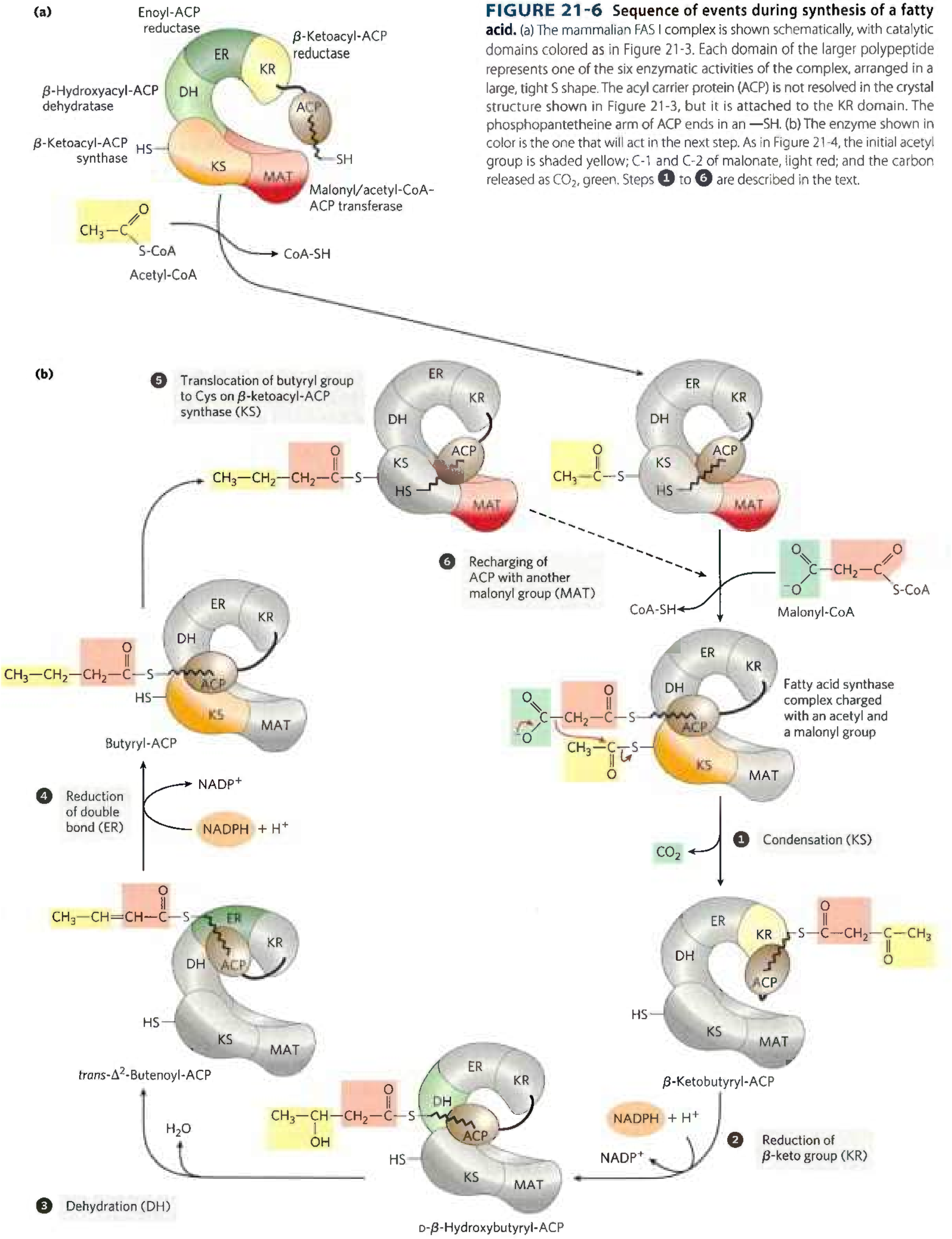
Step ③ Dehydration The elements of water are now removed from C-2 and C-3 of D-β-hydroxybutyryl-ACP to yield a double bond in the product, ***trans*-Δ^2-butenoyl-ACP**. The enzyme that catalyzes this dehydration is **β-hydroxyacyl-ACP dehydratase** (DH).

Step ④ Reduction of the Double Bond Finally, the double bond of *trans*-Δ^2-butenoyl-ACP is reduced (saturated) to form **butyryl-ACP** by the action of **enoyl-ACP reductase** (ER); again, NADPH is the electron donor.

The Fatty Acid Synthase Reactions Are Repeated to Form Palmitate

Production of the four-carbon, saturated fatty acyl–ACP marks completion of one pass through the fatty acid synthase complex. In step ⑤, the butyryl group is transferred from the phosphopantetheine —SH group of ACP to the Cys —SH group of β-ketoacyl-ACP synthase, which initially bore the acetyl group (Fig. 21-6). To start the next cycle of four reactions that lengthens the chain by two more carbons (step ⑥), another malonyl group is linked to the now unoccupied phosphopantetheine

FIGURE 21-6 Sequence of events during synthesis of a fatty acid. (a) The mammalian FAS I complex is shown schematically, with catalytic domains colored as in Figure 21-3. Each domain of the larger polypeptide represents one of the six enzymatic activities of the complex, arranged in a large, tight S shape. The acyl carrier protein (ACP) is not resolved in the crystal structure shown in Figure 21-3, but it is attached to the KR domain. The phosphopantetheine arm of ACP ends in an —SH. (b) The enzyme shown in color is the one that will act in the next step. As in Figure 21-4, the initial acetyl group is shaded yellow; C-1 and C-2 of malonate, light red; and the carbon released as CO_2, green. Steps ❶ to ❻ are described in the text.


(a)
Enoyl-ACP reductase
β-Ketoacyl-ACP reductase
β-Hydroxyacyl-ACP dehydratase
β-Ketoacyl-ACP synthase
ER
KR
DH
ACP
HS
KS
MAT
SH
Malonyl/acetyl-CoA-ACP transferase
CH₃—C(=O)—S-CoA
Acetyl-CoA
CoA-SH
(b)
❺ Translocation of butyryl group to Cys on β-ketoacyl-ACP synthase (KS)
CH₃—CH₂—CH₂—C(=O)—S
CH₃—C(=O)—S
❻ Recharging of ACP with another malonyl group (MAT)
CoA-SH
⁻O—C(=O)—CH₂—C(=O)—S-CoA
Malonyl-CoA
Fatty acid synthase complex charged with an acetyl and a malonyl group
❶ Condensation (KS)
CO₂
β-Ketobutyryl-ACP
S—C(=O)—CH₂—C(=O)—CH₃
NADPH + H⁺
NADP⁺
❷ Reduction of β-keto group (KR)
D-β-Hydroxybutyryl-ACP
CH₃—CH(OH)—CH₂—C(=O)—S
❸ Dehydration (DH)
H₂O
trans-Δ²-Butenoyl-ACP
CH₃—CH=CH—C(=O)—S
❹ Reduction of double bond (ER)
NADPH + H⁺
NADP⁺
Butyryl-ACP
CH₃—CH₂—CH₂—C(=O)—S

—SH group of ACP (**Fig. 21-7**). Condensation occurs as the butyryl group, acting like the acetyl group in the first cycle, is linked to two carbons of the malonyl-ACP group with concurrent loss of CO_2. The product of this condensation is a six-carbon acyl group, covalently bound to the phosphopantetheine —SH group. Its β-keto group is reduced in the next three steps of the synthase cycle to yield the saturated acyl group, exactly as in the first round of reactions—in this case forming the six-carbon product.

Seven cycles of condensation and reduction produce the 16-carbon saturated palmitoyl group, still bound to ACP. For reasons not well understood, chain elongation by the synthase complex generally stops at this point, and free palmitate is released from the ACP by a hydrolytic activity (**thioesterase**; TE) in the multifunctional protein.

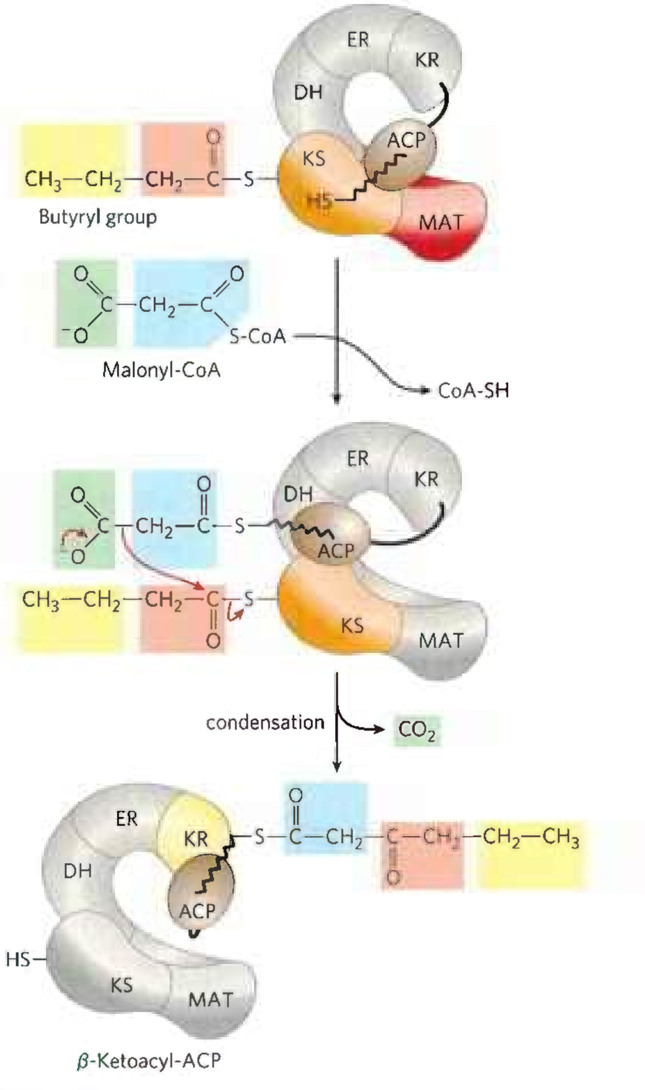

ER
KR
DH
ACP
KS
HS
MAT
$CH_3—CH_2—CH_2—C(=O)—S—$
Butyryl group
$^-O(O=)C—CH_2—C(=O)S\text{-CoA}$
Malonyl-CoA
CoA-SH
condensation
CO_2
$—S—C(=O)—CH_2—C(=O)—CH_2—CH_2—CH_3$
β-Ketoacyl-ACP

FIGURE 21-7 Beginning of the second round of the fatty acid synthesis cycle. The butyryl group is on the Cys —SH group. The incoming malonyl group is first attached to the phosphopantetheine —SH group. Then, in the condensation step, the entire butyryl group on the Cys —SH is exchanged for the carboxyl group of the malonyl residue, which is lost as CO_2 (green). This step is analogous to step ① in Figure 21-6. The product, a six-carbon β-ketoacyl group, now contains four carbons derived from malonyl-CoA and two derived from the acetyl-CoA that started the reaction. The β-ketoacyl group then undergoes steps ② through ④ in Figure 21-6.

We can consider the overall reaction for the synthesis of palmitate from acetyl-CoA in two parts. First, the formation of seven malonyl-CoA molecules:

$$7\ \text{Acetyl-CoA} + 7CO_2 + 7ATP \longrightarrow 7\ \text{malonyl-CoA} + 7ADP + 7P_i \quad (21\text{-}1)$$

then seven cycles of condensation and reduction:

$$\text{Acetyl-CoA} + 7\ \text{malonyl-CoA} + 14NADPH + 14H^+ \longrightarrow \text{palmitate} + 7CO_2 + 8CoA + 14NADP^+ + 6H_2O \quad (21\text{-}2)$$

Notice that only six net water molecules are produced, because one is used to hydrolyze the thioester linking the palmitate product to the enzyme. The overall process (the sum of Eqns 21-1 and 21-2) is

$$8\ \text{Acetyl-CoA} + 7ATP + 14NADPH + 14H^+ \longrightarrow \text{palmitate} + 8CoA + 7ADP + 7P_i + 14NADP^+ + 6H_2O \quad (21\text{-}3)$$

P3 The biosynthesis of fatty acids such as palmitate thus requires acetyl-CoA and the input of chemical energy in two forms: the group transfer potential of ATP and the reducing power of NADPH. The ATP is required to attach CO_2 to acetyl-CoA to make malonyl-CoA; the NADPH molecules are required to reduce the β-keto group and the double bond.

In nonphotosynthetic eukaryotes there is an additional cost to fatty acid synthesis, because acetyl-CoA is generated in the mitochondria and must be transported to the cytosol. As we will see, this extra step consumes two ATP per molecule of acetyl-CoA transported, increasing the energetic cost of fatty acid synthesis to three ATP per two-carbon unit.

Fatty Acid Synthesis Is a Cytosolic Process in Most Eukaryotes but Takes Place in the Chloroplasts in Plants

In most eukaryotes, the fatty acid synthase complex (FAS I) is found in the cytosol, as are the biosynthetic enzymes for nucleotides, amino acids, and glucose. This location segregates synthetic processes from degradative reactions, many of which take place in the mitochondrial matrix. **P3** There is a corresponding segregation of the electron-carrying cofactors used in anabolism (generally a reductive process) and those used in catabolism (generally oxidative).

Usually, NADPH is the electron carrier for anabolic reactions, and NAD^+ serves in catabolic reactions. The liver, the largest mammalian internal organ and a key metabolic center responding to large changes during feasting and fasting, provides a useful focus for this discussion. In hepatocytes, the $[NADPH]/[NADP^+]$ ratio is very high (~75) in the cytosol, furnishing a strongly reducing

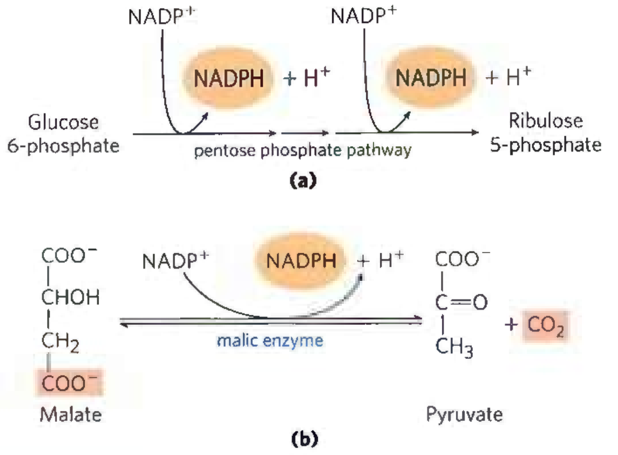

NADP+
NADP+
NADPH + H+
NADPH + H+
Glucose 6-phosphate
pentose phosphate pathway
Ribulose 5-phosphate
(a)
COO−
CHOH
CH2
COO−
Malate
NADP+
NADPH + H+
malic enzyme
COO−
C=O
CH3
+ CO2
Pyruvate
(b)

FIGURE 21-8 Production of NADPH. Two routes to NADPH, catalyzed by (a) the pentose phosphate pathway and (b) malic enzyme.

environment for the reductive synthesis of fatty acids and other biomolecules. The cytosolic [NADH]/[NAD$^+$] ratio is much lower (~8×10^{-4}), so the NAD$^+$-dependent oxidative catabolism of glucose can take place in the same compartment, and at the same time, as fatty acid synthesis. The [NADH]/[NAD$^+$] ratio in the mitochondrion is much higher than that in the cytosol, because of the flow of electrons to NAD$^+$ from the oxidation of fatty acids, amino acids, pyruvate, and acetyl-CoA. This high mitochondrial [NADH]/[NAD$^+$] ratio favors the reduction of oxygen via the respiratory chain.

In hepatocytes and adipocytes, cytosolic NADPH is largely generated by the pentose phosphate pathway (**Fig. 21-8a**; also see Fig. 14-30) and by **malic enzyme** (Fig. 21-8b). The pyruvate produced by the action of malic enzyme reenters the mitochondrion.

In the photosynthetic cells of plants, fatty acid synthesis occurs not in the cytosol but in the chloroplast stroma (**Fig. 21-9**). This makes sense, given that NADPH is produced in chloroplasts by the light-dependent reactions of photosynthesis:

$$H_2O + NADP^+ \xrightarrow{\text{light}} \tfrac{1}{2}O_2 + NADPH + H^+$$

Acetate Is Shuttled out of Mitochondria as Citrate

In nonphotosynthetic eukaryotes, nearly all the acetyl-CoA used in fatty acid synthesis is formed in mitochondria from pyruvate oxidation and from catabolism of the carbon skeletons of amino acids. Acetyl-CoA arising from the oxidation of fatty acids is not a significant source of acetyl-CoA for fatty acid biosynthesis in animals, because the two pathways are reciprocally regulated, as described below.

The inner mitochondrial membrane is impermeable to acetyl-CoA, so an indirect shuttle transfers acetyl group equivalents across the membrane (**Fig. 21-10**). Intramitochondrial acetyl-CoA first reacts with oxaloacetate to form citrate, in the citric acid cycle reaction catalyzed by **citrate synthase** (see Fig. 16-7). Citrate then passes through the inner membrane on the **citrate transporter**. In the cytosol, citrate cleavage by **citrate lyase** regenerates acetyl-CoA and oxaloacetate in an ATP-dependent reaction. Oxaloacetate cannot return to the mitochondrial matrix directly, as there is no oxaloacetate transporter. Instead, cytosolic malate dehydrogenase reduces the oxaloacetate to malate, which can return to the mitochondrial matrix on the **malate–α-ketoglutarate transporter**, in exchange for

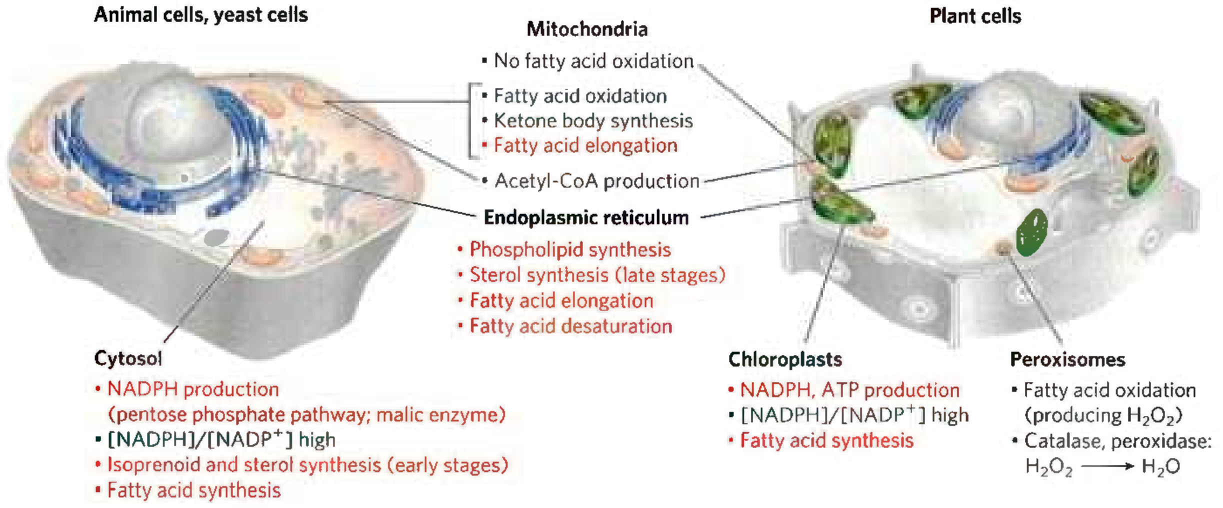

Animal cells, yeast cells
Mitochondria
• No fatty acid oxidation
• Fatty acid oxidation
• Ketone body synthesis
• Fatty acid elongation
• Acetyl-CoA production
Plant cells
Endoplasmic reticulum
• Phospholipid synthesis
• Sterol synthesis (late stages)
• Fatty acid elongation
• Fatty acid desaturation
Cytosol
• NADPH production (pentose phosphate pathway; malic enzyme)
• [NADPH]/[NADP+] high
• Isoprenoid and sterol synthesis (early stages)
• Fatty acid synthesis
Chloroplasts
• NADPH, ATP production
• [NADPH]/[NADP+] high
• Fatty acid synthesis
Peroxisomes
• Fatty acid oxidation (producing H2O2)
• Catalase, peroxidase: H2O2 → H2O

FIGURE 21-9 Subcellular localization of lipid metabolism. Yeast and animal cells differ from higher plant cells in the compartmentation of lipid metabolism. Fatty acid synthesis takes place in the compartment in which NADPH is available for reductive synthesis (i.e., where the [NADPH]/[NADP$^+$] ratio is high); this is the cytosol in animals and yeast, and the chloroplast in plants. Processes in red type are addressed in this chapter.

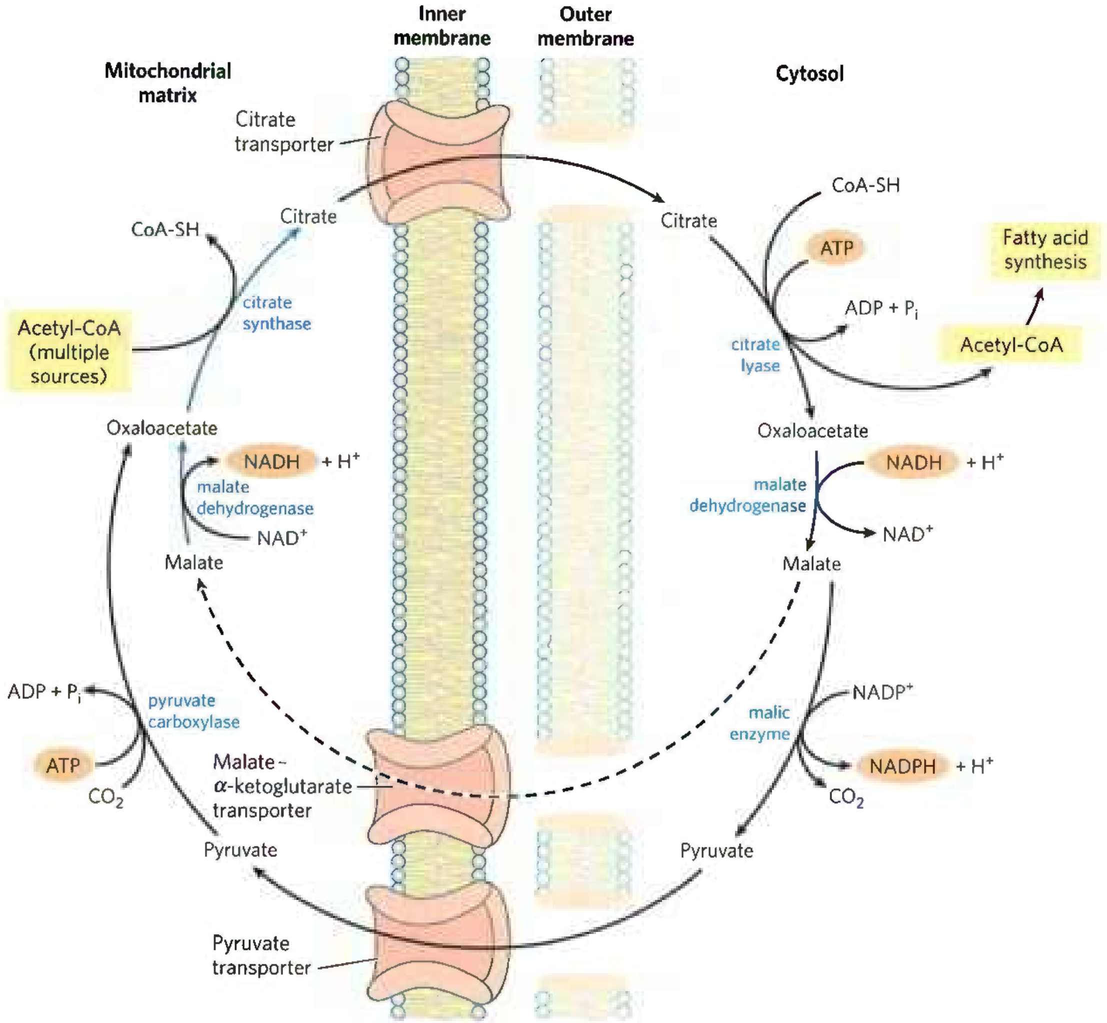

Inner membrane
Outer membrane
Mitochondrial matrix
Cytosol
Citrate transporter
Citrate
CoA-SH
citrate synthase
Acetyl-CoA (multiple sources)
Oxaloacetate
NADH + H^+
malate dehydrogenase
NAD^+
Malate
ADP + P_i
pyruvate carboxylase
ATP
CO_2
Malate–α-ketoglutarate transporter
Pyruvate
Pyruvate transporter
Citrate
CoA-SH
ATP
ADP + P_i
citrate lyase
Fatty acid synthesis
Acetyl-CoA
Oxaloacetate
NADH + H^+
malate dehydrogenase
NAD^+
Malate
$NADP^+$
malic enzyme
NADPH + H^+
CO_2
Pyruvate

FIGURE 21-10 Shuttle for transfer of acetyl groups from mitochondria to the cytosol. The outer mitochondrial membrane is freely permeable to all these compounds. Pyruvate derived from amino acid catabolism in the mitochondrial matrix, or from glucose by glycolysis in the cytosol, is converted to acetyl-CoA in the matrix. Acetyl groups pass out of the mitochondrion as citrate; in the cytosol they are delivered as acetyl-CoA for fatty acid synthesis. Oxaloacetate is reduced to malate, which can be returned to the mitochondrial matrix. The steps converting malate to oxaloacetate and oxaloacetate plus acetyl-CoA to citrate (indicated by blue arrows) are part of the citric acid cycle. The major fate of cytosolic malate, however, is oxidation by malic enzyme to generate cytosolic NADPH; the pyruvate produced returns to the mitochondrial matrix.

citrate. In the matrix, malate is reoxidized to oxaloacetate to complete the shuttle. However, most of the malate produced in the cytosol is used to generate cytosolic NADPH through the activity of malic enzyme (Fig. 21-8b). The pyruvate produced is transported into the mitochondria by the pyruvate transporter (Fig. 21-10), then converted back into oxaloacetate by pyruvate carboxylase in the matrix. In the resulting cycle, two ATP molecules are consumed (by citrate lyase and pyruvate carboxylase) for every molecule of acetyl-CoA delivered to fatty acid synthesis.

Malate thus has two fates in metabolism. In the mitochondrial matrix, malate is part of the citric acid cycle. In the cytosol, malate degradation becomes a significant source of NADPH. After citrate cleavage to generate acetyl-CoA, conversion of the four remaining carbons to pyruvate and CO_2 by malic enzyme generates about half the NADPH required for fatty acid synthesis. The pentose phosphate pathway contributes the rest of the needed NADPH.

Fatty Acid Biosynthesis Is Tightly Regulated

P1 When a cell or an organism has more than enough metabolic fuel to meet its energy needs, the excess is generally converted to fatty acids and stored as lipids such as triacylglycerols. **P4** The reaction catalyzed by acetyl-CoA carboxylase is the rate-limiting step in the biosynthesis of fatty acids, and this enzyme is an important site of regulation. In vertebrates, palmitoyl-CoA, the principal product of fatty acid synthesis, is a feedback inhibitor of the enzyme; citrate is an allosteric activator (**Fig. 21-11a**), increasing V_{max}. Citrate plays a central role in diverting cellular metabolism from the consumption (oxidation) of metabolic fuel to the storage of fuel

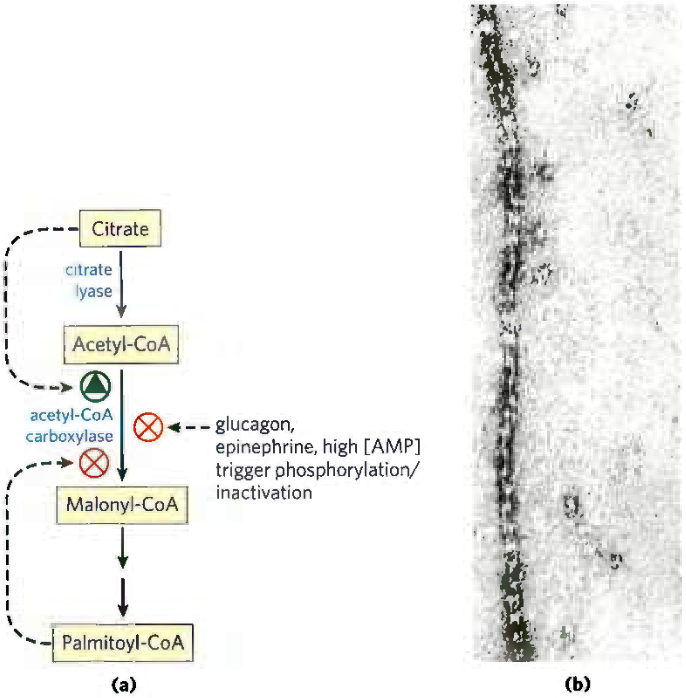


FIGURE 21-11 Regulation of fatty acid synthesis. (a) In the cells of vertebrates, both allosteric regulation and hormone-dependent covalent modification influence the flow of precursors into malonyl-CoA. In plants, acetyl-CoA carboxylase is activated by the changes in [Mg^{2+}] and pH that accompany illumination (not shown here). (b) Filaments of acetyl-CoA carboxylase from chicken hepatocytes (the active, dephosphorylated form), as seen with the electron microscope. [(b) Courtesy James M. Ntambi, PhD, Professor of Biochemistry, Steenbock Professor of Nutritional Sciences, University of Wisconsin–Madison.]

as fatty acids. When the concentrations of mitochondrial acetyl-CoA and ATP increase, citrate is transported out of mitochondria; it then becomes both the precursor of cytosolic acetyl-CoA and an allosteric signal for the activation of acetyl-CoA carboxylase. At the same time, citrate inhibits the activity of phosphofructokinase-1 (see Fig. 14-23), reducing the flow of carbon through glycolysis.

P4 Acetyl-CoA carboxylase is also regulated by covalent modification. Phosphorylation, triggered by the hormones glucagon and epinephrine or by high [AMP], inactivates the enzyme and reduces its sensitivity to activation by citrate, thereby slowing fatty acid synthesis. Phosphorylation occurs on at least three Ser residues and is catalyzed primarily by the AMP-activated protein kinase (AMPK). In its active (dephosphorylated) form, acetyl-CoA carboxylase polymerizes into long filaments (Fig. 21-11b); phosphorylation is accompanied by dissociation into monomeric subunits and loss of activity.

The acetyl-CoA carboxylase of plants and bacteria is not regulated by citrate or by a phosphorylation-dephosphorylation cycle. Instead, P4 the plant enzyme is activated by an increase in stromal pH and [Mg^{2+}], which occurs on illumination of the plant (see Fig. 20-35). Bacteria do not use triacylglycerols as energy stores. In *Escherichia coli*, the primary role of fatty acid synthesis is to provide precursors for membrane lipids; the regulation of this process is complex, employing guanine nucleotides (such as ppGpp; see Fig. 8-42) that coordinate cell growth with membrane formation.

P4 In addition to the moment-by-moment regulation of enzymatic activity, these pathways are regulated at the level of gene expression. For example, when animals ingest an excess of certain polyunsaturated fatty acids, the expression of genes encoding many lipogenic enzymes in the liver is suppressed. This gene regulation is mediated by a family of nuclear receptor proteins called PPARs, which we encountered in Section 17.2.

P4 If fatty acid synthesis and β oxidation were to proceed simultaneously, the two processes would constitute a futile cycle, wasting energy. We noted earlier (see Fig. 17-13) that β oxidation is blocked by malonyl-CoA, which inhibits carnitine acyltransferase I. Thus, during fatty acid synthesis, production of the first intermediate, malonyl-CoA, shuts down β oxidation at the level of a transport system in the inner mitochondrial membrane. This control mechanism illustrates another advantage of segregating synthetic and degradative pathways in different cellular compartments.

Long-Chain Saturated Fatty Acids Are Synthesized from Palmitate

Palmitate, the principal product of the fatty acid synthase system in animal cells, is the precursor of other long-chain fatty acids (**Fig. 21-12**). It may be lengthened to form stearate (18:0) or even longer saturated fatty acids by further additions of acetyl groups, through the action of **fatty acid elongation systems** present in the smooth endoplasmic reticulum (smooth ER) and in mitochondria. The more active elongation system of the ER extends the 16-carbon chain of palmitoyl-CoA by two carbons, forming stearoyl-CoA. Although different enzyme systems are used, and coenzyme A rather than ACP is the acyl carrier in the reaction, the mechanism of elongation in the ER is otherwise identical to that in palmitate synthesis: donation of two carbons by malonyl-CoA, followed by reduction, dehydration, and reduction to the saturated 18-carbon product, stearoyl-CoA.

Two key products of elongation pathways are linoleate, an omega-6 fatty acid (see Chapter 10 for the alternative nomenclature), and α-linolenate, an omega-3 fatty acid. These are precursors for two extensive families of derivative unsaturated fatty acids, the omega-6 and omega-3 families. Humans cannot synthesize linoleate and α-linolenate and must obtain them in the diet. The ratio of omega-6 to omega-3 fatty acids in the diet, if too high, can lead to cardiovascular disease. The importance of this ratio may reflect the multitude of signaling molecules in the omega-6 and omega-3 families (Fig. 21-12), with their equally complex physiological effects. Several of these derivative unsaturated fatty acids are considered below.

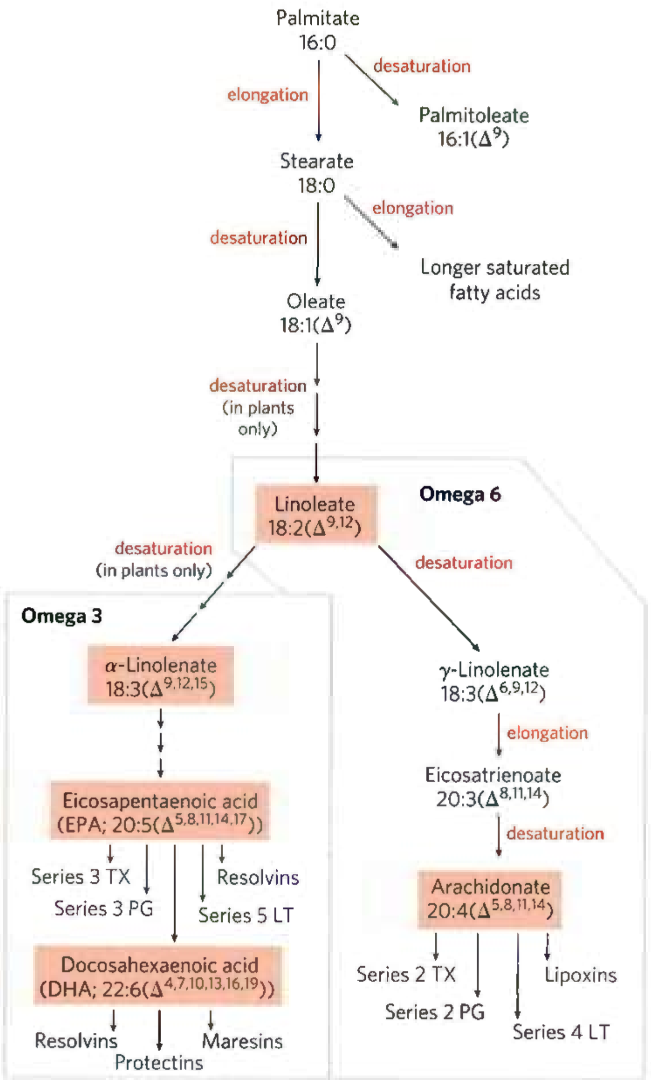

Palmitate
16:0
desaturation
elongation
Palmitoleate
16:1(Δ^9)
Stearate
18:0
elongation
desaturation
Longer saturated
fatty acids
Oleate
18:1(Δ^9)
desaturation
(in plants
only)
Linoleate
18:2($\Delta^{9,12}$)
Omega 6
desaturation
(in plants only)
desaturation
Omega 3
α-Linolenate
18:3($\Delta^{9,12,15}$)
γ-Linolenate
18:3($\Delta^{6,9,12}$)
elongation
Eicosatrienoate
20:3($\Delta^{8,11,14}$)
desaturation
Eicosapentaenoic acid
(EPA; 20:5($\Delta^{5,8,11,14,17}$))
Series 3 TX
Series 3 PG
Resolvins
Series 5 LT
Arachidonate
20:4($\Delta^{5,8,11,14}$)
Docosahexaenoic acid
(DHA; 22:6($\Delta^{4,7,10,13,16,19}$))
Series 2 TX
Series 2 PG
Lipoxins
Series 4 LT
Resolvins
Protectins
Maresins

FIGURE 21-12 Routes of synthesis of unsaturated fatty acids and their derivatives. Palmitate is the precursor of stearate and longer-chain saturated fatty acids, as well as the monounsaturated acids palmitoleate and oleate. Mammals cannot convert oleate to linoleate or α-linolenate (shaded), which are therefore required in the diet as essential fatty acids. Conversion of linoleate to other polyunsaturated fatty acids and eicosanoids is outlined. Unsaturated fatty acids are symbolized by indicating the number of carbons and the number and position of double bonds, as in Table 10-1. Linoleate and α-linolenate are important omega-6 and omega-3 fatty acids, respectively; they are also precursors for a wide range of unsaturated fatty acids that act as signaling molecules. Two-letter abbreviations specify the eicosanoid prostaglandins (PG), thromboxanes (TX), and leukotrienes (LT). Particular classes of unsaturated fatty acids are further delineated by the number of double bonds, which defines subclasses referred to as series. For example, series 2 TX are thromboxanes with two double bonds in the hydrocarbon chain.

Desaturation of Fatty Acids Requires a Mixed-Function Oxidase

Palmitate and stearate serve as precursors of the two most common monounsaturated fatty acids of animal tissues: palmitoleate, 16:1(Δ^9), and oleate, 18:1(Δ^9); both of these fatty acids have a single cis double bond between C-9 and C-10 (see Table 10-1). The double bond is introduced into the fatty acid chain by an oxidative reaction catalyzed by **fatty acyl–CoA desaturase** (**Fig. 21-13**), a **mixed-function oxidase** (**Box 21-1**). Two different substrates, the fatty acid and NADPH, simultaneously undergo two-electron oxidations. The path of electron flow includes a cytochrome (cytochrome b_5) and a flavoprotein (cytochrome b_5 reductase), both of which, like fatty acyl–CoA desaturase, are in the smooth ER. In plants, oleate is produced by a **stearoyl-ACP desaturase (SCD)** that uses reduced ferredoxin as the electron donor in the chloroplast stroma.

The SCD of animals (as studied in mice) has an important role in the development of obesity and the insulin resistance that often accompanies obesity and precedes development of type 2 diabetes mellitus. Mice have four isozymes, SCD1 through SCD4, of which SCD1 is the best understood. Its synthesis is induced by dietary saturated fatty acids, and also by the action of SREBP and LXR, two protein regulators of lipid metabolism that activate transcription of lipid-synthesizing enzymes (described in Section 21.4). Mice with mutant forms of SCD1 are resistant to diet-induced obesity and do not develop diabetes under conditions that cause both obesity and diabetes in mice with normal SCD1. ■

Mammalian hepatocytes can readily introduce double bonds at the Δ^9 position of fatty acids but cannot introduce additional double bonds between C-10 and the methyl-terminal end. Thus, as noted above, mammals cannot synthesize the omega-6 family precursor linoleate, 18:2($\Delta^{9,12}$), or the omega-3 family precursor α-linolenate, 18:3($\Delta^{9,12,15}$). Plants, however, can synthesize both; the desaturases that introduce double bonds at the Δ^{12} and Δ^{15} positions are located in the ER and in chloroplasts. The ER enzymes act not on free fatty acids but on a phospholipid, phosphatidylcholine, that contains at least one oleate linked to the glycerol (**Fig. 21-14**). Both plants and bacteria must synthesize polyunsaturated fatty acids to ensure membrane fluidity at reduced temperatures.

Because they are necessary precursors for the synthesis of other products, linoleate and α-linolenate are **essential fatty acids** for mammals; they must be obtained from dietary plant material. Once ingested, linoleate may be converted to certain other polyunsaturated acids, particularly γ-linolenate, eicosatrienoate, and **arachidonate (eicosatetraenoate)**, all of which can be made only from linoleate (Fig. 21-12). Similarly, α-linolenate is converted to two important derivatives, **eicosapentaenoic acid (EPA)** and **docosahexaenoic acid (DHA)**. Arachidonate, 20:4($\Delta^{5,8,11,14}$), EPA, 20:5($\Delta^{5,8,11,14,17}$), and DHA, 22:6($\Delta^{4,7,10,13,16,19}$), are essential precursors of distinct classes of eicosanoids,

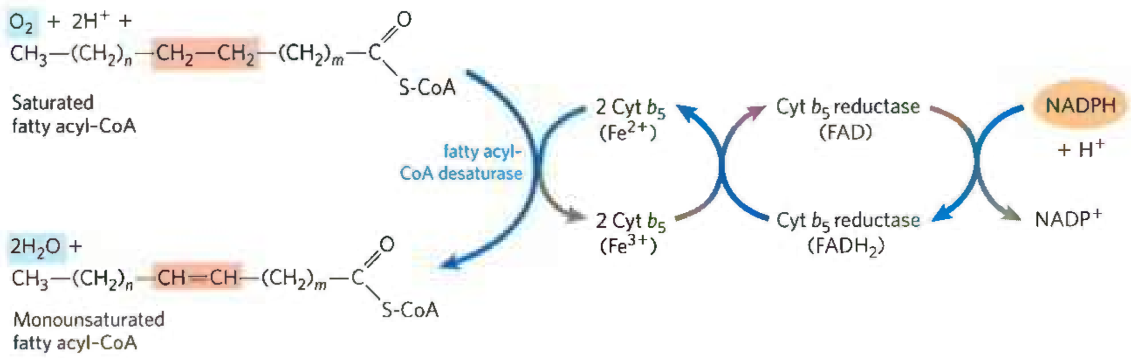

$O_2 + 2H^+ +$
$CH_3-(CH_2)_n-CH_2-CH_2-(CH_2)_m-C(=O)-S\text{-CoA}$
Saturated fatty acyl-CoA
fatty acyl-CoA desaturase
$2H_2O +$
$CH_3-(CH_2)_n-CH=CH-(CH_2)_m-C(=O)-S\text{-CoA}$
Monounsaturated fatty acyl-CoA
2 Cyt b_5 (Fe^{2+})
2 Cyt b_5 (Fe^{3+})
Cyt b_5 reductase (FAD)
Cyt b_5 reductase ($FADH_2$)
NADPH
$+ H^+$
$NADP^+$

FIGURE 21-13 **Electron transfer in the desaturation of fatty acids in vertebrates.** Blue arrows show the path of electrons as two substrates — a fatty acyl-CoA and NADPH — undergo oxidation by molecular oxygen. These reactions take place on the lumenal face of the smooth ER. A similar pathway, but with different electron carriers, occurs in plants.

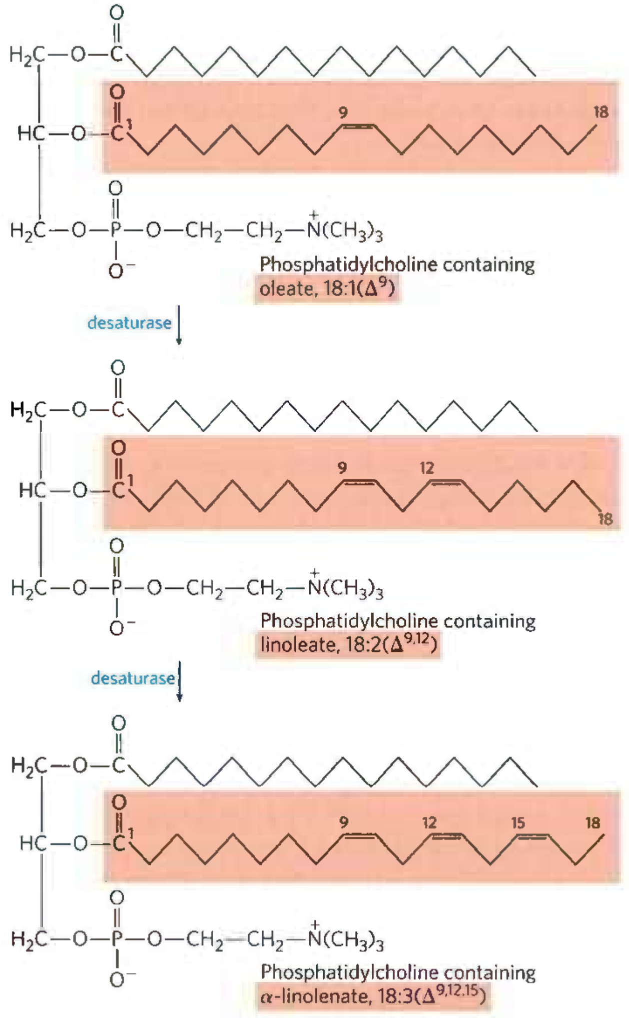

Phosphatidylcholine containing oleate, 18:1(Δ^9)
desaturase
Phosphatidylcholine containing linoleate, 18:2($\Delta^{9,12}$)
desaturase
Phosphatidylcholine containing α-linolenate, 18:3($\Delta^{9,12,15}$)
9
12
15
18
1

FIGURE 21-14 **Action of plant desaturases.** Desaturases in plants oxidize phosphatidylcholine-bound oleate to polyunsaturated fatty acids. Some of the products are released from the phosphatidylcholine by hydrolysis.

lipids with important regulatory functions. The 20- and 22-carbon fatty acids are synthesized from linoleate and α-linolenate by fatty acid elongation reactions analogous to those described on page 753.

Eicosanoids Are Formed from 20- and 22-Carbon Polyunsaturated Fatty Acids

P5 Eicosanoids are a family of very potent biological signaling molecules that act as short-range messengers, affecting tissues near the cells that produce them.

In response to hormonal or other stimuli, phospholipase A_2, present in most types of mammalian cells, attacks membrane phospholipids, releasing arachidonate from the middle carbon of glycerol. Enzymes of the smooth ER then convert arachidonate to **prostaglandins**, beginning with the formation of prostaglandin H_2 (PGH_2), the immediate precursor of many other prostaglandins and thromboxanes (**Fig. 21-15a**). The two reactions that lead to PGH_2 are catalyzed by a bifunctional enzyme, **cyclooxygenase (COX)**, also called **prostaglandin H_2 synthase**. In the first step, the cyclooxygenase activity introduces molecular oxygen to convert arachidonate to PGG_2. The second step, catalyzed by the peroxidase activity of COX, converts PGG_2 to PGH_2.

KEY CONVENTION Prostaglandins with different functional groups on the ring are given different letter designations: A, B, C, D, E, F, G, H, and R. The subscript number following the letter, as in PGH_2 and PGG_2, indicates the number of double bonds. Prostaglandins with two double bonds, all of which are derived from arachidonate, are referred to as series 2 prostaglandins; those with three double bonds, derived from EPA, as series 3 (Fig. 21-12). Similar naming patterns are used for other classes of eicosanoids described below. ■

Series 2 prostaglandins have important roles in the immediate response to stress or injury, including inflammation, pain, swelling, and dilation of blood vessels. Series 3 prostaglandins, in general, act more slowly and usually moderate the responses associated with series 2 prostaglandins.

Mammals have two isozymes of prostaglandin H_2 synthase, COX-1 and COX-2. These have different functions but closely similar amino acid sequences (60% to 65% sequence identity) and similar reaction mechanisms

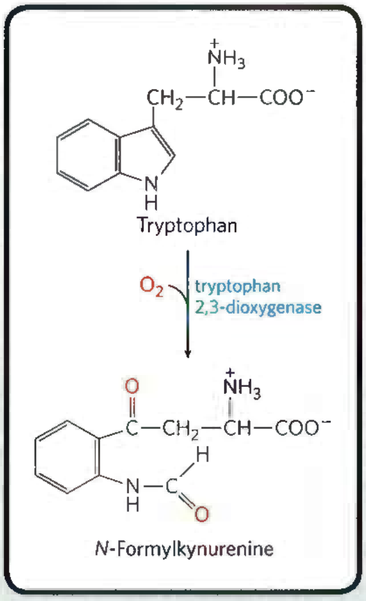
BOX 21-1 MEDICINE

Oxidases, Oxygenases, Cytochrome P-450 Enzymes, and Drug Overdoses

In this chapter we encounter several enzymes that carry out oxidation-reduction reactions in which molecular oxygen is a participant. The stearoyl-CoA desaturase (SCD) that introduces a double bond into a fatty acyl chain (see Fig. 21-13) is one such enzyme.

The nomenclature for enzymes that catalyze reactions of this general type can be confusing. **Oxidase** is the general name for enzymes that catalyze oxidations in which molecular oxygen is the electron acceptor but oxygen atoms do not appear in the oxidized product. The enzyme that creates a double bond in fatty acyl–CoA during the oxidation of fatty acids in peroxisomes (see Fig. 17-14) is an oxidase of this type; a second example is the cytochrome oxidase of the mitochondrial respiratory chain (see Fig. 19-13). In the first case, the transfer of two electrons to H_2O produces hydrogen peroxide, H_2O_2; in the second, two electrons reduce $\frac{1}{2}O_2$ to H_2O. Many, but not all, oxidases are flavoproteins. **Mixed-function oxidases** oxidize two different substrates simultaneously; again, the molecular oxygen atoms do not appear in the oxidized products. Mixed-function oxidases act in fatty acid desaturation (fatty acyl–CoA desaturase; see Fig. 21-13) and in the last step of plasmalogen synthesis (see Fig. 21-30).

Oxygenases catalyze oxidative reactions in which oxygen atoms *are* directly incorporated into the product molecule, forming a new hydroxyl or carboxyl group, for example. **Dioxygenases** catalyze reactions in which both oxygen atoms of O_2 are incorporated into the organic product. An example of a dioxygenase is tryptophan 2,3-dioxygenase, which catalyzes the opening of the five-membered ring of tryptophan in the catabolism of this amino acid. When the reaction takes place in the presence of $^{18}O_2$, the isotopic oxygen atoms are found in the two carbonyl groups of the product (shown in red):



Monooxygenases, more common and more complex in their action, catalyze reactions in which only one of the two oxygen atoms of O_2 is incorporated into the organic product, the other being reduced to H_2O; an example is squalene monooxygenase (see Fig. 21-37). Monooxygenases require two substrates to serve as reductants of the two oxygen atoms of O_2. The main substrate accepts one of the two oxygen atoms, and a cosubstrate furnishes hydrogen atoms to reduce the other oxygen atom to H_2O. The general reaction equation for monooxygenases is

$$AH + BH_2 + O{-}O \longrightarrow A{-}OH + B + H_2O$$

where AH is the main substrate and BH_2 is the cosubstrate. Because most monooxygenases catalyze reactions in which the main substrate becomes hydroxylated, they are also called **hydroxylases**. They are also sometimes called **mixed-function oxygenases** to indicate that they oxidize two different substrates simultaneously.

Monooxygenases are divided into several classes, depending on the nature of the cosubstrate. Some use reduced flavin nucleotides ($FMNH_2$ or $FADH_2$), others use NADH or NADPH, and still others use α-ketoglutarate as cosubstrate. The enzyme that hydroxylates the phenyl ring of phenylalanine to form tyrosine is a monooxygenase that uses tetrahydrobiopterin as cosubstrate (see Fig. 18-23). (This is the enzyme that is defective in the human genetic disease phenylketonuria.)

The most numerous and most complex monooxygenation reactions are those employing a type of heme protein called **cytochrome P-450**. Like mitochondrial cytochrome oxidase, enzymes containing a cytochrome P-450 domain can react with O_2 and bind carbon monoxide, but they can be differentiated from cytochrome oxidase because the carbon monoxide complex of their reduced form absorbs light strongly at 450 nm—thus the name P-450.

Cytochrome P-450 enzymes catalyze hydroxylation reactions in which an organic substrate, RH, is hydroxylated to R—OH, incorporating one oxygen atom of O_2; the other oxygen atom is reduced to H_2O by reducing equivalents that are furnished by NADH or NADPH but are usually passed to cytochrome P-450 by an iron-sulfur protein. Figure 1 shows a simplified outline of the action of cytochrome P-450.

One large family of P-450–containing proteins consists of two general types: those highly specific for a single substrate (like typical enzymes) and those with more promiscuous binding sites that accept a variety of substrates, generally similar in being hydrophobic. In the adrenal cortex, for example, a specific cytochrome P-450 participates in the hydroxylation of steroids to yield the adrenocortical hormones (see Fig. 21-49). There are dozens of P-450 enzymes that act on specific substrates in the biosynthetic pathways to steroid hormones and

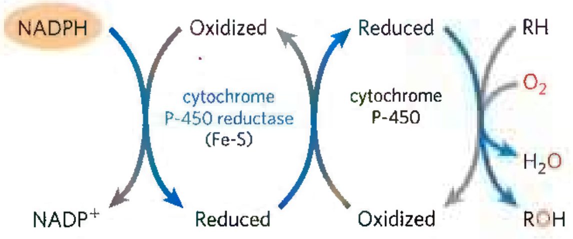

NADPH
Oxidized
Reduced
RH
cytochrome P-450 reductase (Fe-S)
cytochrome P-450
O$_2$
H$_2$O
NADP$^+$
Reduced
Oxidized
ROH

FIGURE 1 Simplified cytochrome P-450 reaction cycle.

eicosanoids (Fig. 2). Cytochrome P-450 enzymes with broader specificity are important in the hydroxylation of many different drugs, such as barbiturates and other xenobiotics (substances foreign to the organism), particularly if they are hydrophobic and relatively insoluble. The environmental carcinogen benzo[*a*]pyrene, found in cigarette smoke, undergoes cytochrome P-450–dependent hydroxylation during detoxification. Hydroxylation of xenobiotics, sometimes combined with the attachment of a polar compound such as glucuronic acid to the hydroxyl group, makes them more soluble in water and allows their excretion in urine. Hydroxylation (and glucuronidation) inactivates most drugs, and the rate at which it occurs can determine how long a given dose of a medication remains in the blood at therapeutic levels.

Humans differ in their levels of drug-metabolizing enzymes, both because of their genetics and because past exposure to substrates can induce the synthesis of higher levels of P-450 enzymes. Ethanol and barbiturate drugs share a P-450 enzyme. Long-term heavy drinking induces synthesis of this enzyme. Then, because the barbiturate is inactivated and cleared faster, larger doses are required to get the same therapeutic effect. If an individual takes this larger-than-usual dose of barbiturate and then also drinks alcohol, competition between the alcohol and the barbiturate for the limited amount of enzyme means that both alcohol and barbiturate are cleared more slowly. The resulting high levels of these two central nervous system depressants can be lethal. Similar complications arise when an individual takes two drugs that happen to be inactivated by the same P-450 enzyme; each drug increases the effective dose of the other by slowing its inactivation. It is therefore essential for physicians and pharmacists to know about all of a patient's prescribed and over-the-counter drugs and supplements, as well as a history of heavy drinking, or smoking, or exposure to environmental toxins.

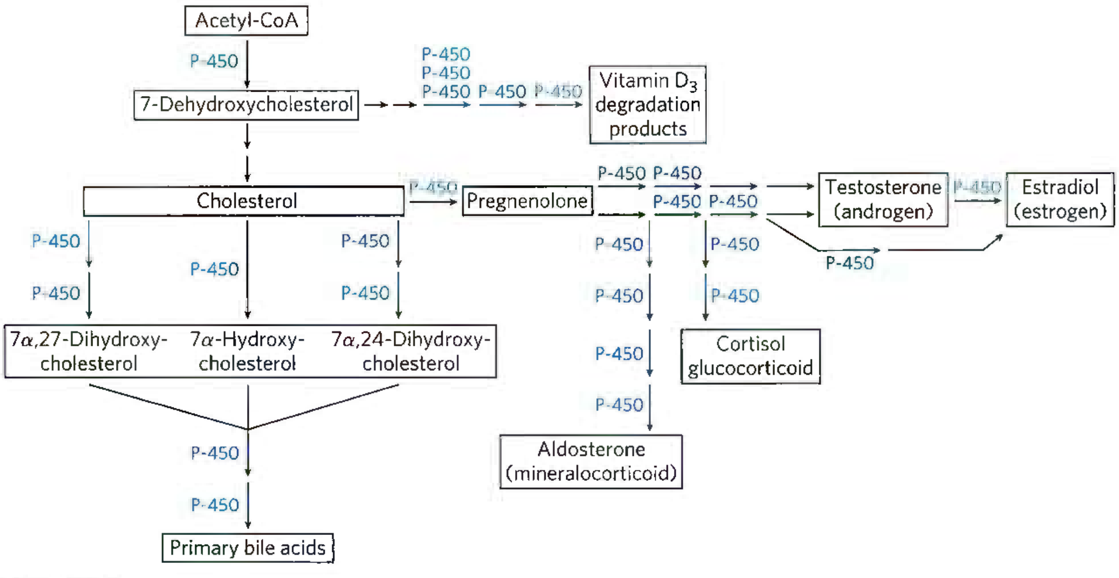

Acetyl-CoA
P-450
7-Dehydroxycholesterol
P-450
P-450
P-450 P-450 P-450
Vitamin D$_3$ degradation products
Cholesterol
P-450
Pregnenolone
P-450 P-450
P-450 P-450
Testosterone (androgen)
P-450
Estradiol (estrogen)
P-450
P-450
P-450
P-450
P-450
P-450
P-450
P-450
P-450
P-450
P-450
7α,27-Dihydroxy-cholesterol
7α-Hydroxy-cholesterol
7α,24-Dihydroxy-cholesterol
P-450
Cortisol glucocorticoid
P-450
P-450
P-450
Aldosterone (mineralocorticoid)
Primary bile acids

FIGURE 2 Pathways of sterol biosynthesis, showing the steps requiring cytochrome P-450 enzymes.

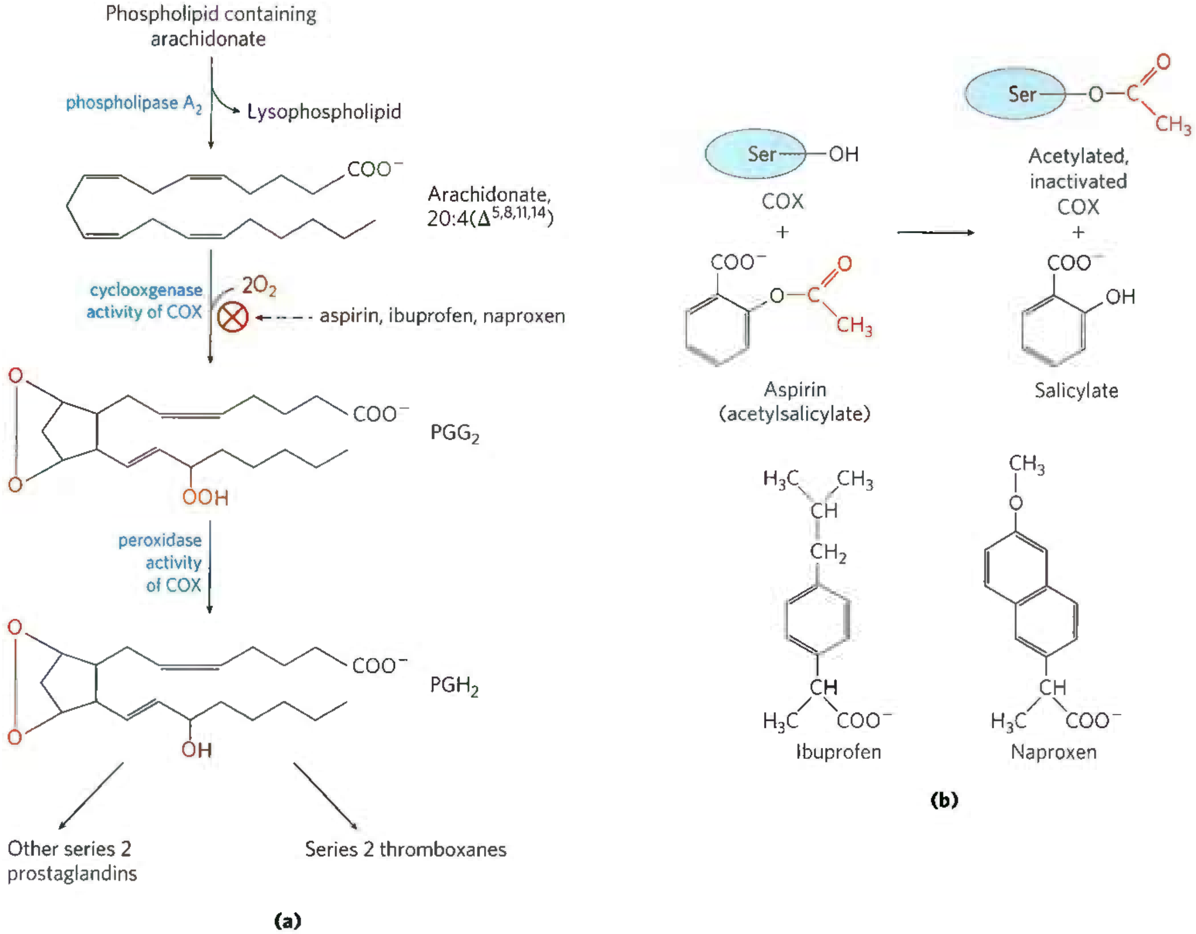

Phospholipid containing arachidonate
phospholipase A_2
Lysophospholipid
COO^-
Arachidonate, 20:4($\Delta^{5,8,11,14}$)
cyclooxygenase activity of COX
$2O_2$
aspirin, ibuprofen, naproxen
O
O
COO^-
PGG_2
OOH
peroxidase activity of COX
O
O
COO^-
PGH_2
OH
Other series 2 prostaglandins
Series 2 thromboxanes
(a)
Ser
OH
COX
+
COO^-
O
C
O
CH_3
Aspirin (acetylsalicylate)
Ser
O
C
O
CH_3
Acetylated, inactivated COX
+
COO^-
OH
Salicylate
H_3C
CH_3
CH
CH_2
CH
H_3C
COO^-
Ibuprofen
CH_3
O
CH
H_3C
COO^-
Naproxen
(b)

FIGURE 21-15 The "cyclic" pathway from arachidonate to prostaglandins and thromboxanes. (a) After arachidonate is released from phospholipids by the action of phospholipase A_2, the cyclooxygenase and peroxidase activities of COX (also called prostaglandin H_2 synthase) catalyze the production of PGH_2, the precursor of other prostaglandins and of thromboxanes. (b) Aspirin inhibits the first reaction by acetylating an essential Ser residue on the enzyme. Ibuprofen and naproxen inhibit the same step, probably by mimicking the structure of the substrate or an intermediate in the reaction.

at both of their catalytic centers. COX-1 is responsible for synthesis of the prostaglandins that regulate the secretion of gastric mucin, and COX-2 is responsible for synthesis of the prostaglandins that mediate inflammation, pain, and fever.

Pain can be relieved by inhibiting COX-2. The first drug widely marketed for this purpose was aspirin (acetylsalicylate; Fig. 21-15b). The name "aspirin" (from *a* for acetyl and *spir* for *Spirsaüre*, the German word for the salicylates prepared from the plant *Spiraea ulmaria*) appeared in 1899 when the drug was introduced by the Bayer company. Aspirin irreversibly inactivates the cyclooxygenase activity of both COX isozymes, by acetylating a Ser residue and blocking each enzyme's active site. The synthesis of prostaglandins and thromboxanes is thereby inhibited. Additional widely used *n*onsteroidal *a*nti-*i*nflammatory *d*rugs (NSAIDs; Fig. 21-15b), ibuprofen and naproxen, inhibit the same pair of enzymes. However, the inhibition of COX-1 can result in undesired side effects, including stomach irritation and more serious conditions. In the 1990s, NSAID compounds that had a greater specificity for COX-2 were developed as advanced therapies for severe pain. Three of these drugs were approved for use worldwide: rofecoxib (Vioxx), valdecoxib (Bextra), and celecoxib (Celebrex). Though initially considered a success, Vioxx and Bextra were withdrawn as field reports and clinical studies connected the drugs with an increased risk of heart attack and stroke. Celebrex is still on the market but is being used with increased caution. The detailed reasons for the problems with these drugs are still not clear but serve as a cautionary note. We are increasingly aware of the complexity of the web of these signaling interactions, and predicting the consequences of targeting specific components with pharmaceutical agents remains an imperfect process.

Thromboxane synthase, present in blood platelets (thrombocytes), converts PGH_2 to thromboxane A_2, from which other series 2 **thromboxanes** are derived (Fig. 21-15a). The series 2 thromboxanes induce constriction of blood vessels and platelet aggregation, early steps in blood clotting. Low doses of aspirin, taken regularly, reduce the probability of heart attacks and strokes by reducing thromboxane production. ■

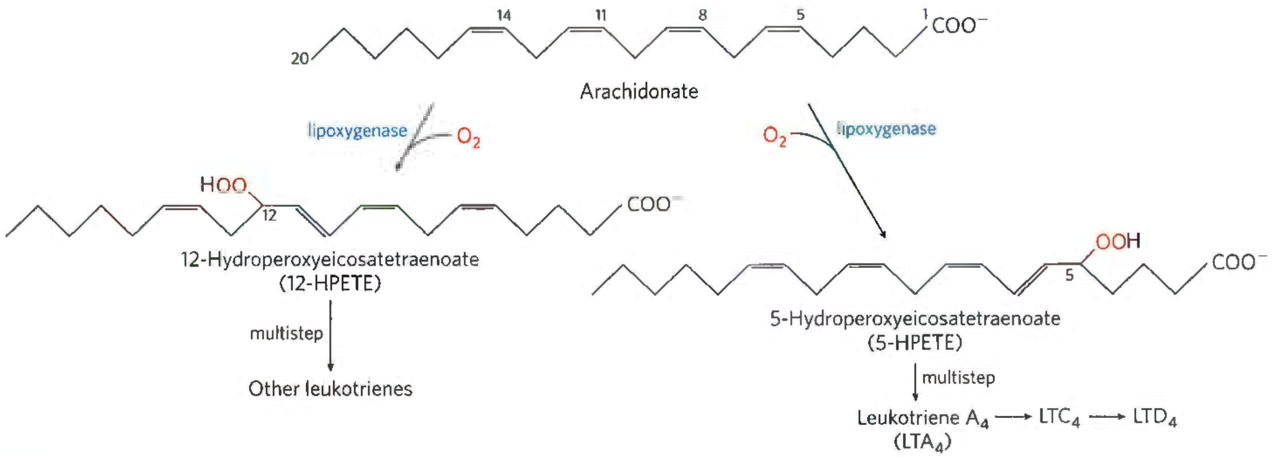


FIGURE 21-16 The "linear" pathway from arachidonate to leukotrienes.

Thromboxanes, like prostaglandins, contain a ring of five or six atoms; the pathway from arachidonate to the series 2 prostaglandins and thromboxanes is sometimes called the "cyclic" pathway, to distinguish it from the "linear" pathway that leads from arachidonate to the **leukotrienes**, which are linear compounds (**Fig. 21-16**). Leukotriene synthesis begins with the action of several lipoxygenases that catalyze the incorporation of molecular oxygen into arachidonate. These enzymes, found in leukocytes and in heart, brain, lung, and spleen, are mixed-function oxidases of the cytochrome P-450 family (see Box 21-1). The various leukotrienes differ in the position of the peroxide group introduced by the lipoxygenases. The linear pathway from arachidonate, unlike the cyclic pathway, is not inhibited by aspirin or other NSAIDs.

Pathogenic organisms, as well as irritants such as air pollution and tobacco smoke, trigger an inflammatory response in the affected tissue, which consists of two phases: initiation and resolution. Eicosanoids of the omega-6 family are critical to initiation—playing key roles in recruiting leukocytes, making blood vessels more permeable, and stimulating chemotaxis and migration of immune system cells. As the source of tissue damage is brought under control, the inflammation must be resolved and the tissue brought back to its normal state. Resolution of inflammation is called **catabasis**, and it is promoted by several classes of signaling molecules; prominent among these are several leukotrienes and prostaglandins. Many eicosanoids of the omega-3 family (including series 3 prostaglandins and thromboxanes) are anti-inflammatory, although the classification is not absolute; individual eicosanoids can be inflammatory in one tissue and anti-inflammatory in another.

Catabasis is also promoted by a set of eicosanoids termed **specialized pro-resolving mediators (SPMs)**. The first family of SPMs to be discovered was the **lipoxins**, followed more recently by resolvins, protectins, and maresins. All SPMs are derived from essential fatty acids (Fig. 21-12). They affect different target cells and tissues in different ways. The sum of their action is to promote removal of debris, microbes, and dead cells, to restore blood vessel integrity, and to regenerate tissue. Particular SPMs also reduce pain and fever, and play roles in resolving the tissue inflammation leading to diabetes, obesity, and asthma. Further research on SPMs thus has potential for the development of new pharmaceutical targets. ■

Plants also derive important signaling molecules from fatty acids. As in animals, a key step in the initiation of signaling is activation of a specific phospholipase. In plants, the fatty acid substrate released by phospholipase action is α-linolenate. A lipoxygenase then catalyzes the first step in a pathway that converts α-linolenate to jasmonate, a substance known to have signaling roles in defense against insects, resistance to fungal pathogens, and maturation of pollen. Jasmonate also affects seed germination, root growth, and fruit and seed development.

SUMMARY 21.1 *Biosynthesis of Fatty Acids and Eicosanoids*

- Malonyl-CoA, a key precursor of fatty acids, is synthesized by the action of acetyl-CoA carboxylase.
- Beginning with malonyl-CoA and acetyl-CoA, fatty acids are synthesized in a repeating cycle of four steps.
- Long-chain saturated fatty acids are synthesized from acetyl-CoA by a cytosolic system of six enzymatic activities plus acyl carrier protein (ACP). There are two types of fatty acid synthase. FAS I, found in vertebrates and fungi, consists of multifunctional polypeptides. FAS II is a dissociated system found in bacteria and plants. Both contain two types of —SH groups (one furnished by the phosphopantetheine of ACP, the other by a Cys residue of β-ketoacyl-ACP synthase) that function as carriers of the fatty acyl intermediates.
- Malonyl-ACP, formed from acetyl-CoA (shuttled out of mitochondria) and CO_2, condenses with an acetyl bound to the Cys —SH to yield acetoacetyl-ACP, with release of CO_2. This is followed by reduction to the D-β-hydroxy derivative, dehydration to the *trans*-Δ^2-unsaturated acyl-ACP, and reduction to butyryl-ACP. NADPH is the

electron donor for both reductions. Fatty acid synthesis is regulated at the level of malonyl-CoA formation.

- Six more molecules of malonyl-ACP react successively at the carboxyl end of the growing fatty acid chain to form palmitoyl-ACP — the end product of the fatty acid synthase reaction. Free palmitate is released by hydrolysis.
- Fatty acid synthesis occurs in the cytosol of animal cells, and in chloroplasts in plants.
- Acetate is exported from the mitochondria as citrate.
- Fatty acid synthesis is tightly regulated, principally by regulation of acetyl-CoA carboxylase.
- Palmitate may be elongated to the 18-carbon stearate.
- Palmitate and stearate can be desaturated to yield palmitoleate and oleate, respectively, by the action of mixed-function oxidases.
- Mammals cannot make linoleate and must obtain it from plant sources; they convert exogenous linoleate to arachidonate, the parent compound of eicosanoids (prostaglandins, thromboxanes, leukotrienes, and specialized pro-resolving mediators), a family of very potent signaling molecules. The synthesis of prostaglandins and thromboxanes is inhibited by NSAIDs that act on the cyclooxygenase activity of prostaglandin H_2 synthase.

21.2 Biosynthesis of Triacylglycerols

P1 Most of the fatty acids synthesized or ingested by an organism have one of two fates, depending on the organism's needs: incorporation into triacylglycerols for the storage of metabolic energy or incorporation into the phospholipid components of membranes. During rapid growth, synthesis of new membranes requires the production of membrane phospholipids; when an organism has a plentiful food supply but is not actively growing, it shunts most of its fatty acids into storage fats. Both pathways begin at the same point: the formation of fatty acyl esters of glycerol. In this section we examine the route to triacylglycerols and its regulation, and the production of glycerol 3-phosphate in the process of glyceroneogenesis.

Triacylglycerols and Glycerophospholipids Are Synthesized from the Same Precursors

Animals can synthesize and store large quantities of triacylglycerols, to be used later as fuel (see Box 17-1). Humans can store only a few hundred grams of glycogen in liver and muscle, barely enough to supply the body's energy needs for 12 hours. However, a 70 kg human stores about 15 kg of triacylglycerol in its tissues, enough to support basal energy needs for as long as 12 weeks (see Table 23-5). Triacylglycerols have the highest energy content of all stored nutrients — more than 38 kJ/g. Whenever carbohydrate is ingested in excess of the organism's capacity to store glycogen, the excess is converted to triacylglycerols and stored in adipose tissue. Plants also manufacture triacylglycerols as an energy-rich fuel, mainly stored in fruits, nuts, and seeds.

In animal tissues, triacylglycerols and glycerophospholipids such as phosphatidylethanolamine share two precursors, fatty acyl–CoA and L-glycerol 3-phosphate, and several biosynthetic steps. The vast majority of the glycerol 3-phosphate is derived from the glycolytic intermediate dihydroxyacetone phosphate (DHAP) by the action of the cytosolic NAD-linked **glycerol 3-phosphate dehydrogenase**; in liver and kidney, a small amount of glycerol 3-phosphate is also formed from glycerol by the action of **glycerol kinase** (Fig. 21-17). The other precursors of triacylglycerols are fatty acyl–CoAs, formed from fatty acids by **acyl-CoA synthetases**, the same enzymes responsible for the activation of fatty acids for β oxidation (see Fig. 17-5).

The first stage in the biosynthesis of triacylglycerols is acylation of the two free hydroxyl groups of L-glycerol 3-phosphate by two molecules of fatty acyl–CoA to yield **diacylglycerol 3-phosphate**, more commonly called **phosphatidic acid**, or phosphatidate (Fig. 21-17). Phosphatidic acid is present in only trace amounts in cells but is a central intermediate in lipid biosynthesis; it can be converted either to a triacylglycerol or to a glycerophospholipid. In the pathway to triacylglycerols, phosphatidic acid is hydrolyzed by **phosphatidic acid phosphatase** (also called lipin) to form a 1,2-diacylglycerol (Fig. 21-18). Diacylglycerols are then converted to triacylglycerols by transesterification with a third fatty acyl–CoA.

Triacylglycerol Biosynthesis in Animals Is Regulated by Hormones

In humans, the amount of body fat stays relatively constant over long periods, although there may be minor short-term changes as caloric intake fluctuates. Biosynthesis and degradation of triacylglycerols are regulated to meet the metabolic requirements of the moment. The rate of triacylglycerol biosynthesis is profoundly altered by the action of several hormones. Insulin, for example, promotes the conversion of carbohydrate to triacylglycerols (Fig. 21-19). People with severe diabetes mellitus, due to failure of insulin secretion or action, not only are unable to use glucose properly but also fail to synthesize fatty acids from carbohydrates or amino acids. If the diabetes is untreated, these individuals have increased rates of fat oxidation and ketone body formation (Chapter 17) and therefore lose weight. ■

Approximately 75% of all fatty acids released by triacylglycerol breakdown (lipolysis) are reesterified to form triacylglycerols rather than used for fuel. This ratio persists even under starvation conditions, when energy metabolism is shunted from the use of carbohydrate to the oxidation of fatty acids. Some of this fatty acid recycling takes place in adipose tissue, with the reesterification occurring before release into the bloodstream; some takes place via a systemic cycle in which free fatty

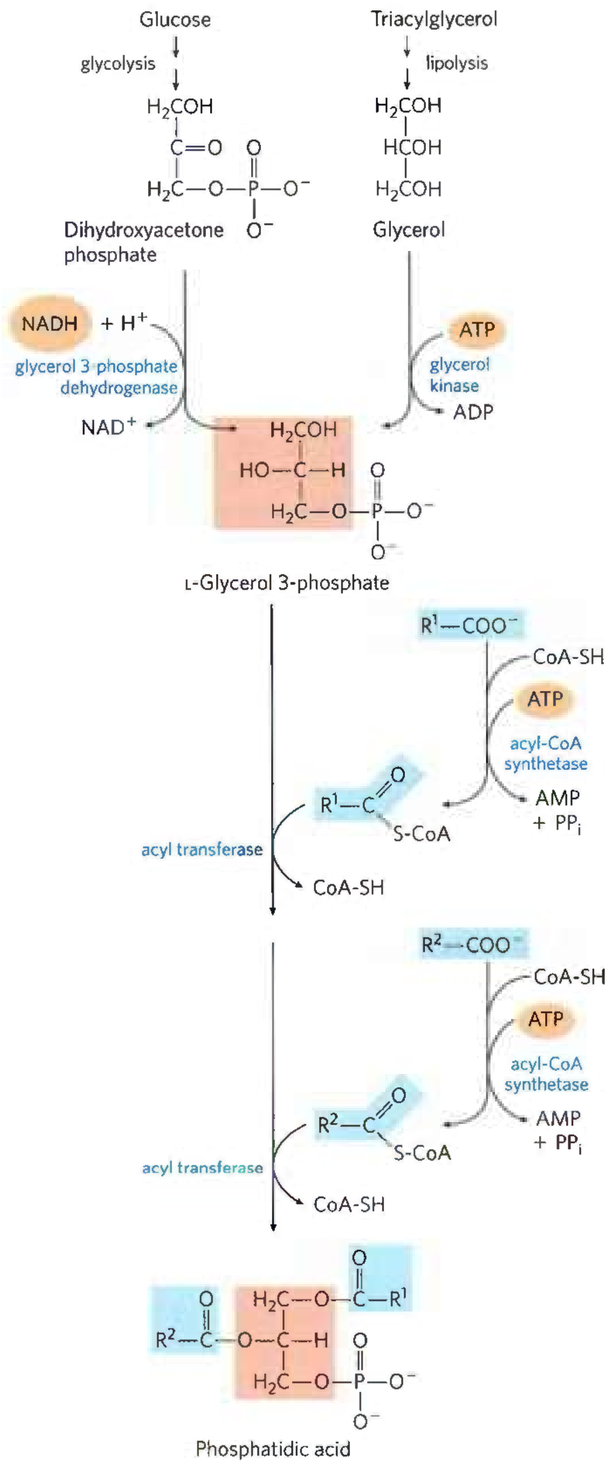

Glucose
glycolysis
H2COH
C=O
H2C—O—P—O−
Dihydroxyacetone phosphate
Triacylglycerol
lipolysis
H2COH
HCOH
H2COH
Glycerol
NADH
+ H+
glycerol 3-phosphate dehydrogenase
NAD+
ATP
glycerol kinase
ADP
H2COH
HO—C—H
H2C—O—P—O−
L-Glycerol 3-phosphate
R1—COO−
CoA-SH
ATP
acyl-CoA synthetase
AMP + PPi
R1—C(=O)S-CoA
acyl transferase
CoA-SH
R2—COO−
CoA-SH
ATP
acyl-CoA synthetase
AMP + PPi
R2—C(=O)S-CoA
acyl transferase
CoA-SH
Phosphatidic acid

FIGURE 21-17 Biosynthesis of phosphatidic acid. A fatty acyl group is activated by formation of the fatty acyl–CoA, then transferred to ester linkage with L-glycerol 3-phosphate, formed in either of the two ways shown. Phosphatidic acid is shown here with the correct stereochemistry (L) at C-2 of the glycerol molecule. (The intermediate product with only one esterified fatty acyl group is lysophosphatidic acid.) To conserve space in subsequent figures (and in Fig. 21-14), both fatty acyl groups of glycerophospholipids, and all three acyl groups of triacylglycerols, are shown projecting to the right.

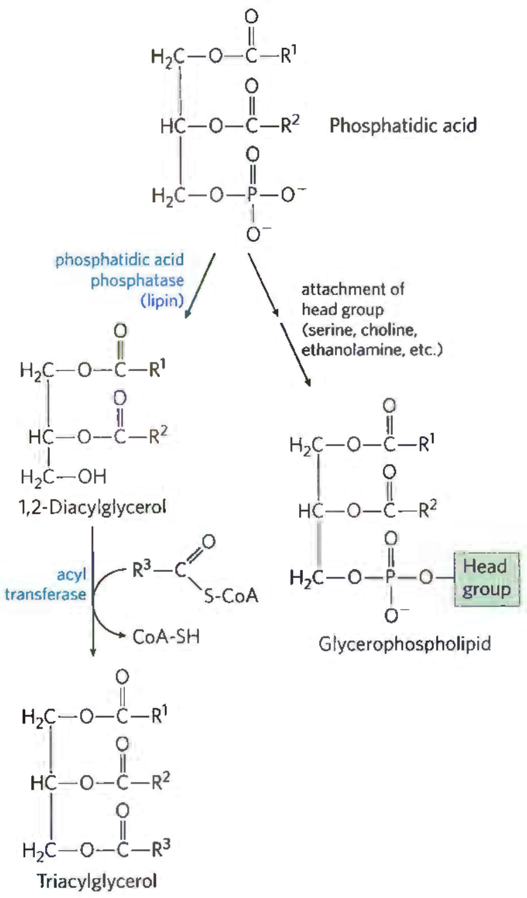

Phosphatidic acid
phosphatidic acid phosphatase (lipin)
attachment of head group (serine, choline, ethanolamine, etc.)
1,2-Diacylglycerol
acyl transferase
R3—C(=O)S-CoA
CoA-SH
Triacylglycerol
Head group
Glycerophospholipid

FIGURE 21-18 Phosphatidic acid in lipid biosynthesis. Phosphatidic acid is the precursor of both triacylglycerols and glycerophospholipids. The mechanisms for head-group attachment in phospholipid synthesis are described later in this section.

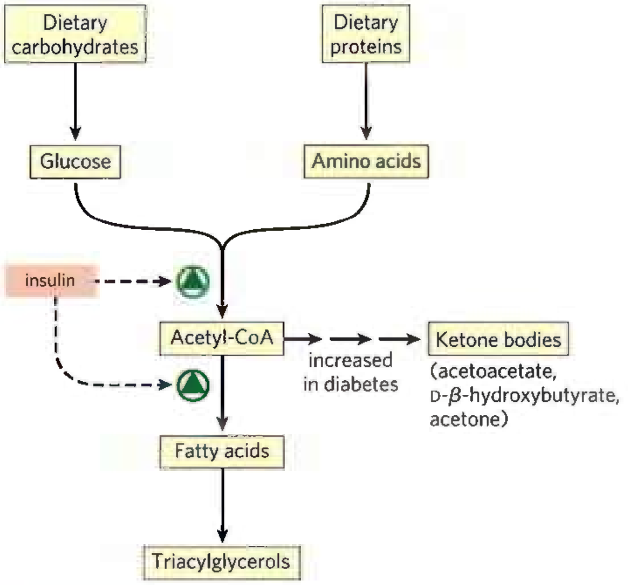

Dietary carbohydrates
Dietary proteins
Glucose
Amino acids
insulin
Acetyl-CoA
increased in diabetes
Ketone bodies
(acetoacetate, D-β-hydroxybutyrate, acetone)
Fatty acids
Triacylglycerols

FIGURE 21-19 Regulation of triacylglycerol synthesis by insulin. Insulin stimulates conversion of dietary carbohydrates and proteins to fat. Individuals with diabetes mellitus either lack insulin or are insensitive to it. This results in diminished fatty acid synthesis, and the acetyl-CoA arising from catabolism of carbohydrates and proteins is shunted instead to ketone body production. People in severe ketosis smell of acetone, so the condition is sometimes mistaken for drunkenness.

acids are transported to the liver, recycled to triacylglycerol, exported again into the blood (transport of lipids in the blood is discussed in Section 21.4), and taken up again by adipose tissue, after release from triacylglycerol by extracellular lipoprotein lipase (**Fig. 21-20**; see also Fig. 17-1). Flux through this **triacylglycerol cycle** between adipose tissue and liver may be low when other fuels are available and the release of fatty acids from adipose tissue is limited, but, as noted above, the proportion of released fatty acids that are reesterified remains roughly constant at 75% under all metabolic conditions. The level of free fatty acids in the blood thus reflects both the rate of release of fatty acids and the balance between the synthesis and breakdown of triacylglycerols in adipose tissue and liver.

When the mobilization of fatty acids is required to meet energy needs, release from adipose tissue is stimulated by the hormones glucagon and epinephrine (see Figs. 17-2, 17-13). Simultaneously, these hormonal signals decrease the rate of glycolysis and increase the rate of gluconeogenesis in the liver (providing glucose for the brain, as further elaborated in Chapter 23). The released fatty acid is taken up by several tissues, including muscle, where it is oxidized to provide energy. Much of the fatty acid taken up by liver is not oxidized but is recycled to triacylglycerol and returned to adipose tissue.

The function of the apparently futile triacylglycerol cycle is not well understood, but as we learn more about how the cycle is sustained via metabolism in two separate organs and is coordinately regulated, some possibilities emerge. For example, the excess capacity in the triacylglycerol cycle—the fatty acid that is eventually reconverted to triacylglycerol rather than oxidized as fuel—could represent an energy reserve in the bloodstream during fasting, one that could be more rapidly mobilized in a "fight or flight" emergency than stored triacylglycerol could be.

The constant recycling of triacylglycerols in adipose tissue even during starvation raises a second question: what is the source of the glycerol 3-phosphate required for this process? As noted above, glycolysis is suppressed under these conditions by the action of glucagon and epinephrine, so little DHAP is available. And glycerol released during lipolysis cannot be converted directly to glycerol 3-phosphate in adipose tissue, which lacks glycerol kinase (Fig. 21-17). So, how is sufficient glycerol 3-phosphate produced? The answer lies in the pathway of glyceroneogenesis, discovered in the 1960s by Lea Reshef, Richard Hanson, and John Ballard, and simultaneously by Eleazar Shafrir and his coworkers. The investigators were intrigued by the presence of two gluconeogenic enzymes, pyruvate carboxylase and phosphoenolpyruvate (PEP) carboxykinase, in adipose tissue, where glucose is not synthesized. Yet, the importance of this pathway was not appreciated until decades later. Glyceroneogenesis is intimately linked to the triacylglycerol cycle and, in a larger sense, to the balance between fatty acid and carbohydrate metabolism.

Adipose Tissue Generates Glycerol 3-Phosphate by Glyceroneogenesis

Glyceroneogenesis is a shortened version of gluconeogenesis, from pyruvate to DHAP (see Fig. 14-16), followed by conversion of the DHAP to glycerol 3-phosphate by cytosolic NAD-linked glycerol 3-phosphate dehydrogenase (**Fig. 21-21**). Glycerol 3-phosphate is subsequently

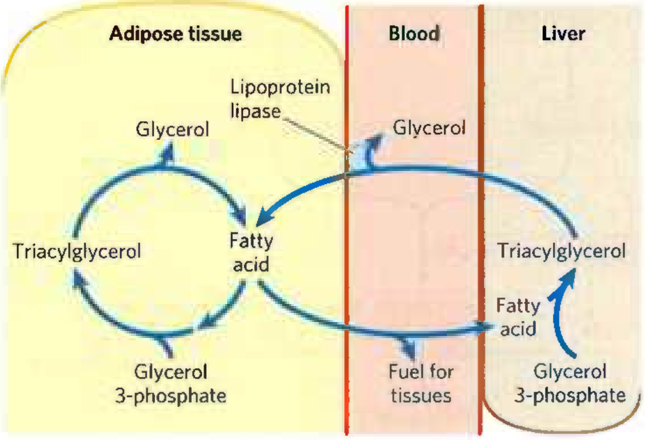


FIGURE 21-20 **The triacylglycerol cycle.** In mammals, triacylglycerol molecules are broken down and resynthesized in a triacylglycerol cycle during starvation. Some of the fatty acids released by lipolysis of triacylglycerol in adipose tissue pass into the bloodstream, and the remainder are used for resynthesis of triacylglycerol. Some of the fatty acids released into the blood are used for energy (in muscle, for example), and some are taken up by the liver and used in triacylglycerol synthesis. The triacylglycerol formed in the liver is transported in the blood back to adipose tissue, where the fatty acid is released by extracellular lipoprotein lipase, taken up by adipocytes, and reesterified into triacylglycerol.

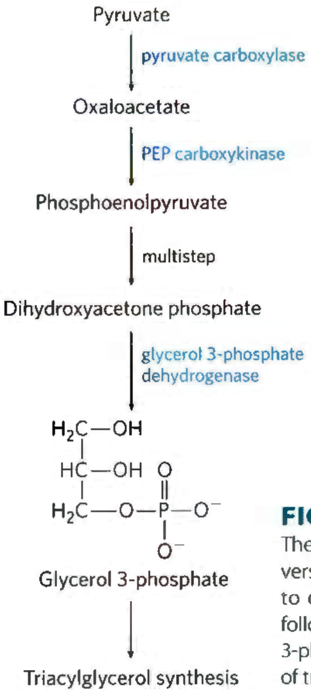
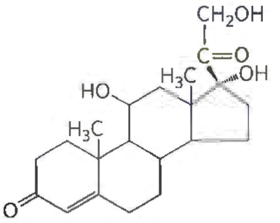


FIGURE 21-21 **Glyceroneogenesis.** The pathway is essentially an abbreviated version of gluconeogenesis, from pyruvate to dihydroxyacetone phosphate (DHAP), followed by conversion of DHAP to glycerol 3-phosphate, which is used for the synthesis of triacylglycerol.

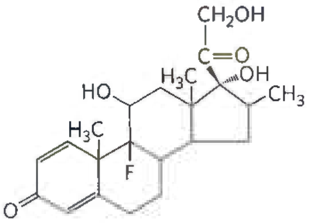
used in triacylglycerol synthesis. There is a link between glyceroneogenesis and type 2 diabetes, as we shall see.

Glyceroneogenesis has multiple roles. In adipose tissue, glyceroneogenesis coupled with reesterification of free fatty acids controls the rate of fatty acid release to the blood. In brown adipose tissue, the same pathway may control the rate at which free fatty acids are delivered to mitochondria for use in thermogenesis. And in fasting humans, glyceroneogenesis in the liver alone supports the synthesis of enough glycerol 3-phosphate to account for up to 65% of fatty acids reesterified to triacylglycerol.

Flux through the triacylglycerol cycle between liver and adipose tissue is controlled to a large degree by the activity of PEP carboxykinase, which limits the rate of both gluconeogenesis and glyceroneogenesis. Glucocorticoid hormones such as cortisol (a biological steroid derived from cholesterol; see Fig. 21-48) and dexamethasone (a synthetic glucocorticoid) regulate the levels of PEP carboxykinase reciprocally in the liver and adipose tissue. Acting through the glucocorticoid receptor, these steroid hormones increase the expression of the gene encoding PEP carboxykinase in the liver, thus increasing gluconeogenesis and glyceroneogenesis (**Fig. 21-22**).


CH₂OH
C=O
H₃C
OH
HO
H₃C
O

Cortisol


CH₂OH
C=O
H₃C
OH
CH₃
HO
H₃C
F
O

Dexamethasone

Stimulation of glyceroneogenesis leads to an increase in the synthesis of triacylglycerol molecules in the liver and their release into the blood. At the same time, glucocorticoids suppress expression of the gene encoding PEP carboxykinase in adipose tissue. This results in a decrease in glyceroneogenesis in adipose tissue; recycling of fatty acids declines as a result, and more free fatty acids are released into the blood. Thus, regulation of glyceroneogenesis in the liver and adipose tissue affects lipid metabolism in opposite ways: a lower rate of glyceroneogenesis in adipose tissue leads to more fatty acid release (rather than recycling), whereas a higher rate in the liver leads to more synthesis and export of triacylglycerols. The net result is an increase in flux through the triacylglycerol cycle. When the glucocorticoids are no longer present, flux through the cycle declines as the expression of PEP carboxykinase increases in adipose tissue and decreases in the liver.

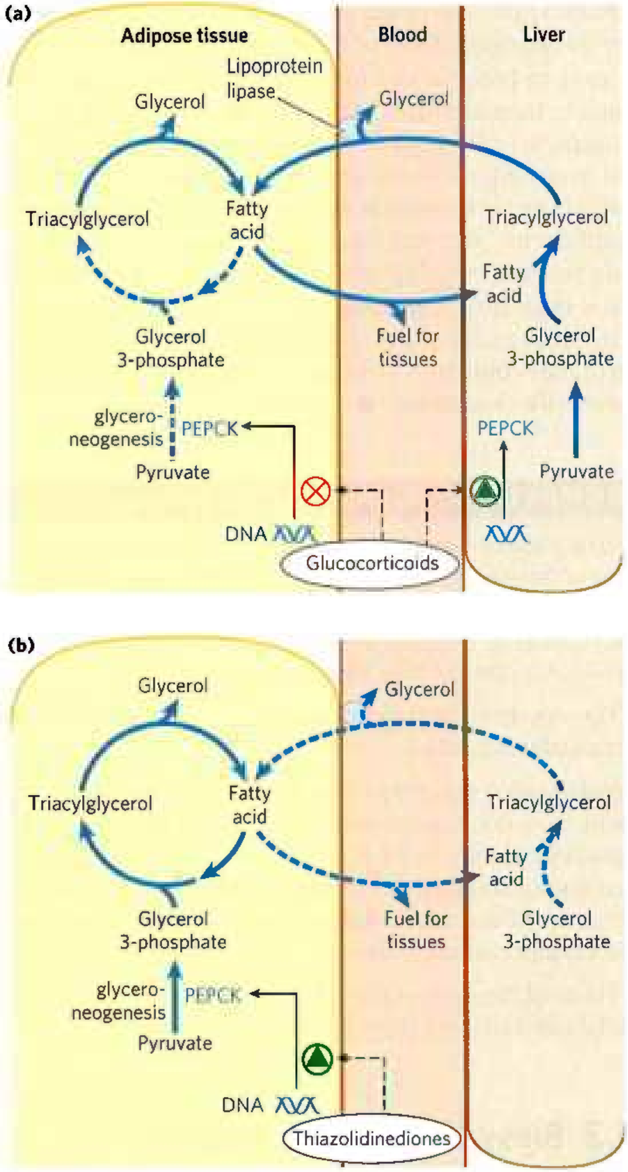

(a)
Adipose tissue
Blood
Liver
Lipoprotein lipase
Glycerol
Glycerol
Triacylglycerol
Fatty acid
Triacylglycerol
Fatty acid
Glycerol 3-phosphate
Fuel for tissues
Glycerol 3-phosphate
glycero-neogenesis
PEPCK
PEPCK
Pyruvate
Pyruvate
DNA
Glucocorticoids
(b)
Glycerol
Glycerol
Triacylglycerol
Fatty acid
Triacylglycerol
Fatty acid
Glycerol 3-phosphate
Fuel for tissues
Glycerol 3-phosphate
glycero-neogenesis
PEPCK
Pyruvate
DNA
Thiazolidinediones

FIGURE 21-22 Regulation of glyceroneogenesis. (a) Glucocorticoid hormones stimulate glyceroneogenesis and gluconeogenesis in the liver, while suppressing glyceroneogenesis in adipose tissue (by reciprocal regulation of the gene expressing PEP carboxykinase (PEPCK) in the two tissues); this increases the flux through the triacylglycerol cycle. The glycerol freed by the breakdown of triacylglycerol in adipose tissue is released to the blood and transported to the liver, where it is primarily converted to glucose, although some is converted to glycerol 3-phosphate by glycerol kinase. (b) A class of drugs called thiazolidinediones is used to treat type 2 diabetes. In this disease, high levels of free fatty acids in the blood interfere with glucose utilization in muscle and promote insulin resistance. Thiazolidinediones activate a nuclear receptor called peroxisome proliferator-activated receptor γ (PPARγ), which induces the activity of PEP carboxykinase. Therapeutically, thiazolidinediones increase the rate of glyceroneogenesis, thus increasing the resynthesis of triacylglycerol in adipose tissue and reducing the amount of free fatty acid in the blood. In both panels, dashed lines identify pathways in which flux declines under the conditions indicated.

Thiazolidinediones Treat Type 2 Diabetes by Increasing Glyceroneogenesis

The connection between glyceroneogenesis and diabetes has stimulated new interest. High levels of free fatty acids in the blood interfere with glucose utilization in muscle and promote the insulin resistance that leads to type 2 diabetes. A class of drugs called **thiazolidinediones** reduces the levels of fatty acids circulating in the blood and increases sensitivity to insulin. Thiazolidinediones promote the increased expression of PEP carboxykinase in adipose tissue (Fig. 21-22), leading to increased synthesis of the precursors of glyceroneogenesis. The therapeutic effect of thiazolidinediones is thus due, at least in part, to the increase in glyceroneogenesis, which in turn increases the resynthesis of triacylglycerol in adipose tissue and reduces the release of free fatty acid from adipose tissue into the blood. Two thiazolidinediones have been available for treatment of type 2 diabetes: rosiglitazone (Avandia) and pioglitazone (Actos). Large-scale trials of rosiglitazone have indicated an increased risk of heart attack, so rosigliatazone has been withdrawn in the United Kingdom, India, South Africa, and many European countries. It remains available in the United States with limitations. ■

SUMMARY 21.2 *Biosynthesis of Triacylglycerols*

- Triacylglycerols are formed by reaction of two molecules of fatty acyl–CoA with glycerol 3-phosphate to form phosphatidic acid; this product is dephosphorylated to a diacylglycerol, then acylated by a third molecule of fatty acyl–CoA to yield a triacylglycerol.
- The synthesis and degradation of triacylglycerols are hormonally regulated.
- Mobilization and recycling of triacylglycerol molecules result in a triacylglycerol cycle. Triacylglycerols are resynthesized from free fatty acids and glycerol 3-phosphate even during starvation. The dihydroxyacetone phosphate precursor of glycerol 3-phosphate is derived from pyruvate via glyceroneogenesis.
- Thiazolidinediones stimulate glyceroneogenesis and can be used to treat type 2 diabetes.

21.3 Biosynthesis of Membrane Phospholipids

In Chapter 10 we introduced two major classes of membrane phospholipids: glycerophospholipids and sphingolipids. Many different phospholipid species can be constructed by combining various fatty acids and polar head groups with the glycerol or sphingosine backbone (see Figs. 10-8, 10-11). All the biosynthetic pathways follow a few basic patterns. In general, the assembly of phospholipids from simple precursors requires (1) synthesis of the backbone molecule (glycerol or sphingosine); (2) attachment of fatty acid(s) to the backbone through an ester or amide linkage; (3) addition of a hydrophilic head group to the backbone through a phosphodiester linkage; and, in some cases, (4) alteration or exchange of the head group or the fatty acids to yield the final phospholipid product.

In eukaryotic cells, phospholipid synthesis occurs primarily on the surfaces of the smooth ER and the inner mitochondrial membrane. Some newly formed phospholipids remain at the site of synthesis, but most are destined for other cellular membranes. Once they arrive, phospholipids can be remodeled within membranes to alter the fatty acid constituents. The process by which water-insoluble phospholipids move from the site of synthesis to the point of their eventual function is not fully understood, but we discuss some mechanisms that have emerged in recent years.

Cells Have Two Strategies for Attaching Phospholipid Head Groups

Stage 1 of glycerophospholipid synthesis is shared with the pathway to triacylglycerols, the formation of glycerol 3-phosphate by one of the two paths shown in Fig. 21-17. In stage 2, fatty acyl groups are esterified to C-1 and C-2 of L-glycerol 3-phosphate to form phosphatidic acid. Usually, the fatty acid at C-1 is saturated and the one at C-2 is unsaturated. A second route to phosphatidic acid is the phosphorylation of a diacylglycerol by a specific kinase.

In stages 3 and 4, the polar head group of glycerophospholipids is attached through a phosphodiester bond, in which each of two alcohol hydroxyls (one on the polar head group and one on C-3 of glycerol) forms an ester with phosphoric acid (**Fig. 21-23**). In the biosynthetic process, one of the hydroxyls is first activated by attachment of a nucleotide, cytidine diphosphate

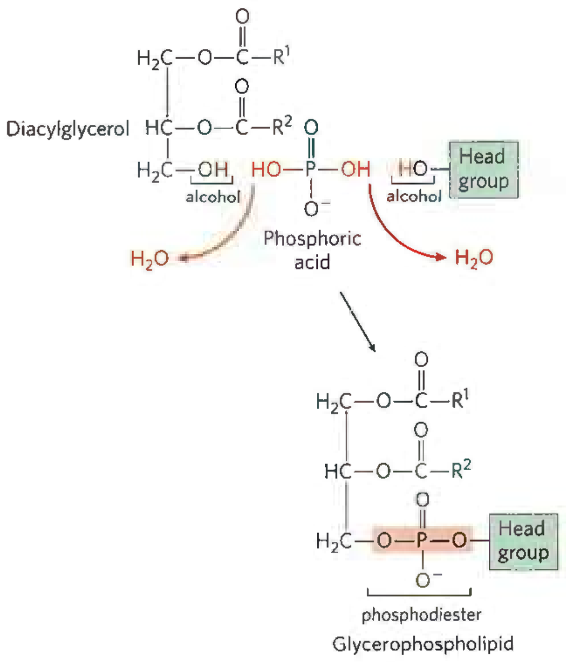


FIGURE 21-23 Final stages of glycerophospholipid biosynthesis: head-group attachment. The phospholipid head group is attached to a diacylglycerol by a phosphodiester bond (shaded light red), formed when phosphoric acid condenses with two alcohols, eliminating two molecules of H_2O.

Eugene P. Kennedy, 1919–2011
[Courtesy of EPK family.]

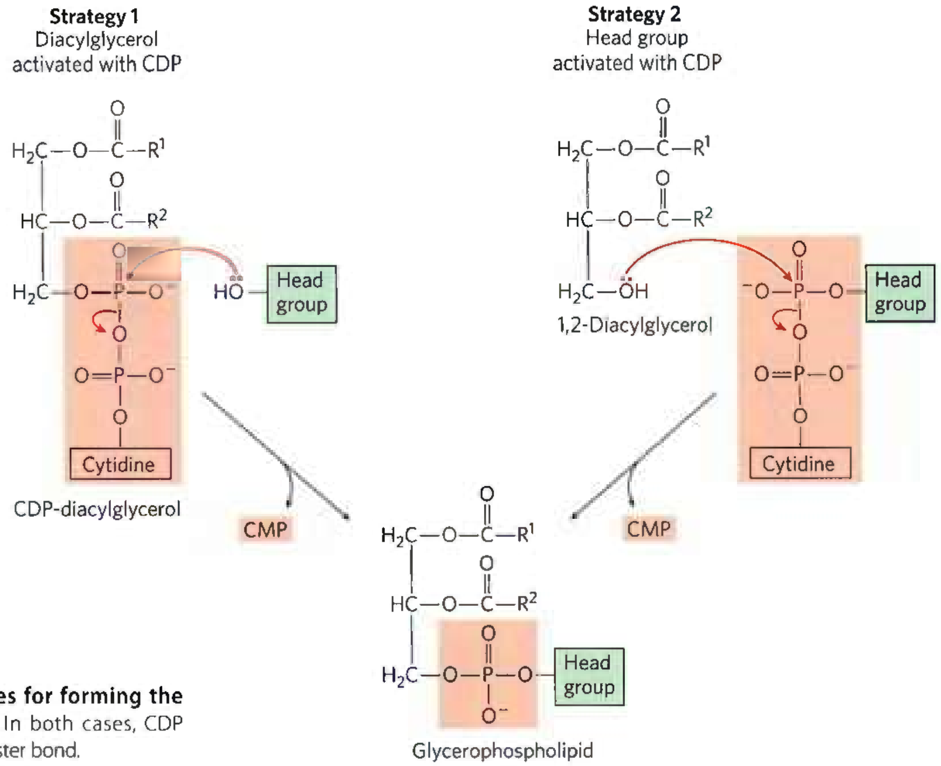


FIGURE 21-24 Two general strategies for forming the phosphodiester bond of phospholipids. In both cases, CDP supplies the phosphate group of the phosphodiester bond.

(CDP). Cytidine monophosphate is then displaced in a nucleophilic attack by the other hydroxyl (**Fig. 21-24**). Two strategies are employed by mammals. The CDP is attached either to the diacylglycerol, forming the activated phosphatidic acid **CDP-diacylglycerol** (strategy 1), or to the hydroxyl of the head group (strategy 2). The central importance of cytidine nucleotides in lipid biosynthesis was discovered by Eugene P. Kennedy in the late 1950s, and this pathway is commonly referred to as the Kennedy pathway. In bacteria, only strategy 1 is used to generate glycerophospholipids.

Pathways for Phospholipid Biosynthesis Are Interrelated

In eukaryotes, many phospholipids are synthesized using strategy 1 in Figure 21-24, and many of the pathways begin with CDP-diacylglycerol. The synthesis of **phosphatidylglycerol** provides our first example. Beginning with CDP-diacylglycerol (**Fig. 21-25**), displacement of CMP through nucleophilic attack by the C-1 hydroxyl of glycerol 3-phosphate yields phosphatidylglycerol 3-phosphate. Phosphatidylglycerol 3-phosphate is processed further by cleavage of the phosphate monoester (with release of P_i) to yield phosphatidylglycerol.

In eukaryotes, **cardiolipin** is a relatively uncommon phospholipid, found almost exclusively in the inner membranes of mitochondria. As described below, cardiolipin is important in bacteria, and its presence in mitochondria is likely yet another relic of the bacterial origin of these organelles. Cardiolipin is essential for the function of some mitochondrial enzymes. It is also synthesized using strategy 1, by condensation of CDP-diacylglycerol with phosphatidylglycerol (Fig. 21-25).

Phosphatidylinositol is similarly synthesized by condensation of CDP-diacylglycerol with inositol (Fig. 21-25). Specific **phosphatidylinositol kinases** then convert phosphatidylinositol to its phosphorylated derivatives. Phosphatidylinositol and its phosphorylated products in the plasma membrane play a central role in signal transduction in eukaryotes (see Figs. 12-11, 12-15, 12-23).

Yeast (but not mammals) use a similar path to produce **phosphatidylserine** by condensation of CDP-diacylglycerol and serine, and they can synthesize **phosphatidylethanolamine** from phosphatidylserine in the reaction catalyzed by phosphatidylserine decarboxylase (Fig. 21-25). This pathway for synthesis of phosphatidylethanolamine occurs primarily in the mitochondria, although this lipid is transported from there to other cellular membranes. Phosphatidylethanolamine may be converted to **phosphatidylcholine** (lecithin) by the addition of three methyl groups to its amino group; *S*-adenosylmethionine is the methyl group donor (see Fig. 18-18) for all three methylation reactions.

In mammals, strategy 2 (Fig. 21-24) is utilized for the synthesis of phosphatidylethanolamine and phosphatidylcholine in the membranes of the ER and nucleus. The activation of the head group to the CDP derivative

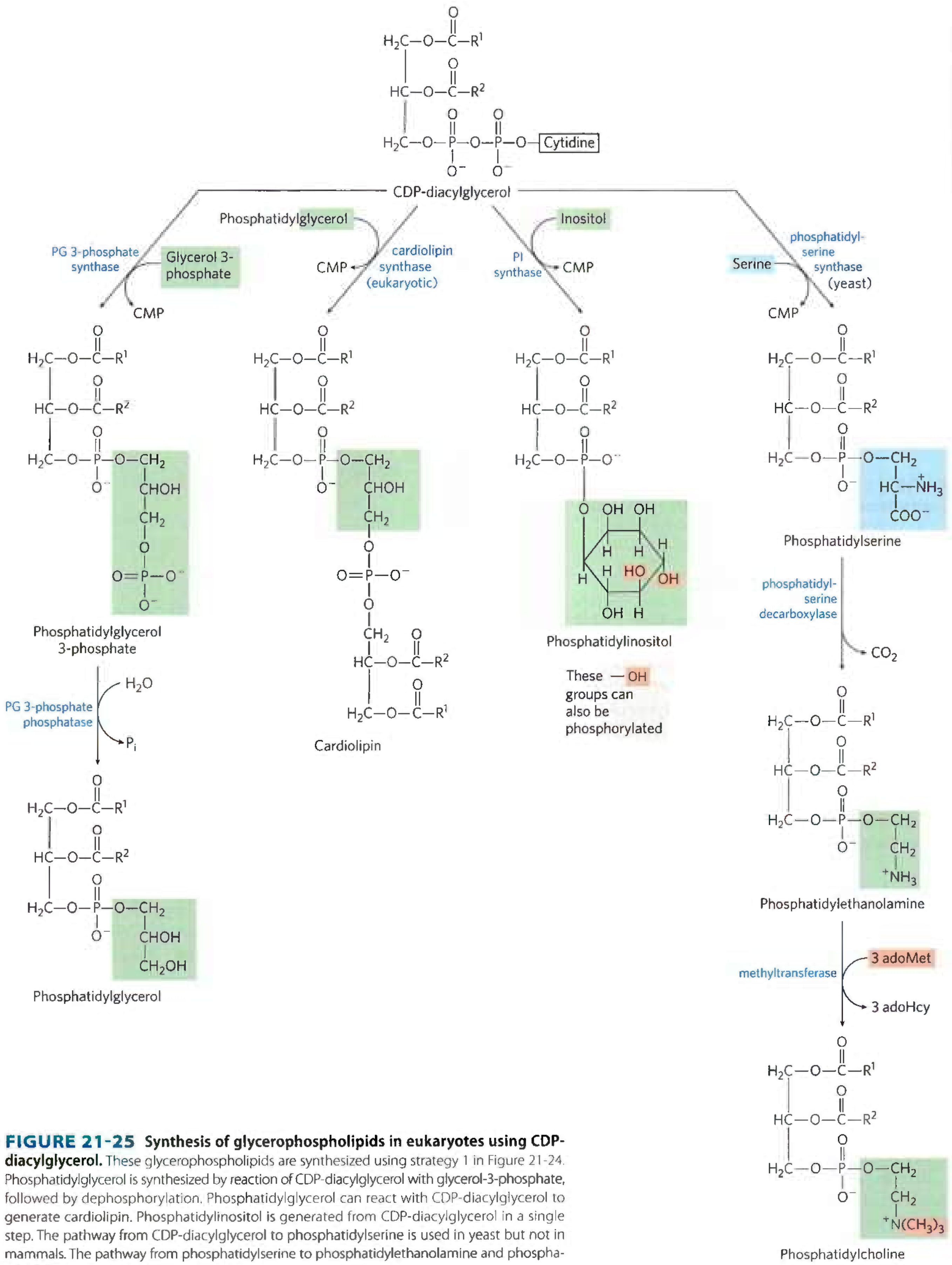
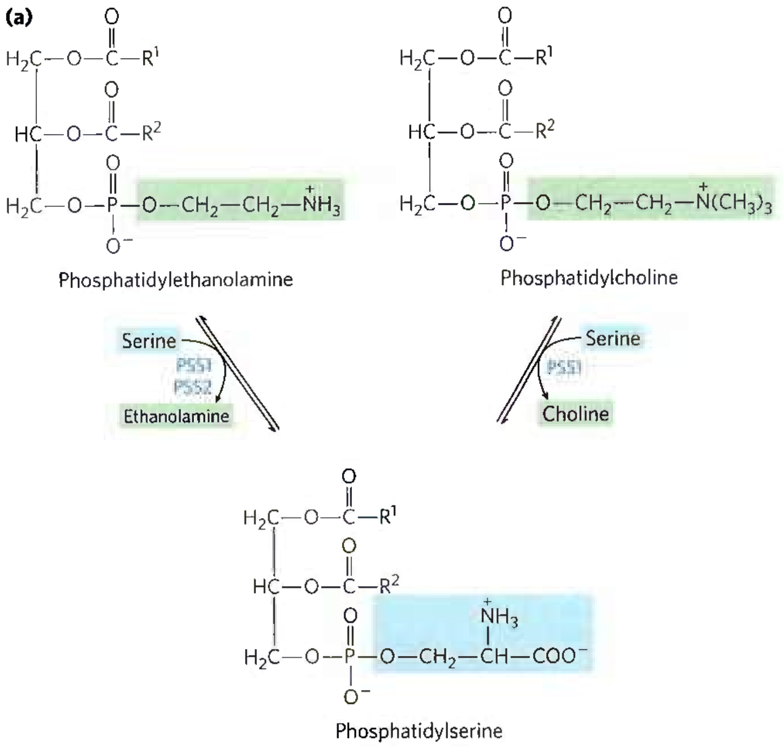

Cytidine
CDP-diacylglycerol
PG 3-phosphate synthase
Glycerol 3-phosphate
CMP
Phosphatidylglycerol 3-phosphate
PG 3-phosphate phosphatase
H2O
Pi
Phosphatidylglycerol
Phosphatidylglycerol
CMP
cardiolipin synthase (eukaryotic)
Cardiolipin
Inositol
PI synthase
CMP
Phosphatidylinositol
These —OH groups can also be phosphorylated
Serine
phosphatidyl-serine synthase (yeast)
CMP
Phosphatidylserine
phosphatidyl-serine decarboxylase
CO2
Phosphatidylethanolamine
methyltransferase
3 adoMet
3 adoHcy
Phosphatidylcholine

FIGURE 21-25 Synthesis of glycerophospholipids in eukaryotes using CDP-diacylglycerol. These glycerophospholipids are synthesized using strategy 1 in Figure 21-24. Phosphatidylglycerol is synthesized by reaction of CDP-diacylglycerol with glycerol-3-phosphate, followed by dephosphorylation. Phosphatidylglycerol can react with CDP-diacylglycerol to generate cardiolipin. Phosphatidylinositol is generated from CDP-diacylglycerol in a single step. The pathway from CDP-diacylglycerol to phosphatidylserine is used in yeast but not in mammals. The pathway from phosphatidylserine to phosphatidylethanolamine and phosphatidylcholine is common to all eukaryotes.


(a)
Phosphatidylethanolamine
Phosphatidylcholine
Serine
PSS1
PSS2
Ethanolamine
Serine
PSS1
Choline
Phosphatidylserine

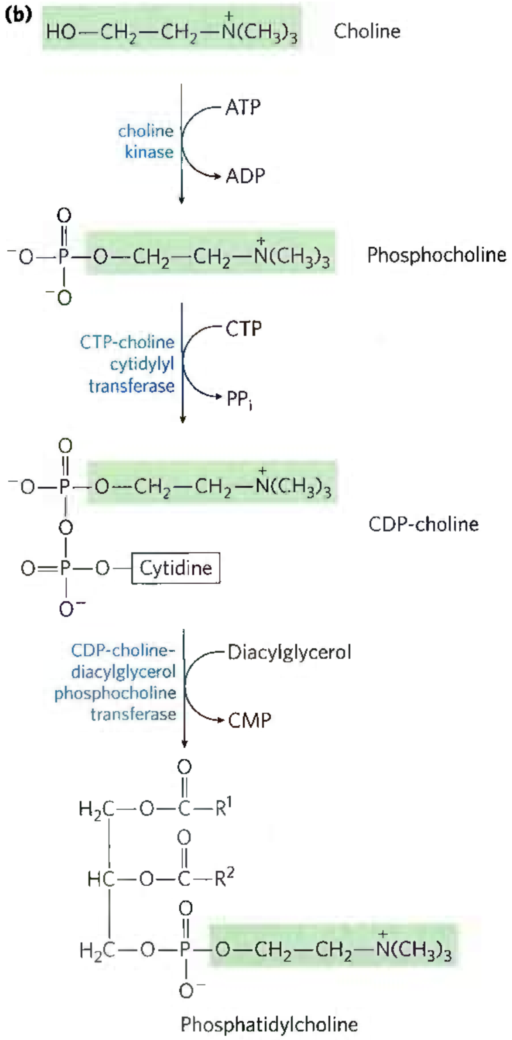

(b)
Choline
choline kinase
ATP
ADP
Phosphocholine
CTP-choline cytidylyl transferase
CTP
PP$_i$
CDP-choline
Cytidine
CDP-choline-diacylglycerol phosphocholine transferase
Diacylglycerol
CMP
Phosphatidylcholine

FIGURE 21-26 Pathways for phosphatidylserine and phosphatidylcholine synthesis in mammals. (a) Phosphatidylserine is synthesized by Ca^{2+}-dependent head-group exchange reactions promoted by phosphatidylserine synthase 1 (PSS1) or phosphatidylserine synthase 2 (PSS2). PSS1 can use either phosphatidylethanolamine or phosphatidylcholine as a substrate. (b) The same strategy shown here for phosphatidylcholine synthesis (strategy 2 in Fig. 21-24) is also used for salvaging ethanolamine in phosphatidylethanolamine synthesis.

is followed by condensation with diacylglycerol as shown for phosphatidylcholine (**Fig. 21-26a**). These pathways serve to salvage free ethanolamine and choline. In contrast, mammalian phosphatidylserine biosynthesis does not utilize either strategy shown in Figure 21-24; instead, it is derived from phosphatidylethanolamine or phosphatidylcholine via one of two head-group exchange reactions carried out in the ER (Fig. 21-26b). These reactions generate free ethanolamine and choline, respectively. The major sources of phosphatidylethanolamine and phosphatidylcholine in all eukaryotic cells are summarized in **Figure 21-27**.

The most prominent phospholipids in bacteria are phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin. The pathway for phosphatidylglycerol synthesis (**Fig. 21-28** on p. 769) is identical to the path employed in mammals (compare to Fig. 21-25), beginning with CDP-diacylglycerol and using strategy 1. Phosphatidylethanolamine is produced in a similar pathway, with phosphatidylserine an intermediate. In bacteria, there are multiple biosynthetic paths to the third prominent phospholipid, cardiolipin, in which two diacylglycerols are joined through a common head group (Fig. 21-28).

Eukaryotic Membrane Phospholipids Are Subject to Remodeling

In principle, the two fatty acyl groups esterified to C-1 and C-2 in a phospholipid may vary in length and degree of desaturation, thus altering the properties of the membrane of which it is a part. Phosphatidylcholine is the major structural phospholipid of mammalian membranes, often representing 40% to 50% of the total. Thus, much of the remodeling centers on phosphatidylcholine. The remodeling occurs largely by a process called the **Lands cycle** (**Fig. 21-29** on p. 770), which replaces the polyunsaturated fatty acyl group at C-2. The fatty acyl moieties are first hydrolyzed by phospholipase A_2 (Fig. 10-14) to generate 1-acyl lysophospholipids. The fatty acid is then replaced by a class of enzymes called **lysophosphatidylcholine acyltransferases**, or **LPCATs**. There are at least four LPCATs in humans, each with distinct tissue distributions and substrate specificities.

The physiological effects of LPCAT enzymes go far beyond the alteration of the lipid composition of membranes. LPCAT3, the most widely distributed version

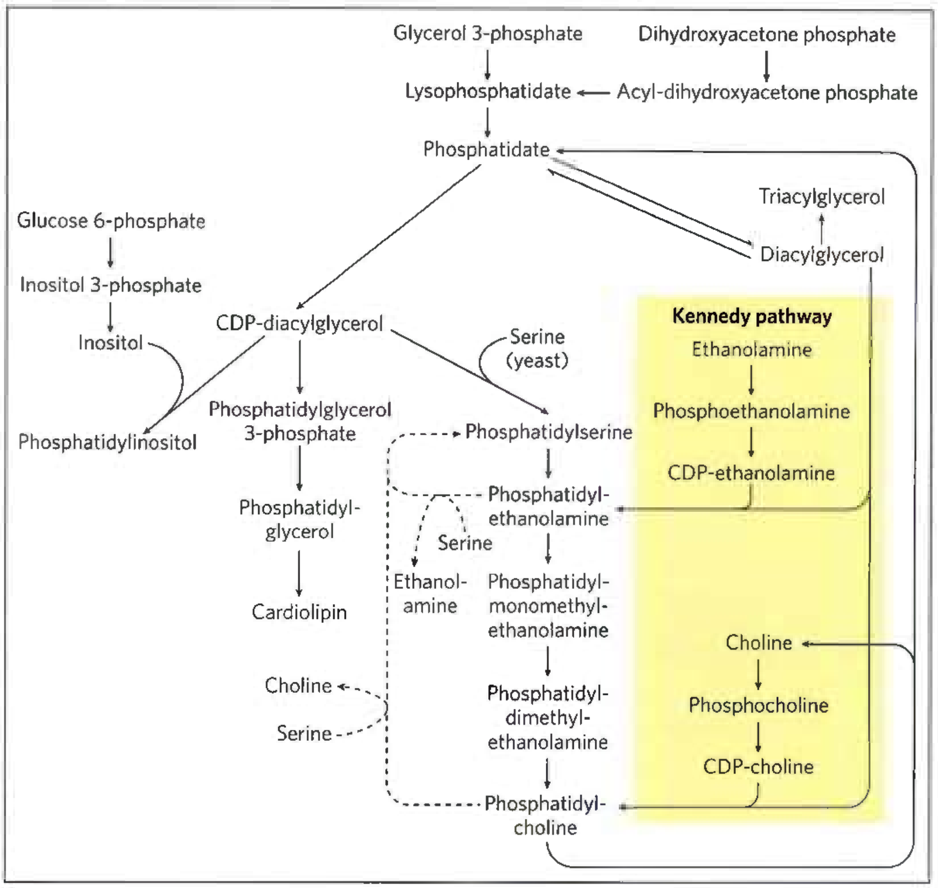

Glycerol 3-phosphate
Dihydroxyacetone phosphate
Lysophosphatidate
Acyl-dihydroxyacetone phosphate
Phosphatidate
Triacylglycerol
Diacylglycerol
Glucose 6-phosphate
Inositol 3-phosphate
Inositol
CDP-diacylglycerol
Serine (yeast)
Phosphatidylinositol
Phosphatidylglycerol 3-phosphate
Phosphatidylserine
Phosphatidylglycerol
Cardiolipin
Phosphatidylethanolamine
Serine
Ethanolamine
Phosphatidylmonomethylethanolamine
Phosphatidyldimethylethanolamine
Phosphatidylcholine
Choline
Serine
Kennedy pathway
Ethanolamine
Phosphoethanolamine
CDP-ethanolamine
Choline
Phosphocholine
CDP-choline

FIGURE 21-27 Summary of the pathways for synthesis of major phospholipids and triacylglycerols in eukaryotes. Phosphatidic acid is formed by transacylation of L-glycerol 3-phosphate with two fatty acyl groups donated from fatty acyl-CoA. The enzyme phosphatidic acid phosphatase (lipin) converts phosphatidic acid to diacylglycerol, which in the Kennedy pathway condenses with a CDP-activated head group (ethanolamine or choline) to form phosphatidylethanolamine or phosphatidylcholine. Alternatively, phosphatidic acid can be activated with a CDP moiety, which is displaced by condensation with a head-group alcohol—inositol, glycerol 3-phosphate, or serine, forming phosphatidylinositol, phosphatidylglycerol, or (only in yeast and fungi) phosphatidylserine. Decarboxylation of phosphatidylserine yields phosphatidylethanolamine, and methylation of phosphatidylethanolamine produces phosphatidylcholine. In mammals, phosphatidylserine and phosphatidylcholine are generated via the head-group exchange pathways, detailed in Fig. 21-26. Lysophosphatidic acid is phosphatidic acid missing one of the two fatty acyl groups. [Information from G. M. Carman and G.-S. Han, *Annu. Rev. Biochem.* 80:859, 2011, Fig. 2.]

of the enzyme, helps to regulate lipogenesis and secretion of very-low-density lipoproteins (VLDLs), described later in this chapter. Mice lacking LPCAT3 have a greatly reduced intake of fatty acids in the intestine, resulting in the release of gut hormones that control appetite. They are unable to survive on a high-fat diet, they resist eating, and they die of starvation unless the diet is changed. LPCATs play a demonstrable but still often mysterious role in processes from atherosclerosis to obesity to cancer, making these enzymes the subjects of increasing interest as research and drug targets. ■

Plasmalogen Synthesis Requires Formation of an Ether-Linked Fatty Alcohol

P5 The biosynthetic pathway to ether lipids, including **plasmalogens** and the **platelet-activating factor** (see Fig. 10-9), requires displacement of an esterified fatty acyl group by a long-chain alcohol to form the ether linkage (**Fig. 21-30** on p. 771). Head-group attachment follows, by mechanisms essentially like those used in formation of the common ester-linked phospholipids. Finally, the characteristic double bond of plasmalogens is introduced by the action of a mixed-function oxidase similar to that responsible for desaturation of fatty acids (Fig. 21-13). The peroxisome is the primary site of plasmalogen synthesis.

Sphingolipid and Glycerophospholipid Synthesis Share Precursors and Some Mechanisms

P5 The biosynthesis of sphingolipids takes place in four stages: (1) synthesis of the 18-carbon amine **sphinganine** from palmitoyl-CoA and serine; (2) attachment of a fatty acid in amide linkage to yield ***N*-acylsphinganine**; (3) desaturation of the sphinganine moiety to form

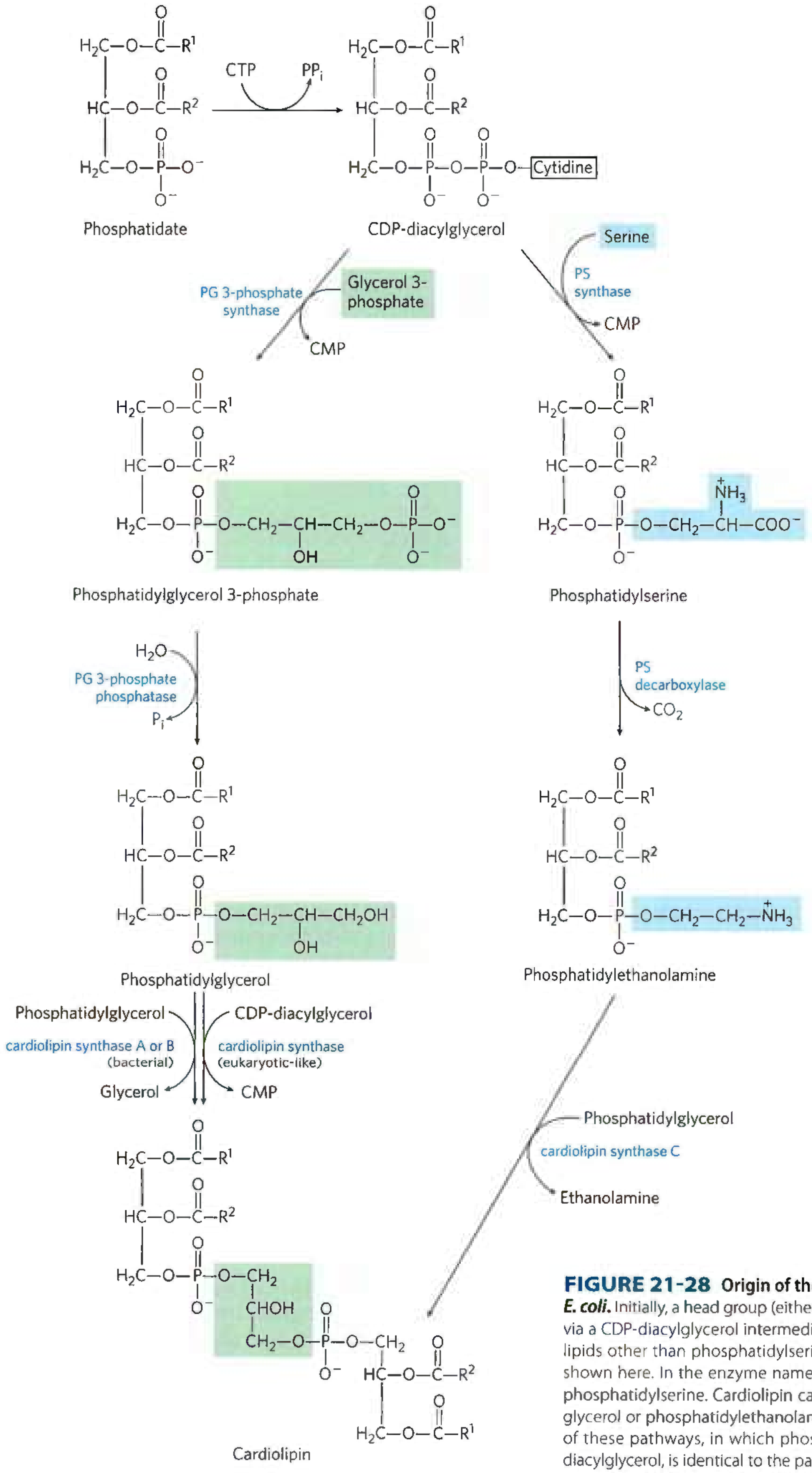

Phosphatidate
CTP
PP$_i$
CDP-diacylglycerol
Cytidine
PG 3-phosphate synthase
Glycerol 3-phosphate
CMP
Serine
PS synthase
CMP
Phosphatidylglycerol 3-phosphate
Phosphatidylserine
H$_2$O
PG 3-phosphate phosphatase
P$_i$
PS decarboxylase
CO$_2$
Phosphatidylglycerol
Phosphatidylethanolamine
Phosphatidylglycerol
CDP-diacylglycerol
cardiolipin synthase A or B (bacterial)
cardiolipin synthase (eukaryotic-like)
Glycerol
CMP
Phosphatidylglycerol
cardiolipin synthase C
Ethanolamine
Cardiolipin

FIGURE 21-28 Origin of the polar head groups of phospholipids in *E. coli*. Initially, a head group (either serine or glycerol 3-phosphate) is attached via a CDP-diacylglycerol intermediate (strategy 1 in Fig. 21-24). For phospholipids other than phosphatidylserine, the head group is further modified, as shown here. In the enzyme names, PG represents phosphatidylglycerol; PS, phosphatidylserine. Cardiolipin can be generated from either phosphatidylglycerol or phosphatidylethanolamine via multiple pathways, as shown. One of these pathways, in which phosphatidylglycerol is condensed with CDP-diacylglycerol, is identical to the pathway used in eukaryotes.

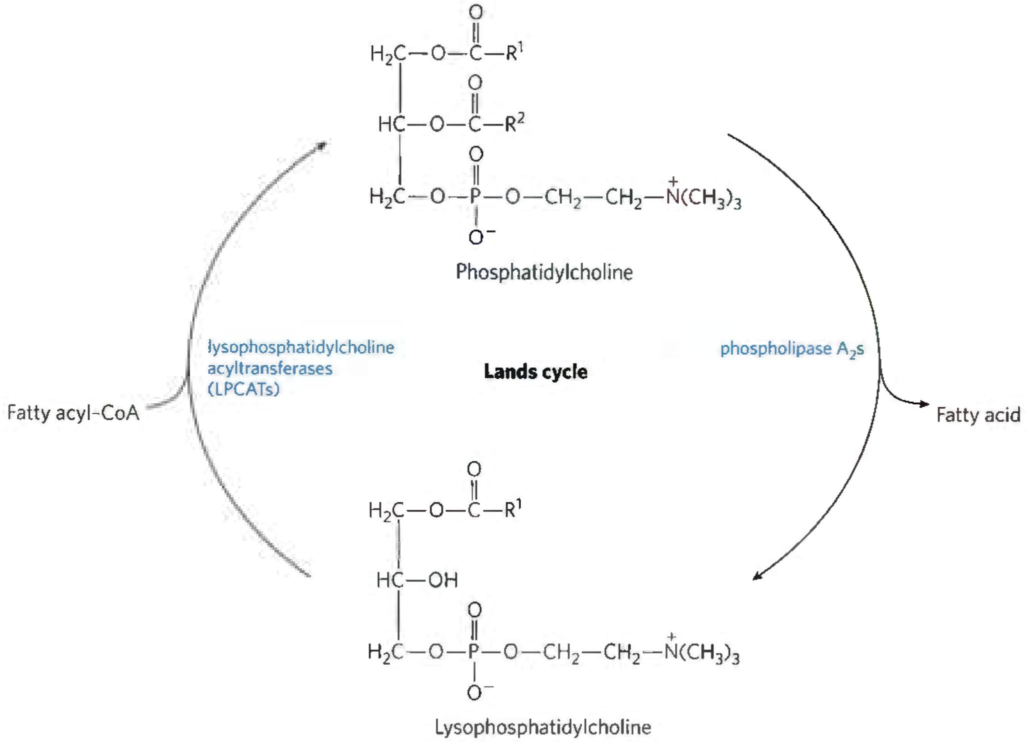


FIGURE 21-29 The Lands cycle for phospholipid remodeling. Phosphatidylcholine, which is the most common phospholipid of eukaryotic membranes, is a major target of this process. Fatty acids at C-2 are removed by phospholipase A_2 enzymes. A new fatty acid is then introduced by the action of a lysophosphatidylphocholine acyltransferase (LPCAT). The four mammalian LPCATs differ in tissue distribution and substrate specificity.

***N*-acylsphingosine** (ceramide); and (4) attachment of a head group to produce a sphingolipid such as a **cerebroside** or **sphingomyelin** (Fig. 21-31 on p. 772). The first few steps of this pathway occur in the ER; the attachment of head groups in stage 4 occurs in the Golgi complex. The pathway shares several features with the pathways leading to glycerophospholipids: NADPH provides reducing power, and fatty acids enter as their activated CoA derivatives. In cerebroside formation, sugars enter as their activated nucleotide derivatives. Head-group attachment in sphingolipid synthesis has several novel aspects. For example, phosphatidylcholine, rather than CDP-choline, serves as the donor of phosphocholine in the synthesis of sphingomyelin.

P5 In glycolipids—the cerebrosides and **gangliosides** (see Fig. 10-11)—the head-group sugar is attached directly to the C-1 hydroxyl of sphingosine in glycosidic linkage rather than through a phosphodiester bond. The sugar donor is a UDP-sugar (UDP-glucose or UDP-galactose).

Polar Lipids Are Targeted to Specific Cellular Membranes

Membrane lipids are insoluble in water, so they cannot simply diffuse from their point of synthesis (the ER) to their point of insertion. Instead, they are transported from the ER to the Golgi complex, where additional synthesis can take place. They are then delivered in membrane vesicles that bud from the Golgi complex and then move to and fuse with the target membrane (see Fig. 11-4). Sphingolipid transfer proteins carry ceramide from the ER to the Golgi complex, where sphingomyelin synthesis occurs. Cytosolic proteins also bind phospholipids and sterols and transport them between cellular membranes (see Fig. 11-7). These mechanisms contribute to establishment of the characteristic lipid compositions of organelle membranes (see Fig. 11-5).

SUMMARY 21.3 *Biosynthesis of Membrane Phospholipids*

- Beginning with diacylglycerol precursors, there are two pathways for adding head groups to phospholipids. Either the diacylglycerol (strategy 1) or the head group (strategy 2) is activated by CDP.
- In eukaryotes, phospholipid biosynthetic strategies vary with subcellular location. The major phospholipids phosphatidylethanolamine and phosphatidylcholine are synthesized using strategy 1 in the mitochondria and strategy 2 in the ER and nucleus. Phosphatidylserine is derived from head-group exchange with phosphatidylethanolamine or phosphatidylcholine.

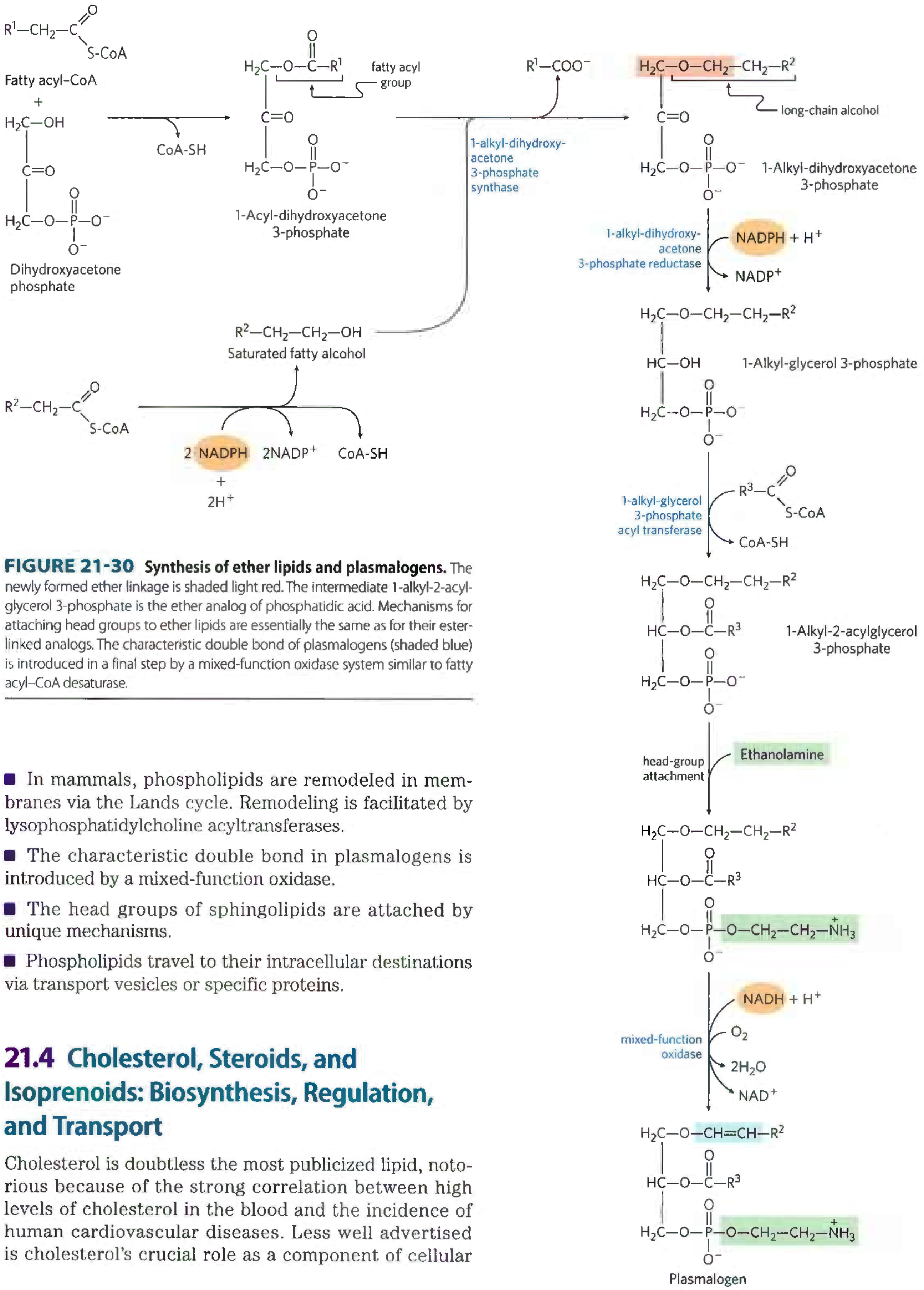

Fatty acyl-CoA
Dihydroxyacetone phosphate
CoA-SH
fatty acyl group
1-Acyl-dihydroxyacetone 3-phosphate
1-alkyl-dihydroxy-acetone 3-phosphate synthase
long-chain alcohol
1-Alkyl-dihydroxyacetone 3-phosphate
Saturated fatty alcohol
2 NADPH + 2H+
2NADP+
CoA-SH
1-alkyl-dihydroxy-acetone 3-phosphate reductase
NADPH + H+
NADP+
1-Alkyl-glycerol 3-phosphate
1-alkyl-glycerol 3-phosphate acyl transferase
CoA-SH
1-Alkyl-2-acylglycerol 3-phosphate
head-group attachment
Ethanolamine
mixed-function oxidase
NADH + H+
O2
2H2O
NAD+
Plasmalogen

FIGURE 21-30 Synthesis of ether lipids and plasmalogens. The newly formed ether linkage is shaded light red. The intermediate 1-alkyl-2-acylglycerol 3-phosphate is the ether analog of phosphatidic acid. Mechanisms for attaching head groups to ether lipids are essentially the same as for their ester-linked analogs. The characteristic double bond of plasmalogens (shaded blue) is introduced in a final step by a mixed-function oxidase system similar to fatty acyl–CoA desaturase.

- In mammals, phospholipids are remodeled in membranes via the Lands cycle. Remodeling is facilitated by lysophosphatidylcholine acyltransferases.
- The characteristic double bond in plasmalogens is introduced by a mixed-function oxidase.
- The head groups of sphingolipids are attached by unique mechanisms.
- Phospholipids travel to their intracellular destinations via transport vesicles or specific proteins.

21.4 Cholesterol, Steroids, and Isoprenoids: Biosynthesis, Regulation, and Transport

Cholesterol is doubtless the most publicized lipid, notorious because of the strong correlation between high levels of cholesterol in the blood and the incidence of human cardiovascular diseases. Less well advertised is cholesterol's crucial role as a component of cellular

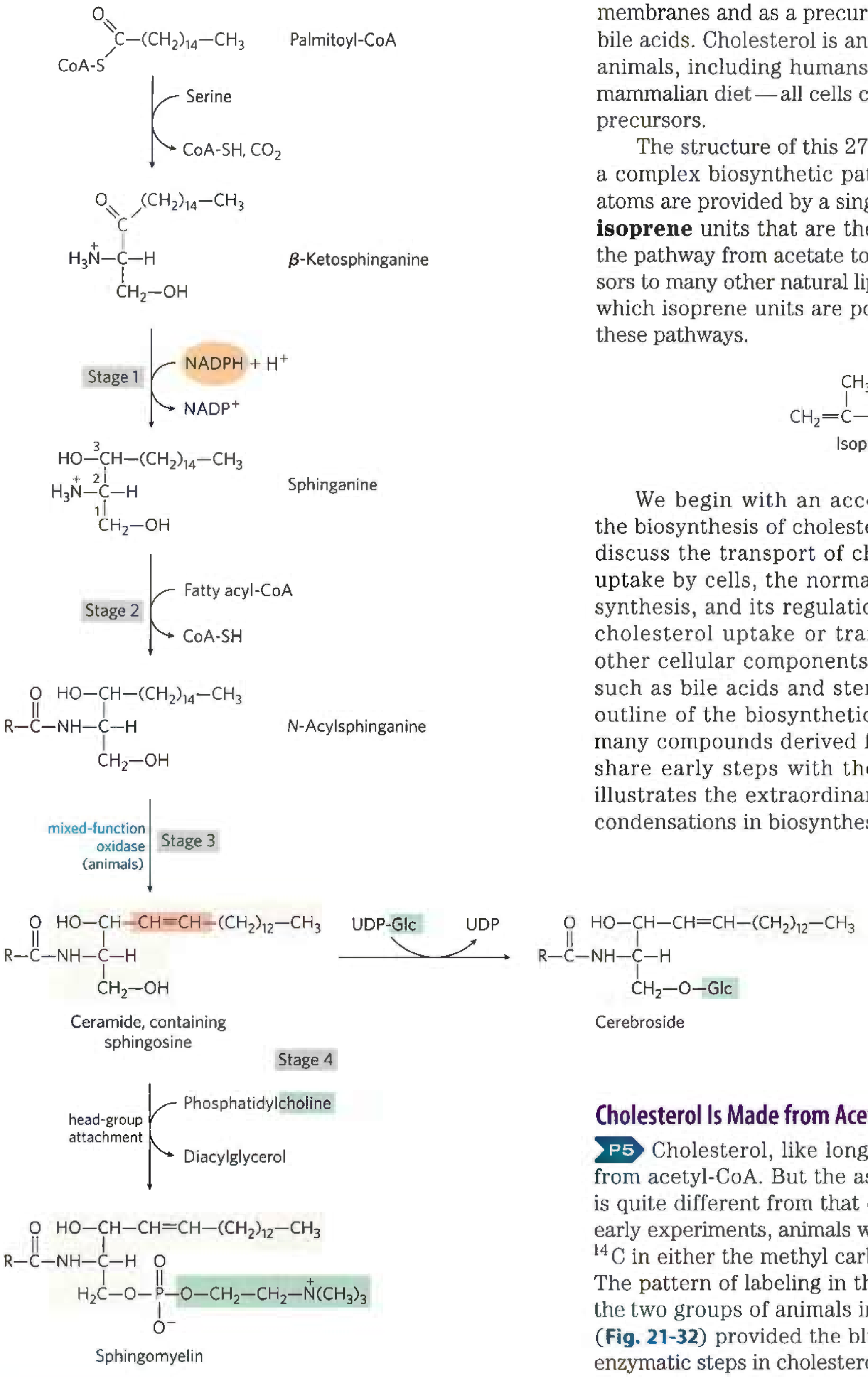
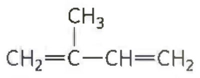

Palmitoyl-CoA
Serine
CoA-SH, CO2
β-Ketosphinganine
Stage 1
NADPH + H+
NADP+
Sphinganine
Stage 2
Fatty acyl-CoA
CoA-SH
N-Acylsphinganine
mixed-function oxidase (animals)
Stage 3
UDP-Glc
UDP
Ceramide, containing sphingosine
Cerebroside
Stage 4
head-group attachment
Phosphatidylcholine
Diacylglycerol
Sphingomyelin

FIGURE 21-31 Biosynthesis of sphingolipids. Condensation of palmitoyl-CoA and serine, forming β-ketosphinganine, followed by reduction with NADPH, yields sphinganine, which is then acylated to *N*-acylsphinganine (a ceramide). The sphingosine is shaded gray. In animals, a double bond (shaded light red) is created by a mixed-function oxidase before the final addition of a head group: phosphatidylcholine, to form sphingomyelin, or glucose, to form a cerebroside.

membranes and as a precursor of steroid hormones and bile acids. Cholesterol is an essential molecule in many animals, including humans, but is not required in the mammalian diet—all cells can synthesize it from simple precursors.

The structure of this 27-carbon compound suggests a complex biosynthetic pathway, but all of its carbon atoms are provided by a single precursor—acetate. The **isoprene** units that are the essential intermediates in the pathway from acetate to cholesterol are also precursors to many other natural lipids, and the mechanisms by which isoprene units are polymerized are similar in all these pathways.



$$CH_2{=}\overset{\displaystyle CH_3}{\overset{|}{C}}{-}CH{=}CH_2$$

Isoprene

We begin with an account of the main steps in the biosynthesis of cholesterol from acetate, and then discuss the transport of cholesterol in the blood, its uptake by cells, the normal regulation of cholesterol synthesis, and its regulation in those with defects in cholesterol uptake or transport. We next consider other cellular components derived from cholesterol, such as bile acids and steroid hormones. Finally, an outline of the biosynthetic pathways to some of the many compounds derived from isoprene units, which share early steps with the pathway to cholesterol, illustrates the extraordinary versatility of isoprenoid condensations in biosynthesis.

Cholesterol Is Made from Acetyl-CoA in Four Stages

P5 Cholesterol, like long-chain fatty acids, is made from acetyl-CoA. But the assembly plan of cholesterol is quite different from that of long-chain fatty acids. In early experiments, animals were fed acetate labeled with ^{14}C in either the methyl carbon or the carboxyl carbon. The pattern of labeling in the cholesterol isolated from the two groups of animals in these tracer experiments (**Fig. 21-32**) provided the blueprint for working out the enzymatic steps in cholesterol biosynthesis.

Synthesis takes place in four stages, as shown in **Figure 21-33**: ① condensation of three acetate units to form a six-carbon intermediate, mevalonate; ② conversion of mevalonate to activated isoprene units; ③ polymerization of six 5-carbon isoprene units to form the 30-carbon linear squalene; and ④ cyclization of squalene to form the

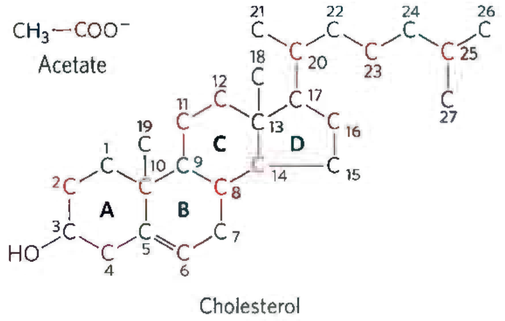

CH3—COO−
Acetate
21
22
24
26
18
20
23
25
12
17
27
11
13
16
19
C
D
1
10
9
14
15
2
8
A
B
3
7
HO
5
4
6
Cholesterol

FIGURE 21-32 Origin of the carbon atoms of cholesterol. This was deduced from tracer experiments with acetate labeled in the methyl carbon (black) or the carboxyl carbon (red). The individual rings in the fused-ring system are designated A through D.

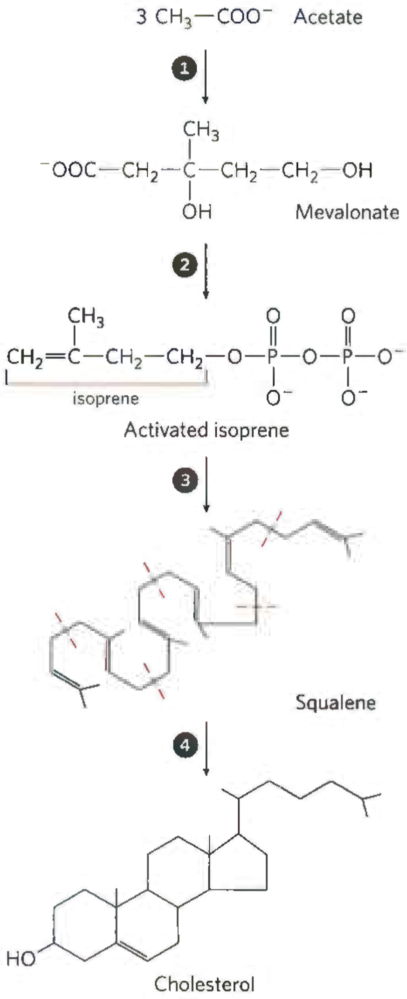

3 CH3—COO− Acetate
1
CH3
−OOC—CH2—C—CH2—CH2—OH
OH
Mevalonate
2
CH3
O
O
CH2=C—CH2—CH2—O—P—O—P—O−
isoprene
O−
O−
Activated isoprene
3
Squalene
4
HO
Cholesterol

FIGURE 21-33 Summary of cholesterol biosynthesis. Isoprene units in squalene are set off by dashed red lines.

four rings of the steroid nucleus, with a further series of changes (oxidations, removal or migration of methyl groups) to produce cholesterol.

Stage ❶ Synthesis of Mevalonate from Acetate The first stage in cholesterol biosynthesis leads to the intermediate **mevalonate** (**Fig. 21-34**). Two molecules of acetyl-CoA condense to form acetoacetyl-CoA, which condenses with a third molecule of acetyl-CoA to yield the six-carbon compound **β-hydroxy-β-methylglutaryl-CoA (HMG-CoA)**. These first two reactions are catalyzed by **acetyl-CoA acetyl transferase** and **HMG-CoA synthase**, respectively. Both reactions are Claisen condensations, and the standard equilibrium in each case favors degradation to acetyl-CoA. However, in cells, the synthetic reactions are facilitated by the rapid utilization of the product HMG-CoA in subsequent reactions. The cytosolic HMG-CoA synthase in this pathway is distinct from the mitochondrial isozyme that catalyzes HMG-CoA synthesis in ketone body formation (see Fig. 17-16).

P3 The third reaction is the committed step: reduction of HMG-CoA to mevalonate, for which two molecules of NADPH each donate two electrons. **HMG-CoA reductase**, an integral membrane protein of the smooth ER, is the major point of regulation on the pathway to cholesterol, as we shall see.

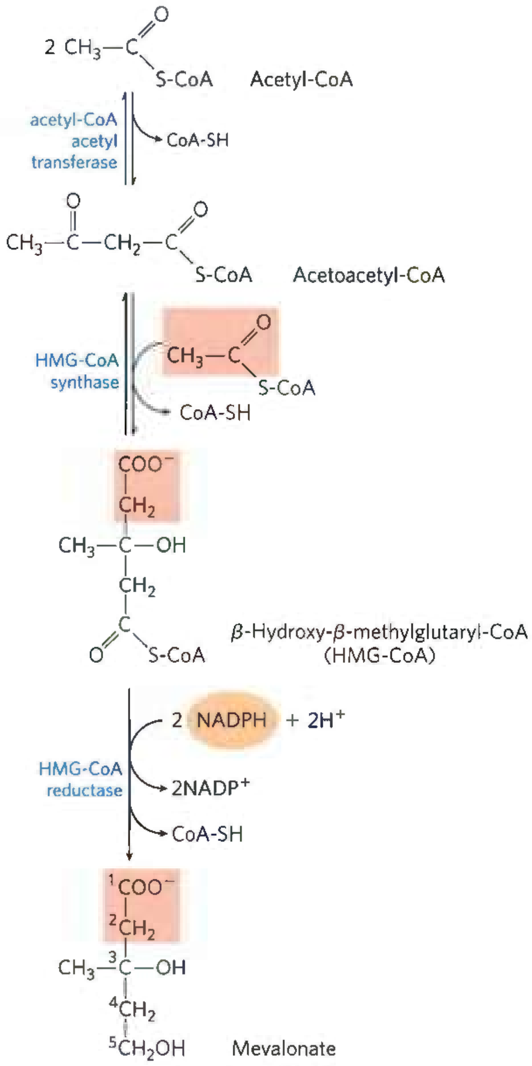

2 CH3—C(=O)—S-CoA
Acetyl-CoA
acetyl-CoA acetyl transferase
CoA-SH
CH3—C(=O)—CH2—C(=O)—S-CoA
Acetoacetyl-CoA
HMG-CoA synthase
CH3—C(=O)—S-CoA
CoA-SH
COO−
CH2
CH3—C—OH
CH2
C
O
S-CoA
β-Hydroxy-β-methylglutaryl-CoA (HMG-CoA)
2 NADPH + 2H+
HMG-CoA reductase
2NADP+
CoA-SH
1COO−
2CH2
CH3—3C—OH
4CH2
5CH2OH
Mevalonate

FIGURE 21-34 Formation of mevalonate from acetyl-CoA. The origin of C-1 and C-2 of mevalonate from acetyl-CoA is shaded light red.

Stage ❷ Conversion of Mevalonate to Two Activated Isoprenes In the next stage, three phosphate groups are transferred from three ATP molecules to mevalonate (**Fig. 21-35**). The phosphate attached to the C-3 hydroxyl group of mevalonate in the intermediate 3-phospho-5-pyrophosphomevalonate is a good leaving group; in the next step, both this phosphate and the nearby carboxyl group leave, producing a double bond in the five-carbon product, **Δ^3-isopentenyl pyrophosphate**. This is the first of the two activated isoprenes central to cholesterol formation. Isomerization of Δ^3-isopentenyl pyrophosphate yields the second activated isoprene, **dimethylallyl pyrophosphate**. Synthesis of isopentenyl pyrophosphate in the cytoplasm of plant cells follows the pathway described here. However, plant chloroplasts and many bacteria use a mevalonate-independent pathway. This alternative pathway does not occur in animals, so it is an attractive target for the development of new antibiotics.

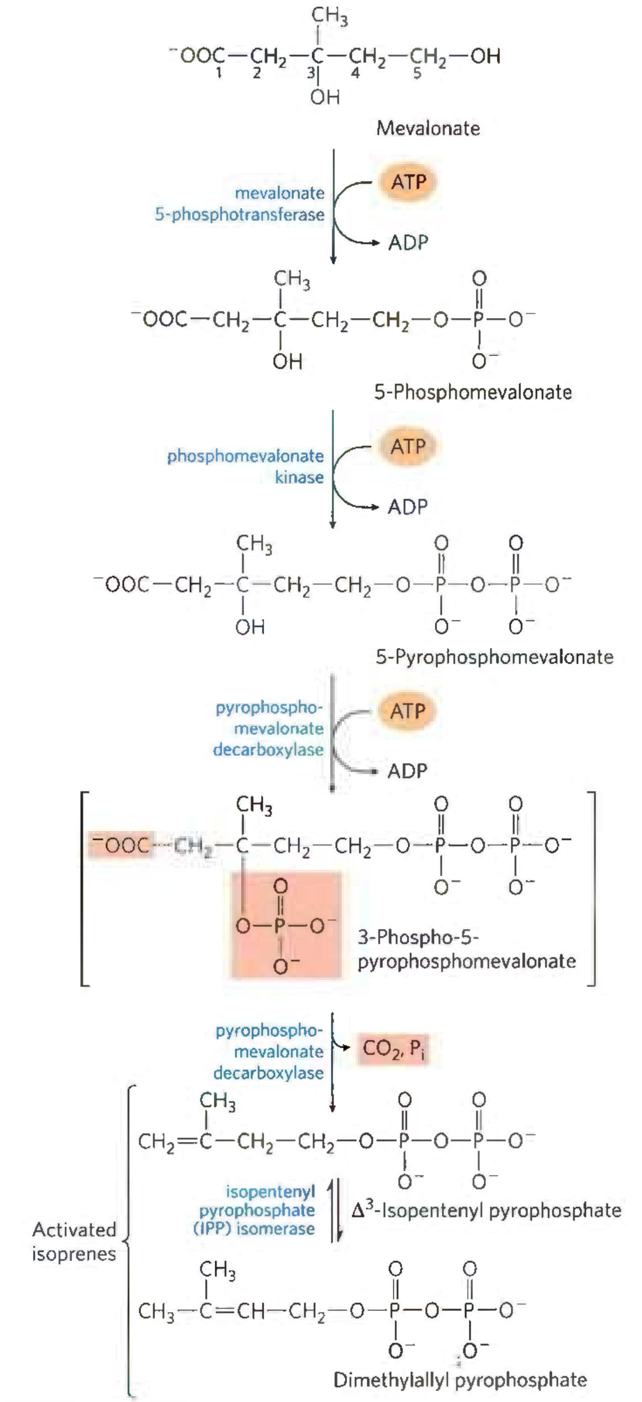

Mevalonate
mevalonate 5-phosphotransferase
ATP
ADP
5-Phosphomevalonate
phosphomevalonate kinase
ATP
ADP
5-Pyrophosphomevalonate
pyrophosphomevalonate decarboxylase
ATP
ADP
3-Phospho-5-pyrophosphomevalonate
pyrophosphomevalonate decarboxylase
CO_2, P_i
isopentenyl pyrophosphate (IPP) isomerase
Δ^3-Isopentenyl pyrophosphate
Activated isoprenes
Dimethylallyl pyrophosphate

FIGURE 21-35 Conversion of mevalonate to activated isoprene units. Six of these activated units combine to form squalene (see Fig. 21-36). The leaving groups of 3-phospho-5-pyrophosphomevalonate are shaded light red. The bracketed intermediate is hypothetical. Both of these isoprene products are required for the next stage in cholesterol biosynthesis.

Stage ❸ Condensation of Six Activated Isoprene Units to Form Squalene Isopentenyl pyrophosphate and dimethylallyl pyrophosphate now undergo a head-to-tail condensation, in which one pyrophosphate group is displaced and a 10-carbon chain, **geranyl pyrophosphate**, is formed (**Fig. 21-36**). (The "head" is the end to which pyrophosphate is joined.) Geranyl pyrophosphate undergoes another head-to-tail condensation with isopentenyl pyrophosphate, yielding the 15-carbon intermediate **farnesyl pyrophosphate**. Finally, two molecules of farnesyl pyrophosphate join head to head, with the elimination of both pyrophosphate groups, to form **squalene**. Squalene has 30 carbons: 24 in the main chain and 6 in the form of methyl group branches.

The common names of these intermediates derive from the sources from which they were first isolated. Geraniol, a component of rose oil, has the aroma of geraniums, and farnesol is an aromatic compound found in flowers of the Farnese acacia tree. Many natural scents of plant origin are synthesized from isoprene units. Squalene was first isolated from the liver of sharks (genus *Squalus*).

Stage ❹ Conversion of Squalene to the Four-Ring Steroid Nucleus When the squalene molecule is represented as in **Figure 21-37**, the relationship of its linear structure to the cyclic structure of the sterols becomes apparent. All sterols have the four fused rings that form the steroid nucleus, and all are alcohols, with a hydroxyl group at C-3—thus the name "sterol." The action of **squalene monooxygenase** adds one oxygen atom from O_2 to the end of the squalene chain, forming an epoxide. This enzyme is another mixed-function oxidase; NADPH reduces the other oxygen atom of O_2 to H_2O. The double bonds of the product, **squalene 2,3-epoxide**, are positioned so that a remarkable concerted reaction can convert the linear squalene epoxide to a cyclic structure. In animal cells, this cyclization results in the formation of **lanosterol**, which contains the four rings characteristic of the steroid nucleus. Lanosterol is finally converted to cholesterol in a series of about 20 reactions that include the migration of some methyl groups and the removal of others. Elucidation of this extraordinary biosynthetic pathway, one of the most complex known,

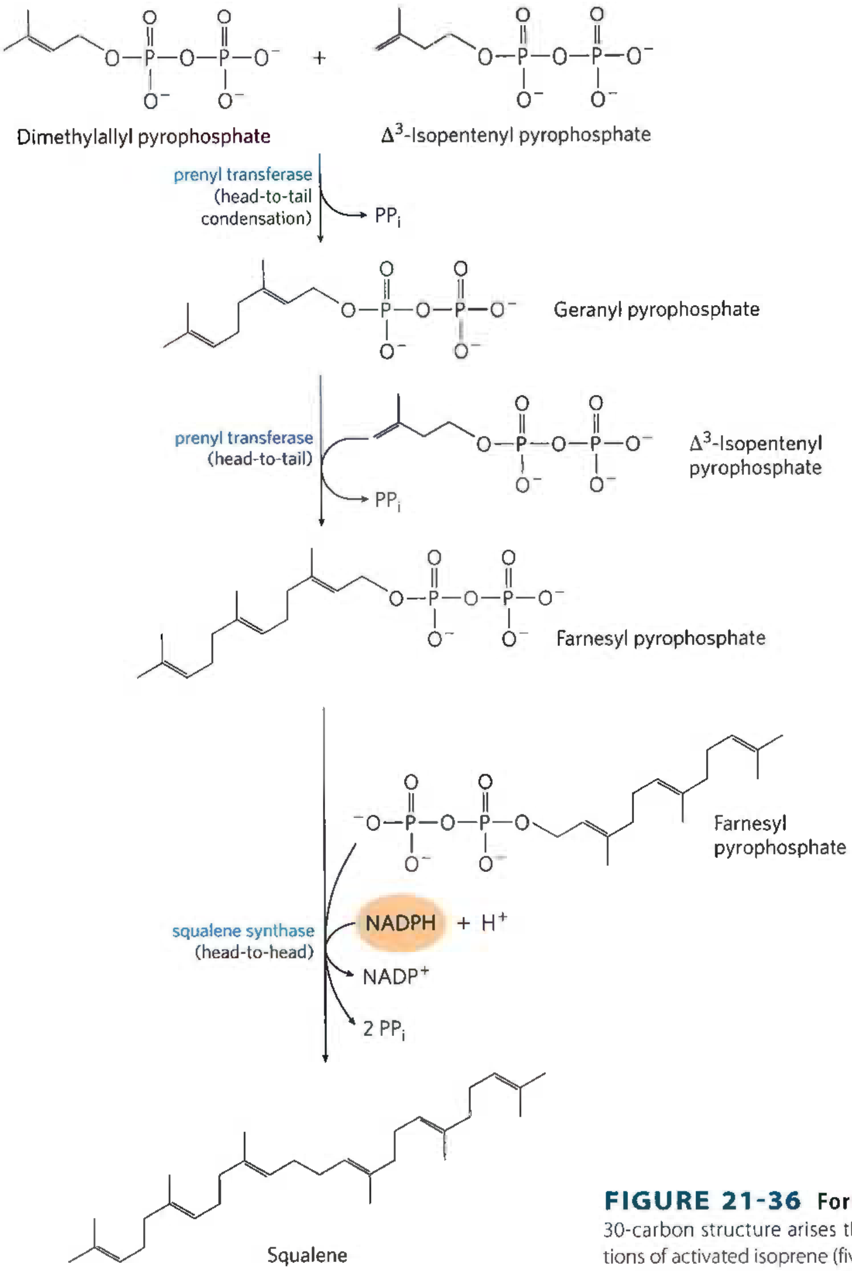

Dimethylallyl pyrophosphate
+
Δ3-Isopentenyl pyrophosphate
prenyl transferase
(head-to-tail condensation)
PPi
Geranyl pyrophosphate
prenyl transferase
(head-to-tail)
Δ3-Isopentenyl pyrophosphate
PPi
Farnesyl pyrophosphate
Farnesyl pyrophosphate
squalene synthase
(head-to-head)
NADPH + H+
NADP+
2 PPi
Squalene

FIGURE 21-36 Formation of squalene. This 30-carbon structure arises through successive condensations of activated isoprene (five-carbon) units.

was accomplished by Konrad Bloch, Feodor Lynen, John Cornforth, and George Popják in the late 1950s.

Cholesterol is the sterol characteristic of animal cells; plants, fungi, and protists make other, closely related sterols instead. They use the same synthetic pathway as far as squalene 2,3-epoxide, at which point the pathways diverge slightly, yielding other sterols, such as stigmasterol in many plants and ergosterol in fungi (Fig. 21-37).

WORKED EXAMPLE 21-1 *Energetic Cost of Squalene Synthesis*

What is the energetic cost of the synthesis of squalene from acetyl-CoA, in number of ATPs per molecule of squalene synthesized?

SOLUTION: In the pathway from acetyl-CoA to squalene, ATP is consumed only in the steps that convert mevalonate to the activated isoprene precursors of squalene. Three ATP molecules are used to create each of the six activated isoprenes required to construct squalene, for a total cost of 18 ATP molecules.

Cholesterol Has Several Fates

Most of the cholesterol synthesis in vertebrates takes place in the liver. A small fraction of the cholesterol made there is incorporated into the membranes of hepatocytes, but most of it is exported in one of three forms: as bile acids, as biliary cholesterol, or as cholesteryl

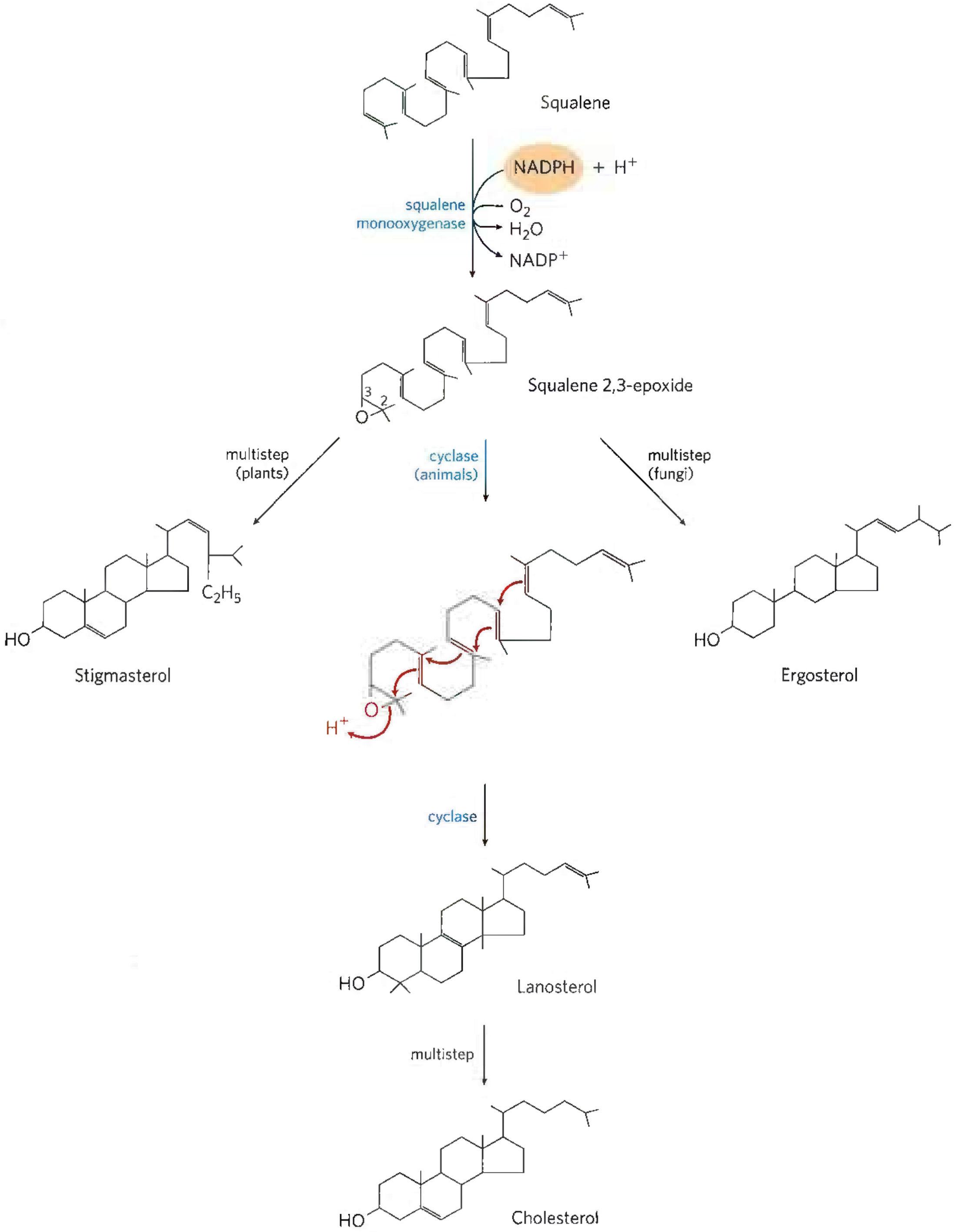

Squalene
NADPH + H+
squalene monooxygenase
O2
H2O
NADP+
Squalene 2,3-epoxide
3
2
O
multistep (plants)
cyclase (animals)
multistep (fungi)
C2H5
HO
Stigmasterol
H+
O
HO
Ergosterol
cyclase
HO
Lanosterol
multistep
HO
Cholesterol

FIGURE 21-37 Ring closure converts linear squalene to the condensed steroid nucleus. The first step in this sequence is catalyzed by a mixed-function oxygenase, for which the cosubstrate is NADPH. The product is an epoxide, which in the next step is cyclized to the steroid nucleus. The final product of these reactions in animal cells is cholesterol; in other organisms, slightly different sterols are produced, as shown.

esters (**Fig. 21-38**). Small quantities of oxysterols such as 25-hydroxycholesterol are formed in the liver and act as regulators of cholesterol synthesis (see below). In other tissues, cholesterol is converted into steroid hormones (in the adrenal cortex and gonads, for example; see Fig. 10-18) or into vitamin D hormone (in the liver and kidney; see Fig. 10-19). Such hormones are extremely potent biological signals acting through nuclear receptor proteins.

Bile acids, one of the three forms of cholesterol exported from the liver, are the principal components of bile, a fluid stored in the gallbladder and excreted into the small intestine to aid in the digestion of fat-containing meals. Bile acids and their salts are relatively hydrophilic

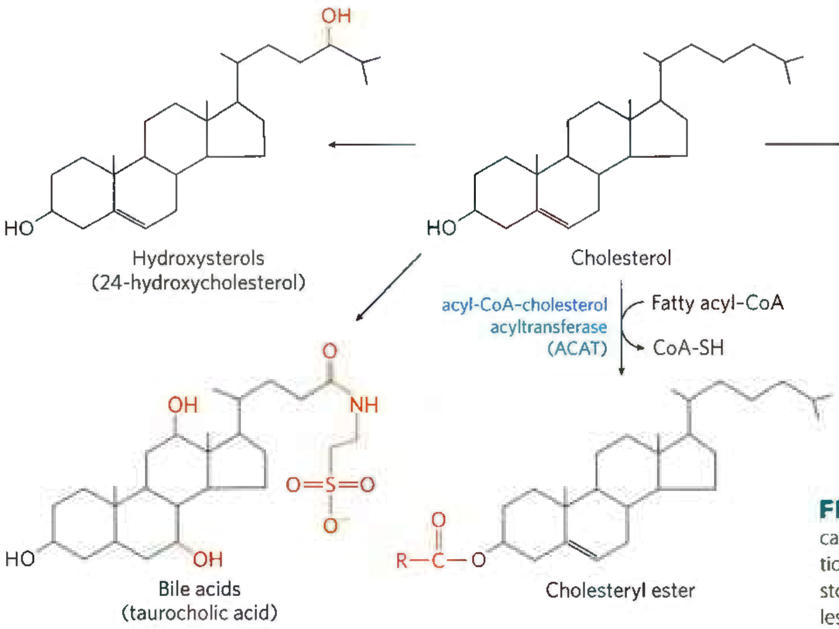


FIGURE 21-38 Metabolic fates of cholesterol. Modifications of the cholesterol structure are shown in red. Esterification converts cholesterol to an even more hydrophobic form for storage and transport; each of the other modifications yields a less hydrophobic product.

cholesterol derivatives that serve as emulsifiers in the intestine, converting large particles of fat into tiny micelles and thereby greatly increasing the surface at which digestive lipases can act (see Fig. 17-1). Bile also contains much smaller amounts of cholesterol (biliary cholesterol). Bile helps remove excess cholesterol from the intestine and facilitates excretion. Dietary fiber can enhance this effect by binding to bile and interfering with bile reabsorption in the intestines, leading to increased bile excretion in the feces. More cholesterol is then used to make bile. The soluble fiber available in oats (oatmeal) and barley is especially effective.

Cholesteryl esters are formed in the liver through the action of **acyl-CoA–cholesterol acyltransferase (ACAT)**. This enzyme catalyzes the transfer of a fatty acid from coenzyme A to the hydroxyl group of cholesterol (Fig. 21-38), converting the cholesterol to a more hydrophobic form that is no longer sufficiently amphipathic to function appropriately in membranes. Cholesteryl esters are transported in secreted lipoprotein particles to other tissues that use cholesterol, or they are stored in the liver in lipid droplets.

Cholesterol and Other Lipids Are Carried on Plasma Lipoproteins

Cholesterol and cholesteryl esters, like triacylglycerols and phospholipids, are essentially insoluble in water, yet they must be moved from the tissue of origin to the tissues in which they will be stored or consumed. They are carried in the blood plasma as **plasma lipoproteins**, macromolecular complexes of specific carrier proteins, called **apolipoproteins**, and various combinations of phospholipids, cholesterol, cholesteryl esters, and triacylglycerols.

Apolipoproteins ("apo" designates the protein in its lipid-free form) combine with lipids to form several classes of lipoprotein particles, spherical complexes with hydrophobic lipids in the core and hydrophilic amino acid side chains at the surface (**Fig. 21-39a, b**). Different combinations of lipids and proteins produce particles of different densities, ranging from chylomicrons to high-density lipoproteins. These particles can be separated by ultracentrifugation (**Table 21-1**) and visualized by electron microscopy (Fig. 21-39c).

Each class of lipoprotein has a specific function, determined by its point of synthesis, lipid composition, and apolipoprotein content. At least 10 distinct apolipoproteins are found in the lipoproteins of human plasma (**Table 21-2**), distinguishable by their size, their reactions with specific antibodies, and their characteristic distribution in the lipoprotein classes. These protein components act as signals, targeting lipoproteins to specific tissues or activating enzymes that act on the lipoproteins. **Figure 21-40** provides an overview of the formation and transport of the lipoproteins in mammals. The numbered steps in the following discussion refer to this figure.

Chylomicrons, discussed in Chapter 17 in connection with the movement of dietary triacylglycerols from the intestine to other tissues, are the first of four classes of lipoproteins we will discuss. These are the largest of the lipoproteins and the least dense, containing a high proportion of triacylglycerols. ❶ Chylomicrons are synthesized from dietary fats in the ER of enterocytes, epithelial cells that line the small intestine. The chylomicrons then move through the lymphatic system and enter the bloodstream via the left subclavian vein. The apolipoproteins of chylomicrons include apoA-IV, apoB-48 (unique to this class of lipoproteins), apoE, apoC-II, and

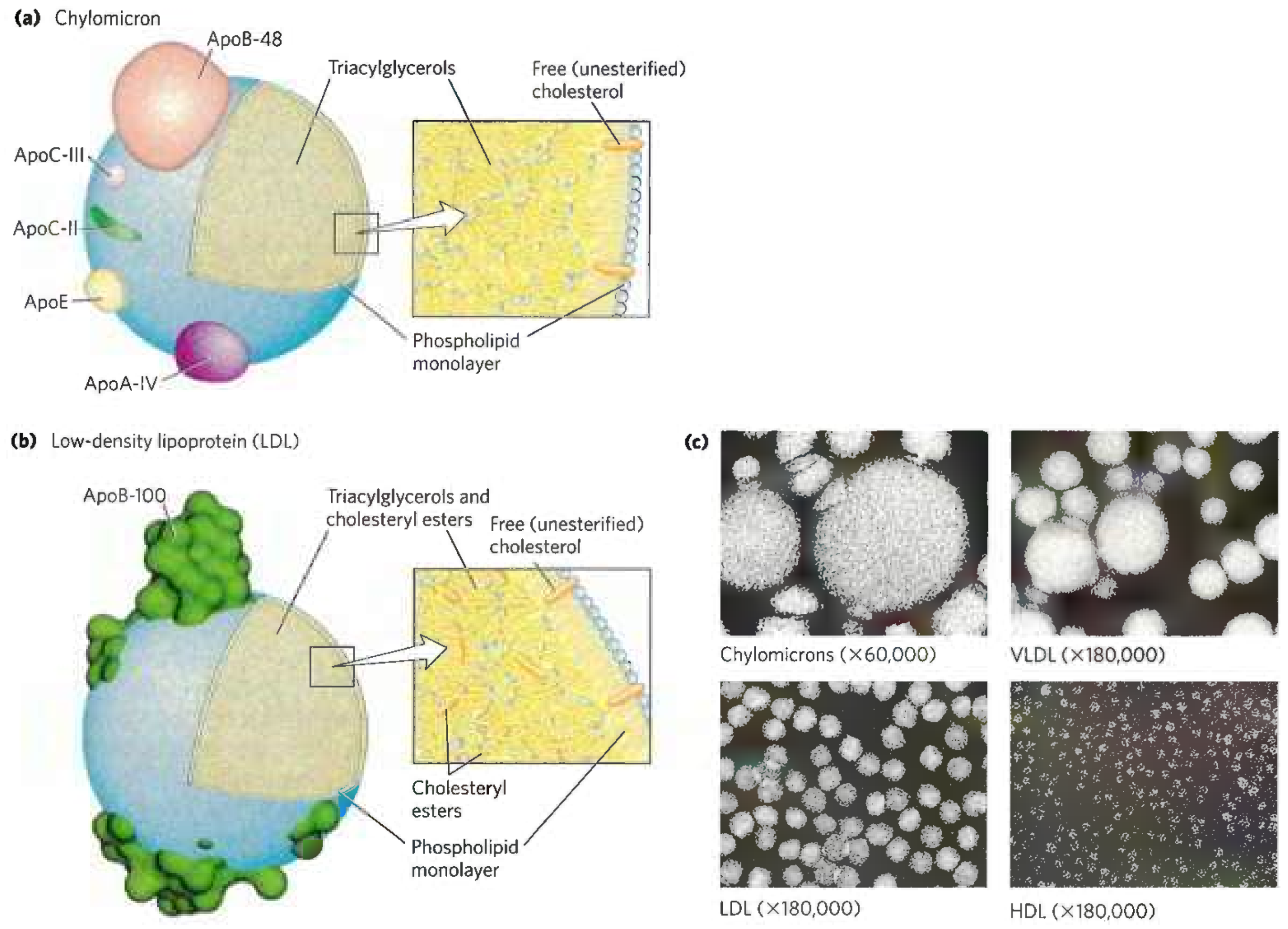

(a) Chylomicron
ApoB-48
Triacylglycerols
Free (unesterified) cholesterol
ApoC-III
ApoC-II
ApoE
ApoA-IV
Phospholipid monolayer
(b) Low-density lipoprotein (LDL)
ApoB-100
Triacylglycerols and cholesteryl esters
Free (unesterified) cholesterol
Cholesteryl esters
Phospholipid monolayer
(c)
Chylomicrons (×60,000)
VLDL (×180,000)
LDL (×180,000)
HDL (×180,000)

FIGURE 21-39 Lipoproteins. (a) Structure of a chylomicron. Apolipoprotein B-48 (apoB-48) defines the chylomicron. Through the life cycle of a chylomicron other apolipoproteins, including apoC-II, apoC-III, and apoE, become part of the particle, acting as signals in the uptake and metabolism of chylomicron contents. Chylomicrons range from about 100 to 500 nm in diameter. (b) Structure of a low-density lipoprotein (LDL). Apolipoprotein B-100 (apoB-100) is one of the largest single polypeptide chains known, with 4,636 amino acid residues (*M*, 512,000). One particle of LDL contains a core with about 1,500 molecules of cholesteryl esters, surrounded by a shell composed of about 500 more molecules of cholesterol, 800 molecules of phospholipids, and one molecule of apoB-100. (c) Four classes of lipoproteins, visualized in the electron microscope after negative staining. The chylomicrons shown here are 50 to 200 nm in diameter; VLDL, 28 to 70 nm; LDL, 20 to 25 nm; and HDL, 8 to 11 nm. Particle sizes given are those measured for these samples; particle sizes vary considerably in different preparations. For properties of lipoproteins, see Table 21-1. [(b) Data for apoB-100 from A. Johs et al., *J. Biol. Chem.* 281:19,732, 2006. (c) Robert Hamilton, Jr., PhD.]

apoC-III (Table 21-2). ❷ ApoC-II activates lipoprotein lipase in the capillaries of adipose, heart, skeletal muscle, and lactating mammary tissues, allowing the release of free fatty acids (FFA) to these tissues. Chylomicrons thus carry dietary fatty acids to tissues where they will be consumed or stored as fuel. ❸ The remnants of chylomicrons, depleted of most of their triacylglycerols but still containing cholesterol, apoE, and apoB-48, move through the bloodstream to the liver. Receptors in the liver bind to the apoE in the chylomicron remnants and

TABLE 21-1 Major Classes of Human Plasma Lipoproteins: Some Properties

		Composition (wt %)				
Lipoprotein	Density (g/mL)	Protein	Phospholipids	Free cholesterol	Cholesteryl esters	Triacylglycerols
Chylomicrons	<1.006	2	9	1	3	85
VLDL	0.95–1.006	10	18	7	12	50
LDL	1.006–1.063	23	20	8	37	10
HDL	1.063–1.210	55	24	2	15	4

Data from D. Kritchevsky, *Nutr. Int.* 2:290, 1986.

TABLE 21-2 Apolipoproteins of the Human Plasma Lipoproteins

Apolipoprotein	Polypeptide molecular weight	Lipoprotein association	Function (if known)
ApoA-I	28,100	HDL	Activates LCAT; interacts with ABC transporter
ApoA-II	17,400	HDL	Inhibits LCAT
ApoA-IV	44,500	Chylomicrons, HDL	Activates LCAT; cholesterol transport/clearance
ApoB-48	242,000	Chylomicrons	Cholesterol transport/clearance
ApoB-100	512,000	VLDL, LDL	Binds to LDL receptor
ApoC-I	7,000	VLDL, HDL	
ApoC-II	9,000	Chylomicrons, VLDL, HDL	Activates lipoprotein lipase
ApoC-III	9,000	Chylomicrons, VLDL, HDL	Inhibits lipoprotein lipase
ApoD	32,500	HDL	
ApoE	34,200	Chylomicrons, VLDL, HDL	Triggers clearance of VLDL and chylomicron remnants
ApoH	50,000	Possibly VLDL, binds phospholipids such as cardiolipin	Roles in coagulation, lipid metabolism, apoptosis, inflammation

Information from D. E. Vance and J. E. Vance (eds), *Biochemistry of Lipids and Membranes*, 5th edn, Elsevier Science Publishing, 2008.

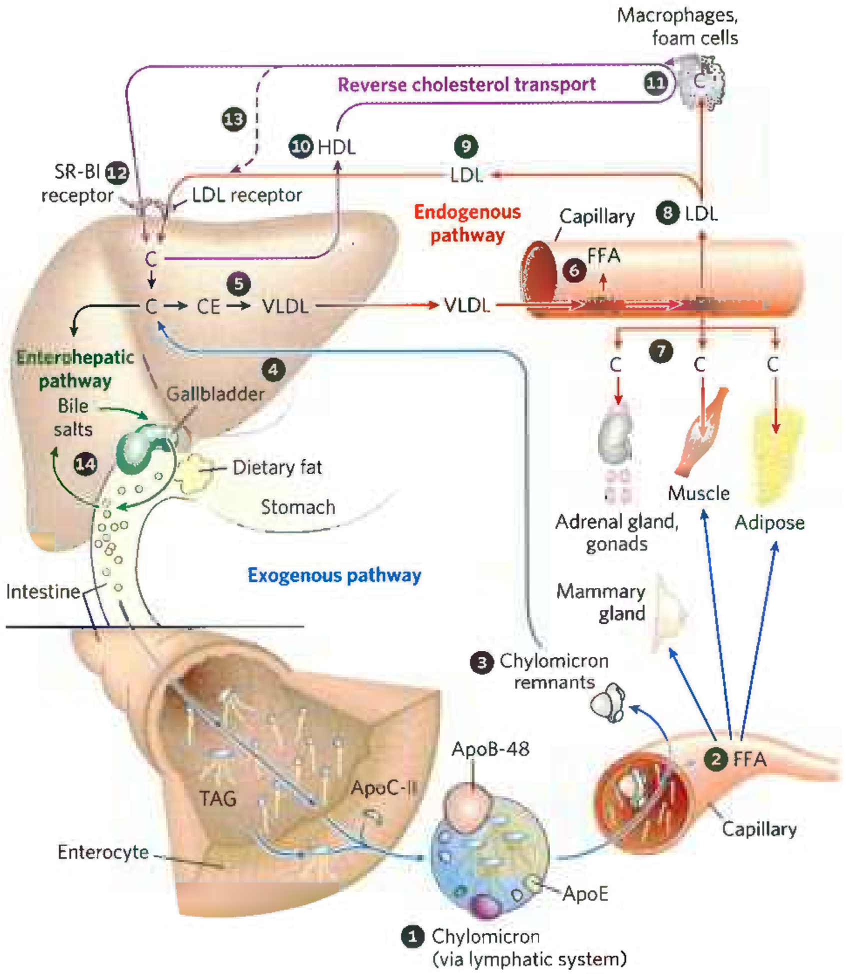

Macrophages, foam cells
Reverse cholesterol transport
⑪
C
⑬
⑩ HDL
⑨ LDL
SR-BI ⑫ receptor
LDL receptor
Endogenous pathway
Capillary
⑧ LDL
⑥ FFA
C
⑤
C → CE → VLDL
VLDL
Enterohepatic pathway
Bile salts
④
Gallbladder
⑦
C
C
C
⑭
Dietary fat
Stomach
Muscle
Adrenal gland, gonads
Adipose
Exogenous pathway
Intestine
Mammary gland
③ Chylomicron remnants
ApoB-48
② FFA
TAG
ApoC-II
Capillary
Enterocyte
ApoE
① Chylomicron (via lymphatic system)

FIGURE 21-40 Lipoproteins and lipid transport. Lipids are transported in the bloodstream as lipoproteins, which exist as several variants that have different functions, different protein and lipid compositions (see Tables 21-1 and 21-2), and thus different densities. Numbered steps are described in the text. In the exogenous pathway (blue arrows), dietary lipids are packaged into chylomicrons; fatty acids from triacylglycerol (TAG) are released by lipoprotein lipase to adipose and muscle tissues, during transport through capillaries. Chylomicron remnants (containing largely protein and cholesterol) are taken up by the liver. Bile salts produced in the liver aid in dispersing dietary fats and are then reabsorbed in the enterohepatic pathway (green arrows). In the endogenous pathway (red arrows), lipids synthesized or packaged in the liver are delivered to peripheral tissues by VLDL. Extraction of lipid from VLDL (along with loss of some apolipoproteins) gradually converts some of it to LDL, which delivers cholesterol to extrahepatic tissues or returns to the liver. Excess cholesterol in extrahepatic tissues is transported back to the liver as HDL in reverse cholesterol transport (purple arrows). C represents cholesterol; CE, cholesteryl ester.

mediate uptake of these remnants by endocytosis. ④ In the liver, the remnants release their cholesterol and are degraded in lysosomes. This pathway from dietary cholesterol to the liver is the **exogenous pathway** (blue arrows in Fig. 21-40).

Very-low-density lipoprotein (VLDL) is the second of the four classes. When the diet contains more fatty acids and cholesterol than are needed immediately as fuel or as precursors to other molecules, ⑤ they are converted to triacylglycerols or cholesteryl esters in the liver and packaged with specific apolipoproteins into VLDL. Excess carbohydrate in the diet can also be converted to triacylglycerols in the liver and exported as VLDL. In addition to triacylglycerols and cholesteryl esters, VLDL contains apoB-100, apoC-I, apoC-II, apoC-III, and apoE (Table 21-2). VLDL is transported in the blood from the liver to muscle and adipose tissue. ⑥ In the capillaries of these tissues, apoC-II activates lipoprotein lipase, which catalyzes the release of free fatty acids from triacylglycerols in the VLDL. Adipocytes take up these fatty acids, reconvert them to triacylglycerols, and store the products in intracellular lipid droplets; myocytes, in contrast, primarily oxidize the fatty acids to supply energy. When the insulin level is high (after a meal), VLDL serves primarily to convey lipids from the diet to adipose tissue for storage. In the fasting state between meals, the fatty acids used to produce VLDL in the liver originate primarily from adipose tissue, and the principal VLDL target is myocytes of the heart and skeletal muscle.

Low-density lipoprotein (LDL), the third class of lipoprotein, is formed when triacylglycerol loss converts some VLDL to VLDL remnants, also called intermediate-density lipoprotein (IDL). Further removal of triacylglycerol from IDL (remnants) produces LDL. Rich in cholesterol and cholesteryl esters, and containing apoB-100 as its major apolipoprotein, ⑦ LDL carries cholesterol to extrahepatic tissues such as muscle, adrenal glands, and adipose tissue. These tissues have plasma membrane LDL receptors that recognize apoB-100 and mediate uptake of cholesterol and cholesteryl esters. ⑧ LDL also delivers cholesterol to macrophages, sometimes converting them into foam cells (see Fig. 21-46). ⑨ LDL not taken up by peripheral tissues and cells returns to the liver and is taken up via **LDL receptors** in the hepatocyte plasma membrane. Cholesterol that enters hepatocytes by this path may be incorporated into membranes, converted to bile acids, or reesterified by ACAT (Fig. 21-38) for storage within cytosolic lipid droplets. This pathway, from VLDL formation in the liver to LDL return to the liver, is the **endogenous pathway** of cholesterol metabolism and transport (red arrows in Fig. 21-40). Accumulation of excess intracellular cholesterol is prevented by reducing the rate of cholesterol synthesis when sufficient cholesterol is available from LDL in the blood. Regulatory mechanisms to accomplish this are described below.

HDL Carries Out Reverse Cholesterol Transport

High-density lipoprotein (HDL), the fourth major lipoprotein in mammals, ⑩ originates in the liver and small intestine as small, protein-rich particles that contain relatively little cholesterol and no cholesteryl esters (Fig. 21-40). HDLs contain primarily apoA-I and other apolipoproteins (Table 21-2). They also contain the enzyme **lecithin-cholesterol acyltransferase (LCAT)**, which catalyzes the formation of cholesteryl esters from lecithin (phosphatidylcholine) and cholesterol (**Fig. 21-41**). LCAT on the surface of nascent (newly forming) HDL particles converts the cholesterol and phosphatidylcholine of chylomicron and VLDL remnants encountered in the bloodstream to cholesteryl esters, which begin to form a core, transforming the disk-shaped nascent HDL to a mature, spherical HDL particle.

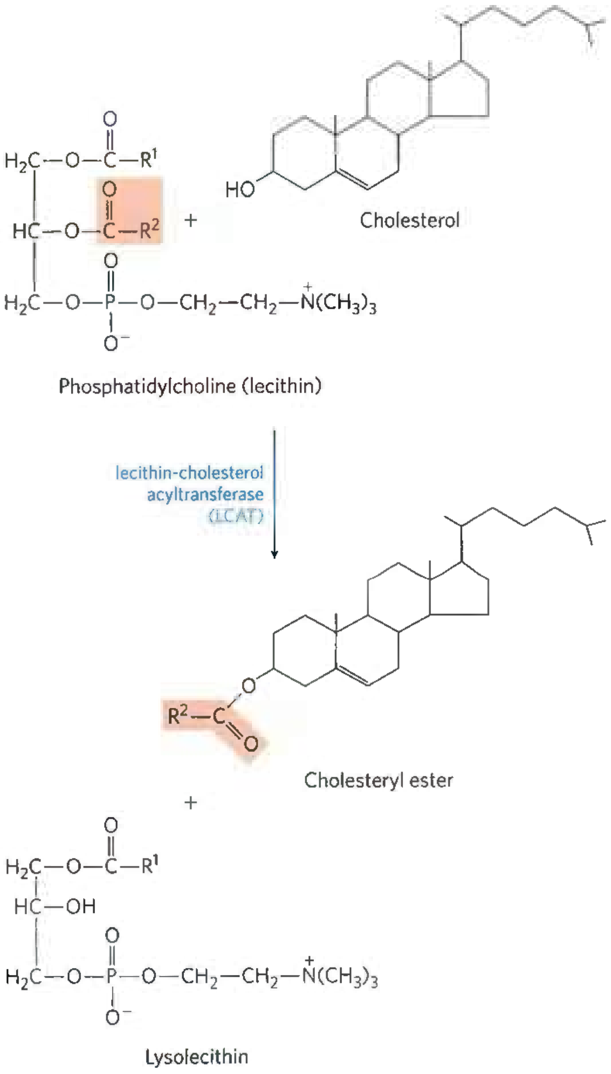

Cholesterol
Phosphatidylcholine (lecithin)
lecithin-cholesterol acyltransferase (LCAT)
Cholesteryl ester
Lysolecithin

FIGURE 21-41 Reaction catalyzed by lecithin-cholesterol acyltransferase (LCAT). This enzyme is present on the surface of HDL and is stimulated by the HDL component apoA-I. Cholesteryl esters accumulate within nascent HDLs, converting them to mature HDLs.

⑪ Nascent HDL can also pick up cholesterol from cholesterol-rich extrahepatic cells (including macrophages and foam cells, formed from macrophages; see below). ⑫ Mature HDL then returns to the liver, where the cholesterol is unloaded via the scavenger receptor SR-BI. ⑬ Some of the cholesteryl esters in HDL can also be transferred to LDL by the cholesteryl ester transfer protein. The HDL circuit is **reverse cholesterol transport** (purple arrows in Fig. 21-40). Much of this cholesterol is converted to bile salts by enzymes sequestered in hepatic peroxisomes; the bile salts are stored in the gallbladder and excreted into the intestine when a meal is ingested. ⑭ Bile salts are reabsorbed by the liver and recirculate through the gallbladder in this **enterohepatic circulation** (green arrows in Fig. 21-40).

The unloading of sterols via SR-BI receptors in liver and other tissues does not occur by endocytosis, the mechanism used for LDL uptake. Instead, when HDL binds to SR-BI receptors in the plasma membranes of hepatocytes or steroidogenic tissues such as the adrenal gland, these receptors mediate partial and selective transfer of cholesterol and other lipids in HDL into the cell. Depleted HDL then dissociates to recirculate in the bloodstream and extract more lipids from remnants of chylomicrons and VLDL, and from cells overloaded with cholesterol, as described below.

Cholesteryl Esters Enter Cells by Receptor-Mediated Endocytosis

Each LDL particle in the bloodstream contains apoB-100, which is recognized by LDL receptors present in the plasma membranes of cells that need to take up cholesterol. **Figure 21-42** shows such a cell. ❶ LDL receptors are synthesized in the ER and transported to the plasma membrane, after modification in the Golgi complex. At the plasma membrane, they are available to bind apoB-100. ❷ Binding of LDL to an LDL receptor initiates endocytosis, which ❸ conveys the LDL and its receptor into the cell within an endosome. ❹ The receptor-containing portions of the endosome membrane bud off and are returned to the cell surface, to function again in LDL uptake. ❺ The endosome fuses with a lysosome, which ❻ contains enzymes that hydrolyze the cholesteryl esters, releasing cholesterol and fatty acids into the cytosol. The apoB-100 protein is also degraded to amino acids that are released to the cytosol. ApoB-100 is also present in VLDL, but its receptor-binding domain is not available for binding to the LDL receptor; conversion of VLDL to LDL exposes the receptor-binding domain of apoB-100.

This pathway for the transport of cholesterol in blood and its **receptor-mediated endocytosis** by target tissues was elucidated by Michael Brown and

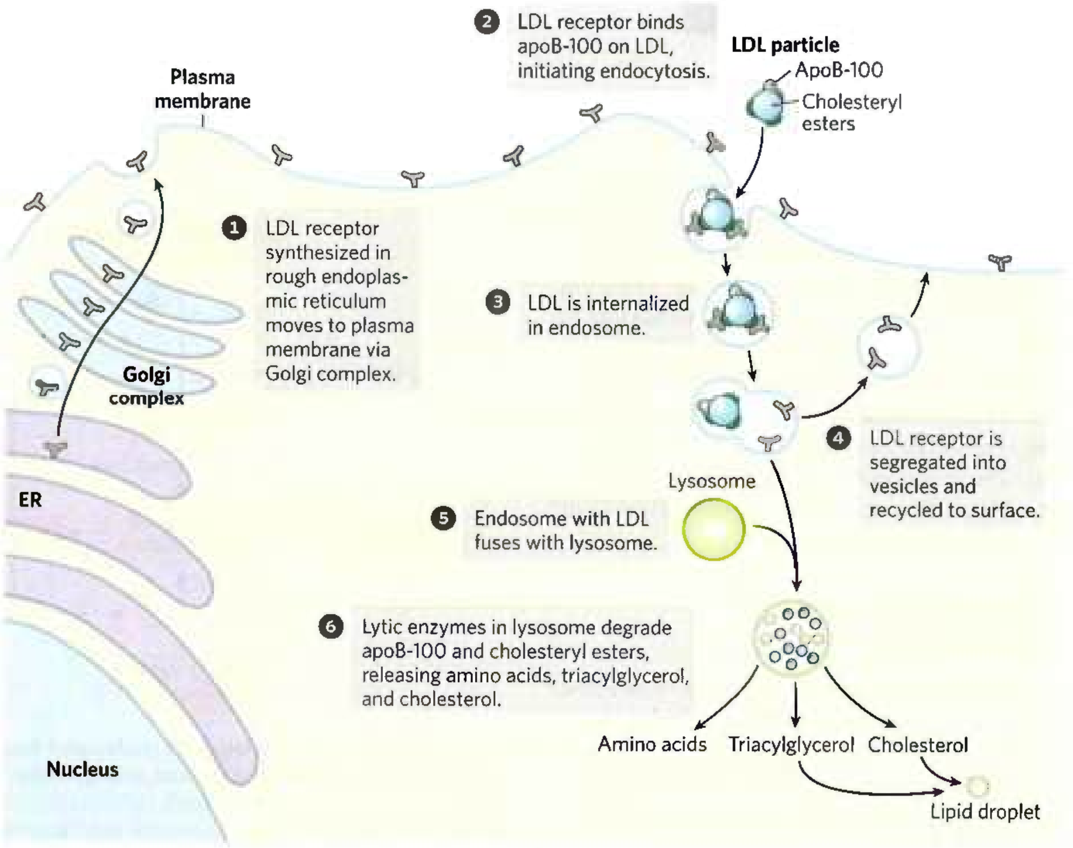

❷ LDL receptor binds apoB-100 on LDL, initiating endocytosis.
LDL particle
ApoB-100
Cholesteryl esters
Plasma membrane
❶ LDL receptor synthesized in rough endoplasmic reticulum moves to plasma membrane via Golgi complex.
❸ LDL is internalized in endosome.
Golgi complex
ER
❹ LDL receptor is segregated into vesicles and recycled to surface.
Lysosome
❺ Endosome with LDL fuses with lysosome.
❻ Lytic enzymes in lysosome degrade apoB-100 and cholesteryl esters, releasing amino acids, triacylglycerol, and cholesterol.
Amino acids
Triacylglycerol
Cholesterol
Lipid droplet
Nucleus

FIGURE 21-42 **Uptake of cholesterol by receptor-mediated endocytosis.**

Joseph Goldstein. They discovered that individuals with the genetic disease **familial hypercholesterolemia (FH)** have mutations in the LDL receptor that prevent the normal uptake of LDL by liver and peripheral tissues. The result of defective LDL uptake is very high blood levels of LDL (and of the cholesterol it carries). Individuals with FH have a greatly increased probability of developing atherosclerosis, a disease of the cardiovascular system in which blood vessels are occluded by cholesterol-rich plaques (see Fig. 21-46).

Michael Brown and Joseph Goldstein
[Mei-Chun Jau. Courtesy of Michael Brown and Joseph Goldstein, University of Texas Southwestern Medical Center.]

Niemann-Pick type-C (NPC) disease is an inherited defect in lipid storage. In this disorder, cholesterol is not transported out of the lysosomes and instead accumulates in lysosomes of liver, brain, and lung, bringing about early death. NPC is the result of a mutation in either of two genes, *NPC1* and *NPC2*, essential to moving cholesterol out of the lysosome and into the cytosol, where it can be further metabolized. *NPC1* encodes a transmembrane lysosomal protein, and *NPC2* encodes a soluble protein. These proteins act in tandem to transfer cholesterol out of the lysosome and into the cytosol for further processing or metabolism. ■

Cholesterol Synthesis and Transport Are Regulated at Several Levels

Cholesterol synthesis is a complex and energy-expensive process. Excess cholesterol cannot be catabolized for use as fuel and must be excreted. Therefore, it is clearly advantageous to an organism to regulate the biosynthesis of cholesterol to complement dietary intake. In mammals, cholesterol production is regulated by intracellular cholesterol concentration, by the supply of ATP, and by the hormones glucagon and insulin. The committed step in the pathway to cholesterol (and a major site of regulation) is the conversion of HMG-CoA to mevalonate (Fig. 21-34), the reaction catalyzed by HMG-CoA reductase.

Short-term (minute-to-minute) regulation of the *activity* of existing HMG-CoA reductase is accomplished by reversible covalent alteration: phosphorylation by the AMP-dependent protein kinase (AMPK), which senses high AMP concentration (indicating low ATP concentration). Thus, when ATP levels drop, the synthesis of cholesterol slows, and catabolic pathways for the generation of ATP are stimulated (**Fig. 21-43**). Hormones that mediate global regulation of lipid and carbohydrate metabolism also act on HMG-CoA reductase; glucagon stimulates its phosphorylation (inactivation), and insulin promotes dephosphorylation, activating the enzyme and favoring cholesterol synthesis. These covalent regulatory mechanisms are probably not as important, quantitatively, as the mechanisms that affect the synthesis and degradation of the enzyme.

In the longer term, the *number of molecules* of HMG-CoA reductase is increased or decreased in response to cellular concentrations of cholesterol. Regulation of HMG-CoA reductase synthesis by cholesterol is mediated by an elegant system of transcriptional regulation of the HMG-CoA gene (**Fig. 21-44**). This gene, along with more than 20 other genes encoding enzymes that mediate the uptake and synthesis of cholesterol and unsaturated fatty acids, is controlled by a small family of proteins called **sterol regulatory element-binding proteins (SREBPs)**. When newly synthesized, these

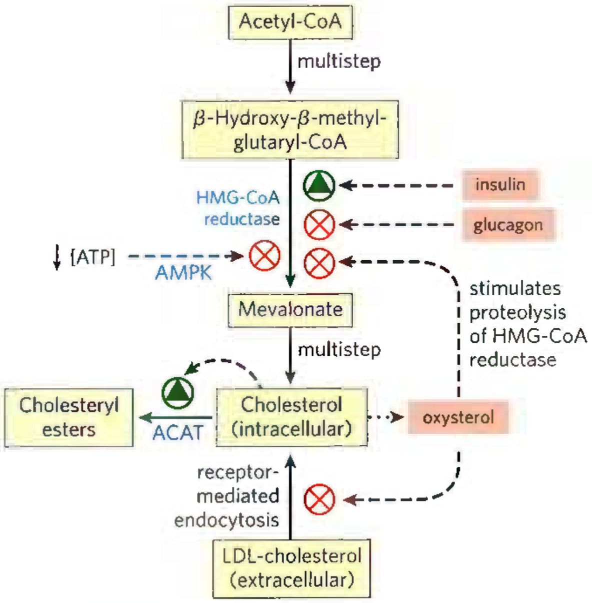
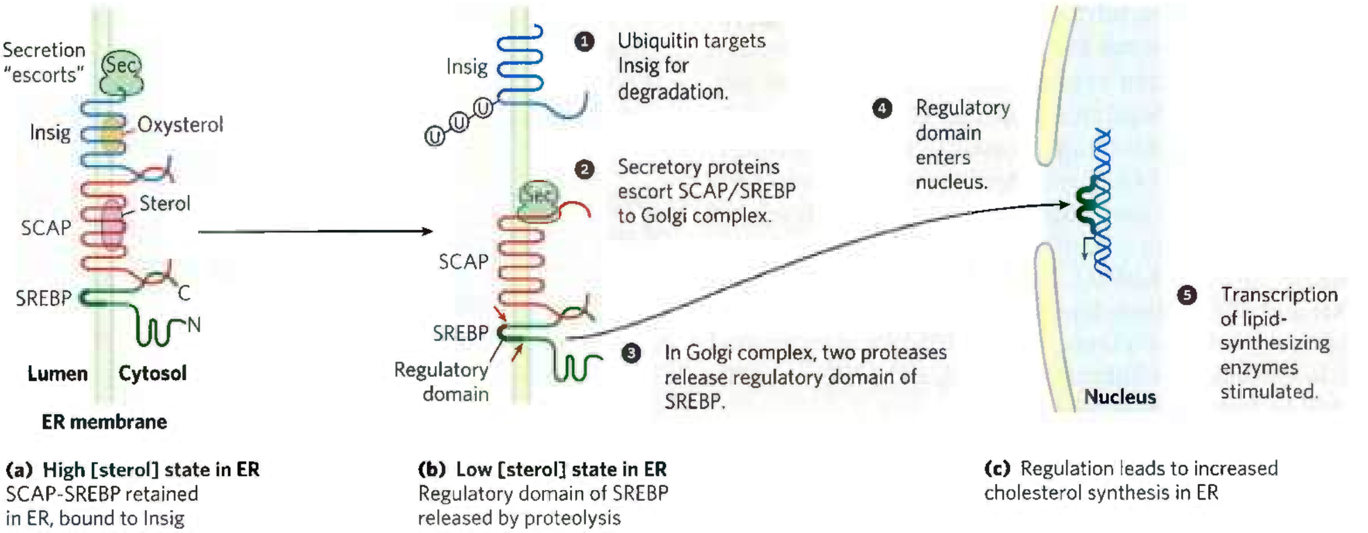

Acetyl-CoA
multistep
β-Hydroxy-β-methyl-glutaryl-CoA
HMG-CoA reductase
insulin
glucagon
↓[ATP]
AMPK
stimulates proteolysis of HMG-CoA reductase
Mevalonate
multistep
Cholesteryl esters
ACAT
Cholesterol (intracellular)
oxysterol
receptor-mediated endocytosis
LDL-cholesterol (extracellular)

FIGURE 21-43 Regulation of cholesterol formation balances synthesis with dietary uptake and energy state. Insulin promotes dephosphorylation (activation) of HMG-CoA reductase; glucagon promotes its phosphorylation (inactivation); and the AMP-dependent protein kinase AMPK, when activated by low [ATP] relative to [AMP], phosphorylates and inactivates HMG-CoA reductase. Oxysterol metabolites of cholesterol (for example, 24(S)-hydroxycholesterol) stimulate proteolysis of HMG-CoA reductase.


Secretion "escorts"
Sec
Insig
Oxysterol
Sterol
SCAP
SREBP
C
N
Lumen
Cytosol
ER membrane
Insig
U
① Ubiquitin targets Insig for degradation.
② Secretory proteins escort SCAP/SREBP to Golgi complex.
SCAP
SREBP
Regulatory domain
③ In Golgi complex, two proteases release regulatory domain of SREBP.
④ Regulatory domain enters nucleus.
Nucleus
⑤ Transcription of lipid-synthesizing enzymes stimulated.

(a) High [sterol] state in ER SCAP-SREBP retained in ER, bound to Insig

(b) Low [sterol] state in ER Regulatory domain of SREBP released by proteolysis

(c) Regulation leads to increased cholesterol synthesis in ER

FIGURE 21-44 Regulation of cholesterol synthesis by SREBP. Sterol regulatory element-binding proteins (SREBPs) are embedded in the ER when first synthesized, in a complex with the protein SREBP cleavage-activating protein (SCAP), which is in turn bound to Insig. (*N* and *C* represent the amino and carboxyl termini of the proteins.) (a) Under normal circumstances (high [sterol]), SCAP and Insig are bound to SREBPs and the SREBPs are inactive. (b) When sterol levels decline, sterol-binding sites on Insig and SCAP are unoccupied, and Insig is targeted for degradation by the attachment of several ubiquitin molecules. The remaining SCAP-SREBP complex migrates to the Golgi complex, and SREBP is cleaved (arrows) to produce a regulatory domain fragment. (c) This domain acts in the nucleus to increase the transcription of sterol-regulated genes. [Information from R. Raghow et al., *Trends Endocrinol. Metab.* 19:65, 2008, Fig. 2.]

proteins are embedded in the ER. Only the soluble regulatory domain fragment of an SREBP functions as a transcription activator, through mechanisms discussed in Chapter 28. When cholesterol and oxysterol levels are high, SREBPs are held in the ER in a complex with another protein called **SREBP cleavage-activating protein (SCAP)**, which in turn is anchored in the ER membrane by its interaction with a third membrane protein, **Insig (*in*sulin-*i*nduced *g*ene protein)** (Fig. 21-44a). SCAP and Insig act as sterol sensors. When sterol levels are high, the Insig-SCAP-SREBP complex is retained in the ER membrane. When the level of sterols in the cell declines (Fig. 21-44b), the SCAP-SREBP complex is escorted by secretory proteins to the Golgi complex. There, two proteolytic cleavages of SREBP release a regulatory fragment, which enters the nucleus and activates transcription of its target genes, including those for HMG-CoA reductase, the LDL receptor protein, and other proteins needed for lipid synthesis. When sterol levels increase sufficiently, the proteolytic release of SREBP amino-terminal domains is again blocked, and proteasome degradation of the existing active domains results in rapid shutdown of the gene targets.

The level of HMG-CoA reductase is also regulated by proteolytic degradation of the enzyme itself. High levels of cellular cholesterol are sensed by Insig, which triggers attachment of ubiquitin molecules to HMG-CoA reductase, leading to its degradation by proteasomes.

Liver X receptor (LXR) is a nuclear transcription factor activated by oxysterol ligands (reflecting high cholesterol levels), which integrates the metabolism of fatty acids, sterols, and glucose. LXRα is expressed primarily in liver, adipose tissue, and macrophages; LXRβ is present in all tissues. When bound to an oxysterol ligand, LXRs form heterodimers with a second type of nuclear receptor, the **retinoid X receptors (RXR)**, and the LXR-RXR dimer activates transcription from a set of genes (**Fig. 21-45**), including those for acetyl-CoA carboxylase,

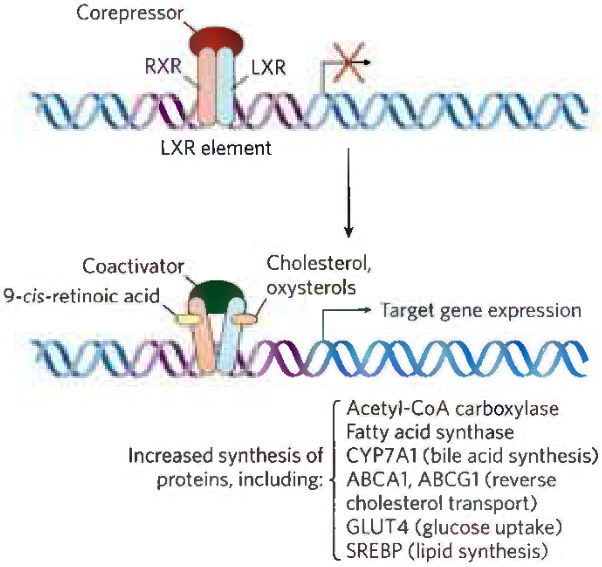

Corepressor
RXR
LXR
LXR element
Coactivator
9-cis-retinoic acid
Cholesterol, oxysterols
Target gene expression
Increased synthesis of proteins, including:
Acetyl-CoA carboxylase
Fatty acid synthase
CYP7A1 (bile acid synthesis)
ABCA1, ABCG1 (reverse cholesterol transport)
GLUT4 (glucose uptake)
SREBP (lipid synthesis)

FIGURE 21-45 Action of RXR-LXR dimer on expression of genes for lipid and glucose metabolism. When their ligands are absent, RXR and LXR associate with a corepressor protein, preventing transcription of the genes associated with the LXR element (LXRE). When their respective ligands are present—9-*cis*-retinoic acid for RXR, cholesterol or oxysterols for LXR—the dimer dissociates from the corepressor, then associates with a coactivator protein. This complex binds to the LXR element and turns on expression of the associated genes. Regulation of gene expression is a topic discussed in more detail in Chapter 28. [Information from A. C. Calkin and P. Tontonoz, *Nat. Rev. Mol. Cell Biol.* 13:213, 2012, Fig. 1.]

the first enzyme in fatty acid synthesis; fatty acid synthase; the cytochrome P-450 enzyme CYP7A1, required for sterol conversion to bile acids; apoproteins that participate in cholesterol transport (apoC-I, apoC-II, apoD, and apoE); the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, required for reverse cholesterol transport (see below); GLUT4, the insulin-stimulated glucose transporter of muscle and adipose tissue; and an SREBP called SREBP1C. The transcriptional regulators LXR and SREBP therefore work together to achieve and maintain cholesterol homeostasis; SREBPs are activated by low levels of cellular cholesterol, and LXRs are activated by high cholesterol levels.

Regulation by LXRs is complemented by the activity of **farnesoid X receptor (FXR)**, which also forms a heterodimer with RXR, with an effect that is often reciprocal to that of LXR-RXR. Although farnesol is a ligand for this receptor, FXR responds primarily to bile acids. High levels of bile acids can be toxic. FXR, expressed mainly in the intestine, liver, kidney, and adrenal glands, provides essential control of bile acid levels by increasing or decreasing expression of multiple genes. FXR represses many of the genes that are activated by LXR.

Finally, two other regulatory mechanisms influence cellular cholesterol level: (1) high cellular concentrations of cholesterol activate ACAT, which increases esterification of cholesterol for storage, and (2) high cellular cholesterol levels diminish (via SREBP) transcription of the gene that encodes the LDL receptor, reducing production of the receptor and thus the uptake of cholesterol from the blood.

Dysregulation of Cholesterol Metabolism Can Lead to Cardiovascular Disease

As noted earlier, cholesterol cannot be catabolized by animal cells. Excess cholesterol can be removed only by excretion or by conversion to bile salts. When the sum of cholesterol synthesized and cholesterol obtained in the diet exceeds the amount required for the synthesis of membranes, bile salts, and steroids, pathological accumulations of cholesterol (plaques) can obstruct blood vessels, a condition called **atherosclerosis**. Heart failure due to occluded coronary arteries is a leading cause of death in industrialized societies. Atherosclerosis is linked to high levels of cholesterol in the blood, and particularly to high levels of LDL-cholesterol ("bad cholesterol"); there is a *negative* correlation between HDL ("good cholesterol") levels and arterial disease. Plaque formation in blood vessels is initiated when LDL containing partially oxidized fatty acyl groups adheres to and accumulates in the extracellular matrix of epithelial cells lining arteries (**Fig. 21-46**). Immune cells (monocytes) are

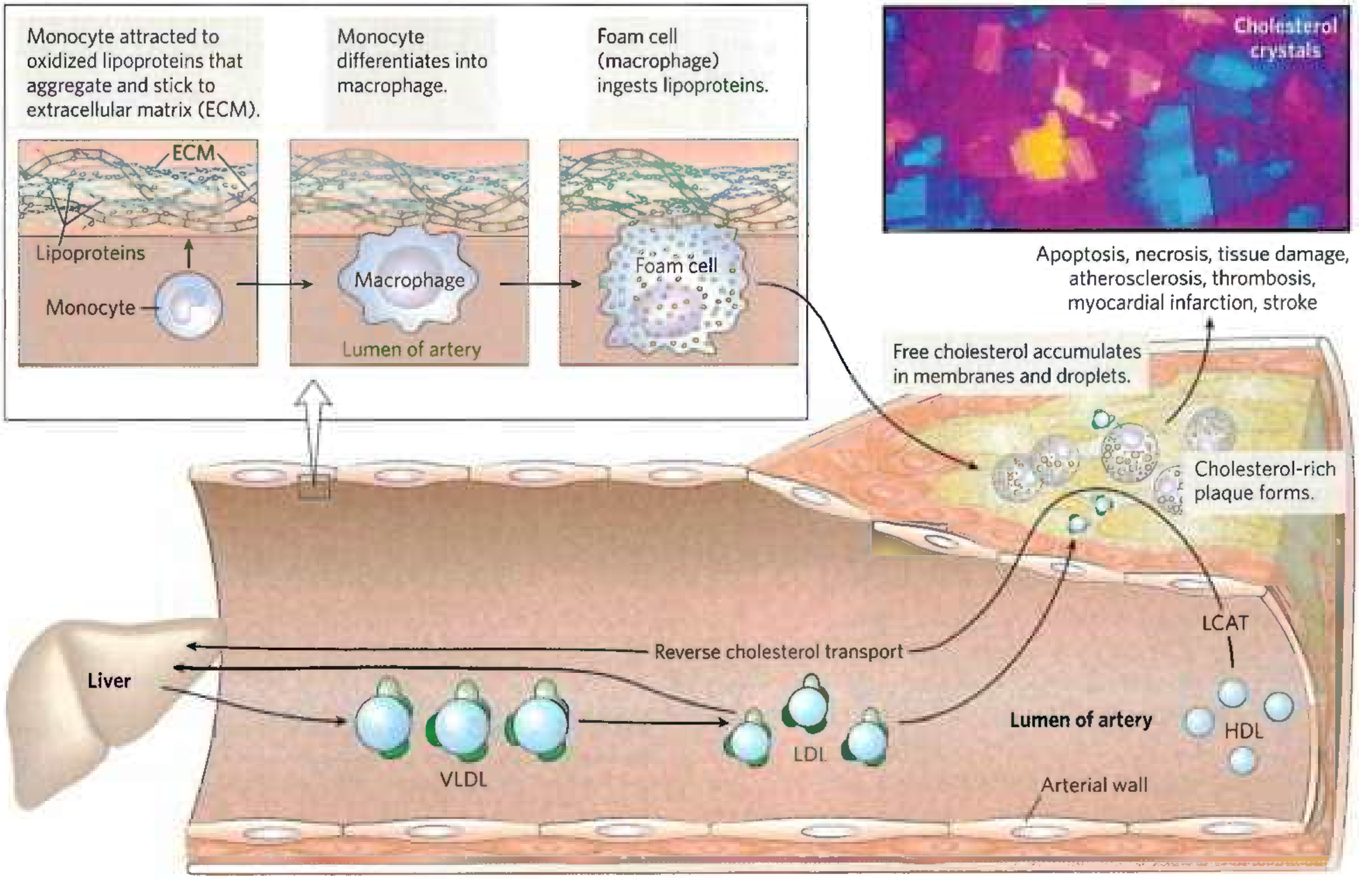

Monocyte attracted to oxidized lipoproteins that aggregate and stick to extracellular matrix (ECM).
Monocyte differentiates into macrophage.
Foam cell (macrophage) ingests lipoproteins.
ECM
Lipoproteins
Monocyte
Macrophage
Lumen of artery
Foam cell
Cholesterol crystals
Apoptosis, necrosis, tissue damage, atherosclerosis, thrombosis, myocardial infarction, stroke
Free cholesterol accumulates in membranes and droplets.
Cholesterol-rich plaque forms.
LCAT
Reverse cholesterol transport
Liver
VLDL
LDL
Lumen of artery
HDL
Arterial wall

FIGURE 21-46 Formation of atherosclerotic plaques. Excess lipid derived from the diet is deposited on arterial walls, a process facilitated by the conversion of monocytes to foam cells and incorporation of foam cells into growing plaques. Some of this deposition is countered by HDL and reverse cholesterol transport. The LCAT reaction is shown in Figure 21-41. The inset shows a polarized light micrograph of cholesterol crystals. [Ralph C. Eagle, Jr./Science Source.]

attracted to regions with such LDL accumulations, and they differentiate into macrophages, which take up the oxidized LDL and the cholesterol they contain. Macrophages cannot limit their uptake of sterols, and with increasing accumulation of cholesteryl esters and free cholesterol, the macrophages become **foam cells** (they appear foamy when viewed under the microscope). As excess free cholesterol accumulates in foam cells and their membranes, the cells undergo apoptosis. Over long periods of time, arteries become progressively occluded as plaques consisting of extracellular matrix material, scar tissue formed from smooth muscle tissue, and foam cell remnants gradually become larger. Within the cholesterol-rich plaques, cholesterol can crystallize (Fig. 21-46, inset). Occasionally, a plaque breaks loose from the site of its formation and is carried through the blood to a narrowed region of an artery in the brain or the heart, causing a stroke or a heart attack. Cholesterol crystals that break loose from plaques can cause vascular injury.

In familial hypercholesterolemia, blood levels of cholesterol are extremely high and severe atherosclerosis develops in childhood. Affected individuals have a defective LDL receptor and lack receptor-mediated uptake of cholesterol carried by LDL. Consequently, cholesterol is not cleared from the blood; it accumulates in foam cells and contributes to the formation of atherosclerotic plaques. Endogenous cholesterol synthesis continues despite the excessive cholesterol in the blood, because extracellular cholesterol cannot enter cells to regulate intracellular synthesis (Fig. 21-44). Drugs in a class called **statins**, some isolated from natural sources and some synthesized industrially, are used to treat patients with elevated serum cholesterol caused by familial hypercholesterolemia and other conditions. The statins resemble mevalonate (**Box 21-2**) and are competitive inhibitors of HMG-CoA reductase.

An alternative approach to controlling serum cholesterol levels is to activate LXRs, which has the overall effect of decreasing cholesterol absorption and promoting its excretion. This is the mode of action of a drug called ezetimibe. Ezetimibe is not as effective in lowering serum cholesterol as statins. However, in combination with statins, it can lower risk of stroke or heart attack in high-risk patients. Because LXR activation also activates SREBP1C, causing the liver to increase its production of fatty acids and triacylglycerols, new classes of drugs that target only intestinal LXRs are being developed. ■

Reverse Cholesterol Transport by HDL Counters Plaque Formation and Atherosclerosis

HDL plays a critical role in the reverse cholesterol transport pathway (**Fig. 21-47**), reducing the potential damage from foam cell buildup. Depleted HDL (low in cholesterol) picks up cholesterol stored in extrahepatic tissues, including foam cells at nascent plaques, and carries it to the liver.

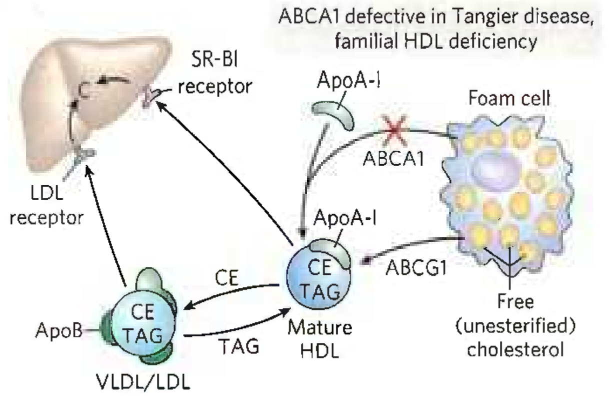


FIGURE 21-47 Reverse cholesterol transport. ApoA-I and HDLs pick up excess cholesterol (C) from peripheral cells, with the participation of ABCA1 and ABCG1 transporters, and return it to the liver. In individuals with genetically defective ABCA1, the failure of reverse cholesterol transport leads to severe and early cardiovascular diseases: Tangier disease and familial HDL deficiency disease. CE, cholesteryl esters; TAG, triacylglycerols. [Information from A. R. Tall et al., *Cell Metab.* 7:365, 2008, Fig. 1.]

Cholesterol movement out of cells requires transporters. The human genome encodes 48 transporters of the ATP-binding cassette (ABC) class, and about half of these promote lipid transport. Two of them transport cholesterol out of cells. In this process, apoA-I interacts with the transporter ABCA1 in a cholesterol-rich cell. ABCA1 transports a load of cholesterol from inside the cell to the outer surface of the plasma membrane, where lipid-free or lipid-poor apoA-I picks it up, then transports it to the liver. Another transporter, ABCG1, interacts with mature HDL, facilitating the movement of cholesterol out of the cell and into the HDL. This efflux process is particularly critical to reverse cholesterol transport away from foam cells at the sites of plaques in the blood vessels of individuals with cardiovascular disease.

In **familial HDL deficiency**, HDL levels are very low, and in **Tangier disease** they are almost undetectable (Fig. 21-47). Both genetic disorders are the result of mutations in the ABCA1 protein. ApoA-I in cholesterol-depleted HDL cannot take up cholesterol from cells that lack ABCA1 protein, and apoA-I and cholesterol-poor HDL are rapidly removed from the blood and destroyed. Both familial HDL deficiency and Tangier disease are very rare (worldwide, fewer than 100 families with Tangier disease are known), but the existence of these diseases establishes a role for ABCA1 and ABCG1 proteins in the regulation of plasma HDL levels. ■

Steroid Hormones Are Formed by Side-Chain Cleavage and Oxidation of Cholesterol

Humans derive all their steroid hormones from cholesterol (**Fig. 21-48**). P5 Two classes of steroid hormones are synthesized in the cortex of the adrenal gland: **mineralocorticoids**, which control the reabsorption of inorganic ions (Na^+, Cl^-, and HCO_3^-) by the kidney, and **glucocorticoids**, which help regulate gluconeogenesis and

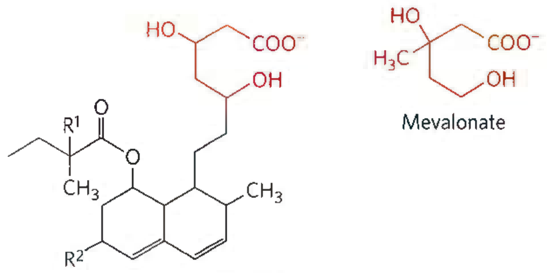
BOX 21-2 MEDICINE

The Lipid Hypothesis and the Development of Statins

Coronary heart disease is the leading cause of death in developed countries. The coronary arteries that bring blood to the heart become narrowed due to the formation of fatty deposits called atherosclerotic plaques, containing cholesterol, fibrous proteins, calcium deposits, blood platelets, and cell debris. Developing the link between artery occlusion (atherosclerosis) and blood cholesterol levels was a project of the twentieth century, triggering a dispute that was resolved only with the development of effective cholesterol-lowering drugs. The Framingham Heart Study, a longitudinal study begun in 1948 and continuing today, was aimed at identifying factors correlated with cardiovascular disease. About 5,000 participants from the city of Framingham, Massachusetts, underwent periodic physical examinations and lifestyle interviews. By 2002, participants of the third generation were included in the study. This monumental study led to the identification of risk factors for cardiovascular disease, including smoking, obesity, physical inactivity, diabetes, high blood pressure, and high blood cholesterol.

In 1913, N. N. Anitschkov, an experimental pathologist in Saint Petersburg, Russia, published a study showing that rabbits fed a diet rich in cholesterol developed lesions very similar to the atherosclerotic plaques seen in aging humans. Anitschkov continued his work over the next few decades, publishing it in prominent western journals. Nevertheless, the work was not accepted as a model for human atherosclerosis, due to a prevailing view that the disease was simply a consequence of aging and could not be prevented. The link between serum cholesterol and atherosclerosis (the lipid hypothesis) was gradually strengthened, however, until researchers in the 1960s openly suggested that therapeutic intervention might be helpful. Controversy persisted until the results of a large study of cholesterol lowering, sponsored by the U.S. National Institutes of Health, was published in 1984: the Coronary Primary Prevention Trial. This study conclusively showed a statistically significant decrease in heart attacks and strokes as a result of decreasing blood cholesterol level. The study made use of a bile acid–binding resin, cholestyramine, to control cholesterol. The results triggered a search for more effective therapeutic interventions. Some controversy persisted until development of the statins in the late 1980s and 1990s.

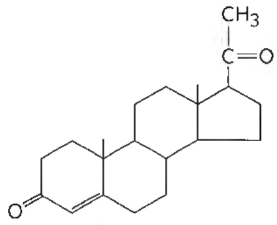
Dr. Akira Endo, working at the Sankyo company in Tokyo, discovered the first statin and reported the work in 1976. Endo had been interested in cholesterol metabolism for some time, and he speculated in 1971 that the fungi being screened at that time for new antibiotics might also contain an inhibitor of cholesterol synthesis. Over a period of several years, he screened more than 6,000 fungal cultures until a positive result emerged. The compound that resulted was named compactin (Fig. 1). This compound eventually proved effective in reducing cholesterol levels in dogs and monkeys, and the work came to the attention of Michael Brown and Joseph Goldstein at the University of Texas–Southwestern Medical School. Brown and Goldstein began to work with Endo, and they confirmed his results. Some dramatic results in the first limited clinical trials convinced several pharmaceutical firms to join the hunt for statins. A team at Merck, led by Alfred Alberts



$R^1 = H$	$R^2 = H$	Compactin
$R^1 = CH_3$	$R^2 = CH_3$	Simvastatin (Zocor)
$R^1 = H$	$R^2 = OH$	Pravastatin (Pravachol)
$R^1 = H$	$R^2 = CH_3$	Lovastatin (Mevacor)

FIGURE 1 Statins as inhibitors of HMG-CoA reductase. A comparison of the structures of mevalonate and four pharmaceutical compounds (statins) that inhibit HMG-CoA reductase.

reduce the inflammatory response. Sex hormones are produced in male and female gonads and the placenta. They include **progesterone**, which regulates the female reproductive cycle, and **androgens** (such as testosterone) and **estrogens** (such as estradiol), which influence the development of secondary sexual characteristics in males and females, respectively. Steroid hormones are effective at very low concentrations and are therefore synthesized in relatively small quantities. In comparison with the bile salts, their production consumes relatively little cholesterol.

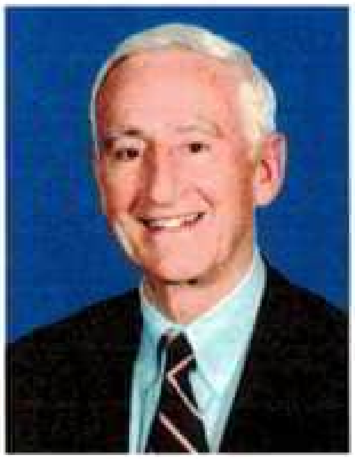


Progesterone

Synthesis of steroid hormones requires removal of some or all of the carbons in the "side chain" on C-17

Akira Endo
[Courtesy of Akira Endo, Ph.D.]

Alfred Alberts (1931–2018)
[Courtesy of Eli Alberts.]


P. Roy Vagelos
[Courtesy of P. Roy Vagelos.]

and P. Roy Vagelos, began screening fungal cultures and found a positive result after screening just 18 cultures. The new statin was eventually called lovastatin (Fig. 1). In 1980, a rumor that compactin, at very high doses, was carcinogenic in dogs almost sidelined the race to develop statins, but the benefits to people with familial hypercholesterolemia were already evident. After much consultation with experts around the world and with the U.S. Food and Drug Administration, Merck proceeded carefully to develop lovastatin. Extensive testing over the next two decades revealed no carcinogenic effects from lovastatin, or from the newer generations of statins that have appeared since.

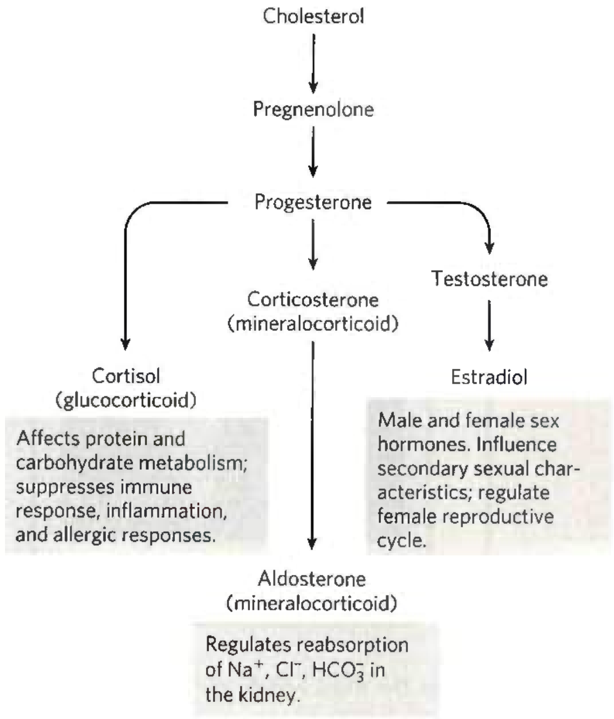
Statins inhibit HMG-CoA reductase, in part, by mimicking the structure of mevalonate (Fig. 1), and thus inhibit cholesterol synthesis. Lovastatin treatment lowers serum cholesterol by as much as 30% in individuals with hypercholesterolemia resulting from one defective copy of the gene for the LDL receptor. When combined with an edible resin that binds bile acids and prevents their reabsorption from the intestine, the statin is even more effective.

Statins are now the most widely used drugs for lowering serum cholesterol levels. Side effects are always a concern with drugs, but in the case of statins, many of the side effects are positive. These drugs can improve blood flow, enhance the stability of atherosclerotic plaques (so they don't rupture and obstruct blood flow), reduce platelet aggregation, and reduce vascular inflammation. In patients taking statins for the first time, some of these effects occur before cholesterol levels drop and may be related to a secondary inhibition of isoprenoid synthesis. Not all effects of statins are positive. Some individuals, usually among those taking statins in combination with other cholesterol-lowering drugs, experience muscle pain or weakness that can become severe and even debilitating. For these patients, the effects may sometimes be ameliorated by diet supplementation with coenzyme Q_{10}, a coenzyme that statin therapy depletes in the serum. A fairly long list of other side effects has been documented; most are rare. However, for the vast majority of people, the statin-mediated decrease in risks associated with coronary heart disease can be dramatic. As with all medications, statins should be used only in consultation with a physician.

of the D ring of cholesterol. Side-chain removal takes place in the mitochondria of steroidogenic tissues. Removal requires the hydroxylation of two adjacent carbons in the side chain (C-20 and C-22) followed by cleavage of the bond between them (**Fig. 21-49**). Formation of the various hormones also requires the introduction of oxygen atoms. All the hydroxylation and oxygenation reactions in steroid biosynthesis are catalyzed by mixed-function oxygenases (see Box 21-1) that use NADPH, O_2, and mitochondrial cytochrome P-450.

Intermediates in Cholesterol Biosynthesis Have Many Alternative Fates

In addition to its role as an intermediate in cholesterol biosynthesis, isopentenyl pyrophosphate is the activated precursor of a huge array of biomolecules with diverse biological roles (**Fig. 21-50**). **P5** They include vitamins A, E, and K; plant pigments such as carotene and the phytol chain of chlorophyll; natural rubber; many essential oils, such as the fragrant principles of lemon oil, eucalyptus, and musk; insect juvenile hormone, which controls


Cholesterol
Pregnenolone
Progesterone
Corticosterone (mineralocorticoid)
Testosterone
Cortisol (glucocorticoid)
Estradiol
Aldosterone (mineralocorticoid)

Affects protein and carbohydrate metabolism; suppresses immune response, inflammation, and allergic responses.

Male and female sex hormones. Influence secondary sexual characteristics; regulate female reproductive cycle.

Regulates reabsorption of Na^+, Cl^-, HCO_3^- in the kidney.

FIGURE 21-48 Some steroid hormones derived from cholesterol. The structures of some of these compounds are shown in Figure 10-18.

metamorphosis; dolichols, which serve as lipid-soluble carriers in complex polysaccharide synthesis; and ubiquinone and plastoquinone, electron carriers in mitochondria and chloroplasts. Collectively, these molecules are called isoprenoids. More than 20,000 different isoprenoid molecules have been discovered in nature, and hundreds of new ones are reported each year.

Prenylation, the covalent attachment of an isoprenoid (see Fig. 27-35), is a common mechanism by which proteins are anchored to the inner surface of cellular membranes in mammals (see Fig. 11-16). In some of these proteins, the attached lipid is the 15-carbon farnesyl group; others have the 20-carbon geranylgeranyl group. Different enzymes attach the two types of lipids. It is possible that prenylation targets proteins to different membranes, depending on which lipid is attached. Protein prenylation is another important role for the isoprene derivatives of the pathway to cholesterol.

SUMMARY 21.4 *Cholesterol, Steroids, and Isoprenoids: Biosynthesis, Regulation, and Transport*

- Cholesterol is formed from acetyl-CoA in a complex series of reactions, through the intermediates β-hydroxy-β-methylglutaryl-CoA (HMG-CoA), mevalonate, and two activated isoprenes, dimethylallyl pyrophosphate and isopentenyl pyrophosphate. Condensation of isoprene units produces the noncyclic squalene, which is cyclized to yield the steroid ring system and side chain.

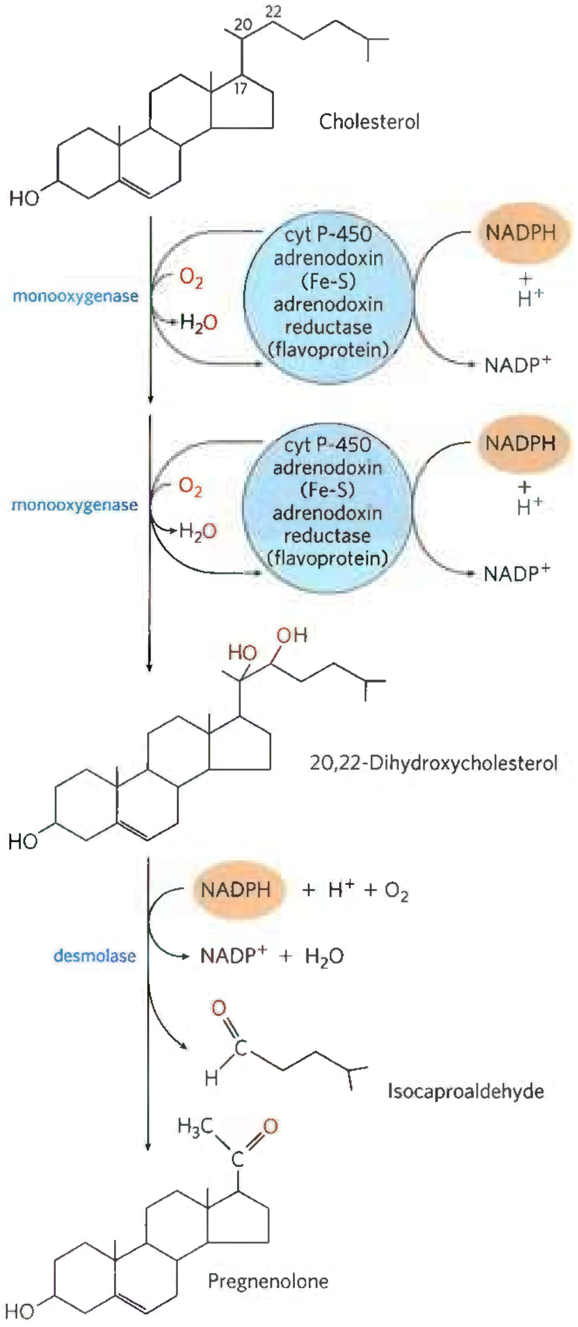

20
22
17
HO
Cholesterol
monooxygenase
O_2
H_2O
cyt P-450
adrenodoxin (Fe-S)
adrenodoxin reductase (flavoprotein)
NADPH
+ H^+
$NADP^+$
monooxygenase
O_2
H_2O
cyt P-450
adrenodoxin (Fe-S)
adrenodoxin reductase (flavoprotein)
NADPH
+ H^+
$NADP^+$
HO
OH
20,22-Dihydroxycholesterol
desmolase
NADPH + H^+ + O_2
$NADP^+$ + H_2O
O
C
H
Isocaproaldehyde
H_3C
O
C
Pregnenolone

FIGURE 21-49 Side-chain cleavage in the synthesis of steroid hormones. Cytochrome P-450 acts as electron carrier in this monooxygenase system that oxidizes adjacent carbons. The process also requires the electron-transferring proteins adrenodoxin and adrenodoxin reductase. This system for cleaving side chains is found in mitochondria of the adrenal cortex, where active steroid production occurs. Pregnenolone is the precursor of all other steroid hormones (see Fig. 21-48).

- Synthesized primarily in the liver, cholesterol is exported as bile acids, biliary cholesterol, or cholesteryl esters.
- Cholesterol and cholesteryl esters are carried in the blood as plasma lipoproteins. VLDL carries cholesterol, cholesteryl esters, and triacylglycerols from the liver to other tissues, where the triacylglycerols are degraded by

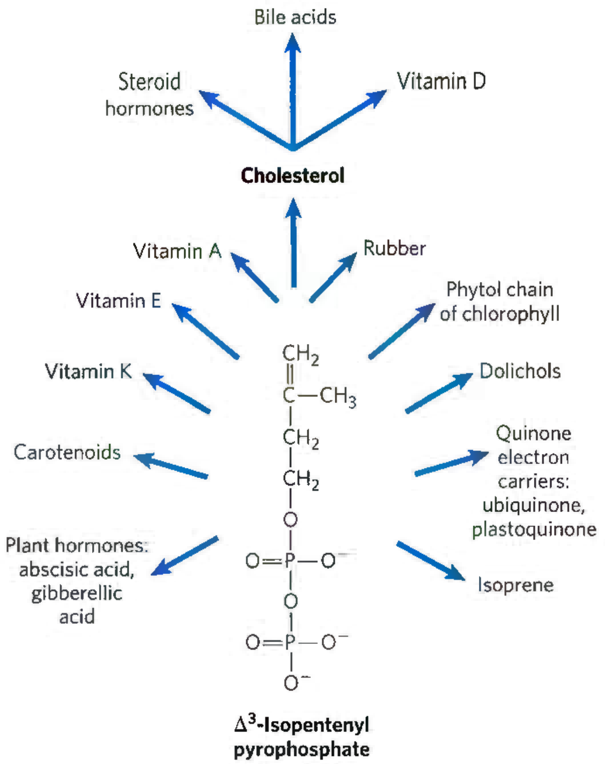

Bile acids
Steroid hormones
Vitamin D
Cholesterol
Vitamin A
Rubber
Vitamin E
Phytol chain of chlorophyll
Vitamin K
Dolichols
Carotenoids
Quinone electron carriers: ubiquinone, plastoquinone
Plant hormones: abscisic acid, gibberellic acid
Isoprene
Δ3-Isopentenyl pyrophosphate

FIGURE 21-50 Overview of isoprenoid biosynthesis. The structures of most of the end products shown here are given in Chapter 10.

lipoprotein lipase, converting VLDL to LDL. Cholesterol scavenging and transport back to the liver are mediated by HDL. Much of the lipid carried to the liver is utilized in bile salts.

- The LDL, rich in cholesterol and its esters, is taken up by receptor-mediated endocytosis, in which the apolipoprotein B-100 of LDL is recognized by receptors in the plasma membrane.
- Cholesterol synthesis and transport are under complex regulation by hormones, cellular cholesterol content, and energy level (AMP concentration). HMG-CoA reductase is regulated allosterically and by covalent modification. Furthermore, a complex of three proteins—Insig, SCAP, and SREBP—sense cholesterol levels and trigger increased synthesis or degradation of HMG-CoA reductase in response. The number of LDL receptors per cell is also regulated by cholesterol content.
- Dietary conditions or genetic defects in cholesterol metabolism may lead to atherosclerosis and heart disease.
- In reverse cholesterol transport, HDL removes cholesterol from peripheral tissues, carrying it to the liver. By reducing the cholesterol content of foam cells, HDL protects against atherosclerosis.
- The steroid hormones (glucocorticoids, mineralocorticoids, and sex hormones) are produced from cholesterol by alteration of the side chain and introduction of oxygen atoms into the steroid ring system. In addition to cholesterol, a wide variety of isoprenoid compounds are derived from mevalonate through condensations of isopentenyl pyrophosphate and dimethylallyl pyrophosphate.
- Prenylation of certain proteins targets them for association with cellular membranes and is essential for their biological activity.

KEY TERMS

Terms in bold are defined in the glossary.

malonyl-CoA 744
acetyl-CoA carboxylase 744
fatty acid synthase 745
acyl carrier protein (ACP) 747
4′-phosphopantetheine 747
fatty acid elongation systems 753
fatty acyl–CoA desaturase 754
mixed-function oxidases 754
stearoyl-ACP desaturase (SCD) 754
essential fatty acids 754
arachidonate 754
eicosatetraenoate 754
prostaglandin (PG) 755
cyclooxygenase (COX) 755
prostaglandin H_2 synthase 755
mixed-function oxygenases 756
cytochrome P-450 756
thromboxane synthase 758
thromboxane (TX) 758
leukotriene (LT) 759
catabasis 759
specialized pro-resolving mediator (SPM) 759
lipoxin 759
glycerol 3-phosphate dehydrogenase 760
phosphatidic acid 760
triacylglycerol cycle 762
glyceroneogenesis 762
thiazolidinediones 763
phosphatidylglycerol 765
cardiolipin 765
phosphatidylinositol 765
phosphatidylserine 765
phosphatidylethanolamine 765
phosphatidylcholine 765
Lands cycle 767
lysophosphatidylcholine acyltransferases (LPCATs) 767
plasmalogen 768
platelet-activating factor 768
cerebroside 770
sphingomyelin 770
ganglioside 770
isoprene 772
mevalonate 773
β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) 773
HMG-CoA synthase 773
HMG-CoA reductase 773
squalene 774
bile acids 776
cholesteryl esters 777
apolipoprotein 777
chylomicron 777
exogenous pathway 780
very-low-density lipoprotein (VLDL) 780
low-density lipoprotein (LDL) 780
LDL receptors 780
endogenous pathway 780
high-density lipoprotein (HDL) 780
reverse cholesterol transport 781
enterohepatic circulation 781
receptor-mediated endocytosis 781
sterol regulatory element-binding proteins (SREBPs) 782
SREBP cleavage-activating protein (SCAP) 783

insulin-induced gene protein (Insig) 783
liver X receptor (LXR) 783
retinoid X receptor (RXR) 783
farnesoid X receptor (FXR) 784
atherosclerosis 784
foam cell 785
statin 785

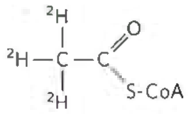
PROBLEMS

1. **Pathway of Carbon in Fatty Acid Synthesis** Using your knowledge of fatty acid biosynthesis, provide an explanation for the two experimental observations.
(a) A biochemist adds uniformly labeled [^{14}C]acetyl-CoA to a soluble liver fraction, which yields palmitate uniformly labeled with ^{14}C.
(b) In a second experiment, the biochemist adds a *trace* of uniformly labeled [^{14}C]acetyl-CoA in the presence of an excess of unlabeled malonyl-CoA to a soluble liver fraction, which yields palmitate labeled with ^{14}C only in C-15 and C-16.

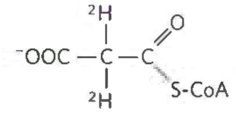
2. **Synthesis of Fatty Acids from Glucose** After a person has ingested large amounts of sucrose, the body transforms the glucose and fructose that exceed caloric requirements to fatty acids for triacylglycerol synthesis. This fatty acid synthesis consumes acetyl-CoA, ATP, and NADPH. How do cells produce acetyl-CoA, ATP, and NADPH from glucose?

3. **Net Equation of Fatty Acid Synthesis** Write the net equation for the biosynthesis of palmitate in rat liver, starting from mitochondrial acetyl-CoA and cytosolic NADPH, ATP, and CO_2.

4. **Pathway of Hydrogen in Fatty Acid Synthesis** A researcher has prepared a solution that contains all the enzymes and cofactors necessary for fatty acid biosynthesis from added acetyl-CoA and malonyl-CoA.
(a) She then adds [2-^{2}H]acetyl-CoA (labeled with deuterium, the heavy isotope of hydrogen) and an excess of unlabeled malonyl-CoA as substrates.



How many deuterium atoms incorporate into every molecule of palmitate? What are their locations? Explain.
(b) In a separate experiment, the researcher adds unlabeled acetyl-CoA and [2-^{2}H]malonyl-CoA as substrates.



How many deuterium atoms incorporate into every molecule of palmitate? What are their locations? Explain.

5. **Energetics of β-Ketoacyl-ACP Synthase** The condensation reaction catalyzed by β-ketoacyl-ACP synthase (see Fig. 21-6) synthesizes a four-carbon unit by combining a two-carbon unit and a three-carbon unit, with the release of CO_2. What is the thermodynamic rationale for this process relative to one that simply combines two two-carbon units?

6. **Modulation of Acetyl-CoA Carboxylase** Acetyl-CoA carboxylase is the principal regulation point in the biosynthesis of fatty acids. Following are some of the properties of the enzyme:
(a) Addition of citrate or isocitrate raises the V_{max} of the enzyme as much as 10-fold.
(b) The enzyme exists in two interconvertible forms that differ markedly in their activities:

$$\text{Protomer (inactive)} \rightleftharpoons \text{filamentous polymer (active)}$$

Citrate and isocitrate bind preferentially to the filamentous form, and palmitoyl-CoA binds preferentially to the protomer.

Explain how these properties are consistent with the regulatory role of acetyl-CoA carboxylase in the biosynthesis of fatty acids.

7. **Shuttling of Acetyl Groups across the Inner Mitochondrial Membrane** The acetyl group shuttle transfers acetyl-CoA, produced by oxidative decarboxylation of pyruvate in the mitochondrion, to the cytosol as outlined in Figure 21-10.
(a) Write the overall equation for the transfer of one acetyl group from the mitochondrion to the cytosol.
(b) What is the cost of this process in ATPs per acetyl group?
(c) In Chapter 17 we encountered an acyl group shuttle in the transfer of fatty acyl–CoA from the cytosol to the mitochondrion in preparation for β oxidation (see Fig. 17-6). One result of that shuttle is separation of the mitochondrial and cytosolic pools of CoA. Does the acetyl group shuttle also accomplish this? Explain.

8. **Oxygen Requirement for Desaturases** The biosynthesis of palmitoleate (see Fig. 21-12), a common unsaturated fatty acid with a cis double bond in the Δ^9 position, uses palmitate as a precursor. Can palmitoleate synthesis be carried out under strictly anaerobic conditions? Explain.

9. **Energy Cost of Triacylglycerol Synthesis** Use a net equation for the biosynthesis of tripalmitoylglycerol (tripalmitin) from glycerol and palmitate to show how many ATPs are required per molecule of tripalmitin formed.

10. **Turnover of Triacylglycerols in Adipose Tissue** A researcher adds [^{14}C]glucose to the balanced diet of adult rats. She finds no increase in the total amount of stored triacylglycerols, but the triacylglycerols become labeled with ^{14}C. Explain.

11. **Energy Cost of Phosphatidylcholine Synthesis** Write the sequence of steps and the net reaction for the biosynthesis of phosphatidylcholine by the salvage pathway from oleate, palmitate, dihydroxyacetone phosphate, and choline. Starting from these precursors, what is the cost (in number of ATPs) of the synthesis of phosphatidylcholine by the salvage pathway?

12. **Salvage Pathway for Synthesis of Phosphatidylcholine** A young rat maintained on a diet deficient in methionine fails to thrive unless choline is included in the diet. Explain.

13. **Energetics of Acetyl-CoA Condensation to Form Acetoacetyl-CoA** The formation of a thioester of acetoacetate

is catalyzed by fatty acid synthase during fatty acid synthesis, and by acetyl-CoA acetyltransferase in the first step of cholesterol biosynthesis. Both are Claisen condensations. However, in fatty acid synthesis, malonyl-CoA forms in an earlier step so that decarboxylation facilitates the condensation. In the cholesterol biosynthesis pathway, the condensation occurs between two acetyl-CoA molecules, and no decarboxylation occurs to facilitate the reaction. Suggest a reason why the thermodynamic augmentation of decarboxylation is needed in fatty acid synthesis, but not in the first steps of cholesterol biosynthesis.

14. Synthesis of Isopentenyl Pyrophosphate A researcher adds [2-^{14}C]acetyl-CoA to a rat liver homogenate that is synthesizing cholesterol. Where will the ^{14}C label appear in Δ^3-isopentenyl pyrophosphate, the activated form of an isoprene unit?

15. Activated Donors in Lipid Synthesis In the biosynthesis of complex lipids, components are assembled by transfer of the appropriate group from an activated donor. For example, the activated donor of acetyl groups is acetyl-CoA. For each of the following groups, give the form of the activated donor: **(a)** phosphate; **(b)** D-glucosyl; **(c)** phosphoethanolamine; **(d)** D-galactosyl; **(e)** fatty acyl; **(f)** methyl; **(g)** the two-carbon group in fatty acid biosynthesis; **(h)** Δ^3-isopentenyl.

16. Importance of Fats in the Diet When young rats are placed on a completely fat-free diet, they grow poorly, develop a scaly dermatitis, lose hair, and soon die. These symptoms can be prevented if linoleate or plant material is included in the diet. What makes linoleate an essential fatty acid? Why can plant material be substituted?

17. Regulation of Cholesterol Biosynthesis Cholesterol in humans can be obtained from the diet or synthesized de novo. An adult human on a low-cholesterol diet typically synthesizes 600 mg of cholesterol per day in the liver. If the amount of cholesterol in the diet is large, de novo synthesis of cholesterol is drastically reduced. How is this regulation brought about?

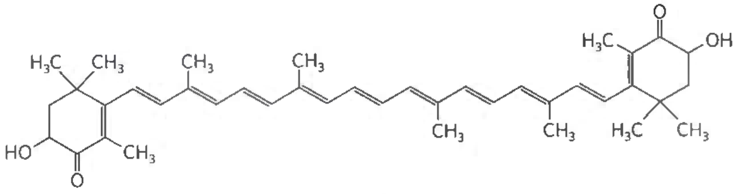
18. Lowering Serum Cholesterol Levels with Statins Patients treated with a statin drug generally exhibit a dramatic lowering of serum cholesterol. However, the amount of the enzyme HMG-CoA reductase present in cells can increase substantially. Suggest a simple explanation for this effect.

19. Roles of Thiol Esters in Cholesterol Biosynthesis Draw a mechanism for each of the three reactions shown in Figure 21-34, detailing the pathway for the synthesis of mevalonate from acetyl-CoA.

20. Potential Side Effects of Treatment with Statins Although the benefits of taking statins have become clear, side effects have yet to be documented in detail. Some physicians have suggested that patients being treated with statins should also take a supplement of coenzyme Q. Suggest a rationale for this recommendation.

DATA ANALYSIS PROBLEM

21. Engineering *E. coli* to Produce Large Quantities of an Isoprenoid There are more than 20,000 naturally occurring isoprenoids, some of which are medically or commercially important and produced industrially. The production methods include in vitro enzymatic synthesis, an expensive and low-yield process. In 1999, Wang, Oh, and Liao reported on their experiments to engineer the easily grown bacterium *E. coli* to produce large amounts of astaxanthin, a commercially important isoprenoid. Astaxanthin is a red-orange carotenoid pigment (an antioxidant) produced by marine algae. Marine animals such as shrimp, lobster, and some fish that feed on the algae get their orange color from the ingested astaxanthin. Astaxanthin is composed of eight isoprene units; its molecular formula is $C_{40}H_{52}O_4$.



Astaxanthin

(a) Circle the eight isoprene units in the astaxanthin molecule. Hint: Use the projecting methyl groups as a guide.

Astaxanthin is synthesized by the pathway shown on the next page, starting with Δ^3-isopentenyl pyrophosphate (IPP). Steps ❶ and ❷ are shown in Figure 21-36, and the reaction catalyzed by IPP isomerase is shown in Figure 21-35.

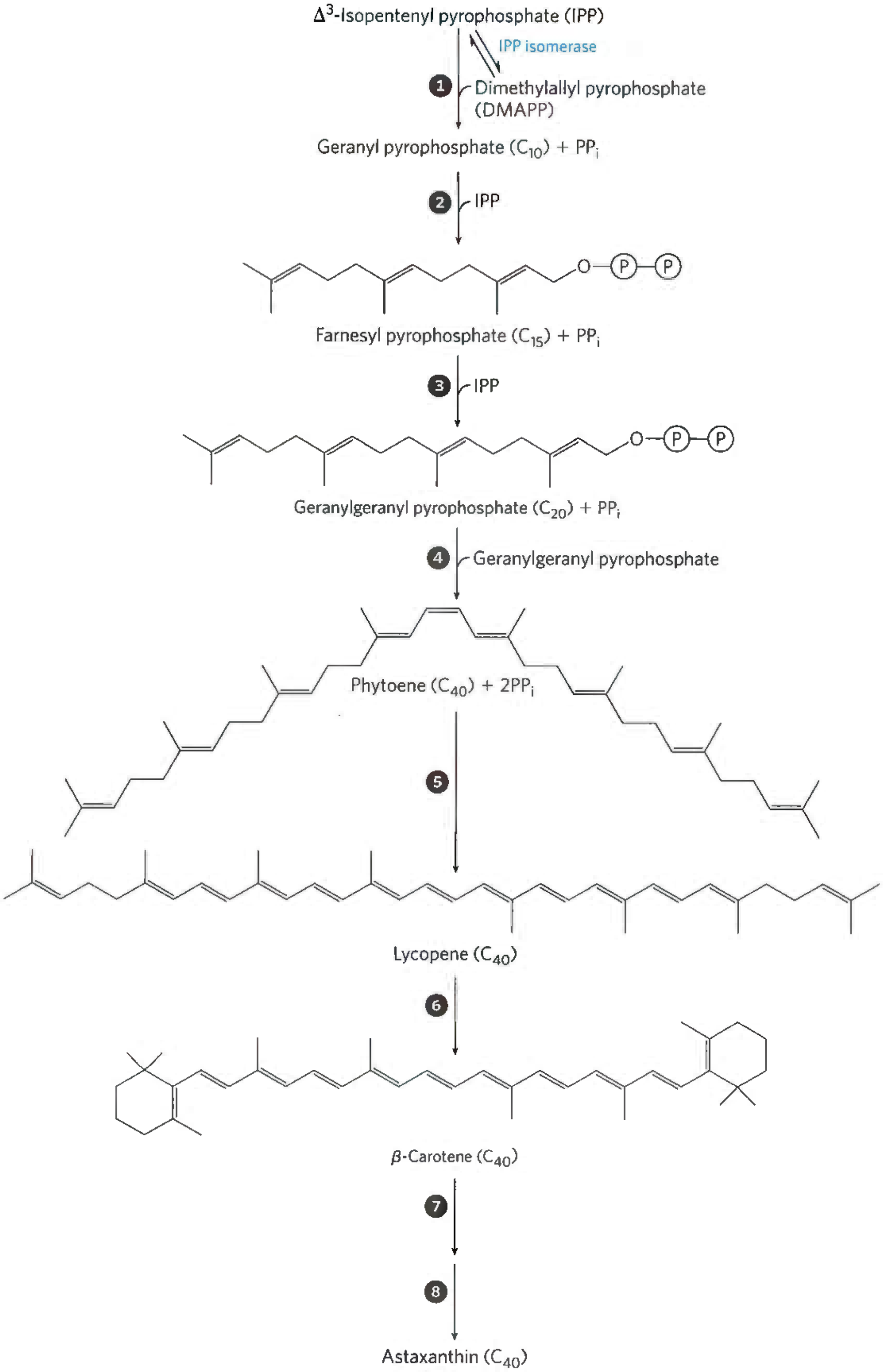
(b) In step ❹ of the pathway, two molecules of geranylgeranyl pyrophosphate are linked to form phytoene. Is this a head-to-head joining or is it a head-to-tail joining? (See Figure 21-36 for details.)

(c) Briefly describe the chemical transformation in step ❺.

(d) The synthesis of cholesterol (Fig. 21-37) includes a cyclization (ring closure) that requires a net oxidation by O_2. Does the cyclization in step ❻ of the astaxanthin synthetic pathway require a net oxidation of the substrate (lycopene)? Explain your reasoning.

E. coli does not make large quantities of many isoprenoids, and does not synthesize astaxanthin. It is known to synthesize small amounts of IPP, DMAPP, geranyl pyrophosphate, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate. Wang and colleagues cloned several of the *E. coli* genes that encode enzymes needed for astaxanthin synthesis, in plasmids that allowed their overexpression. These genes included *idi*, which encodes IPP isomerase, and *ispA*, which encodes a prenyl transferase that catalyzes steps ❶ and ❷.

To engineer an *E. coli* capable of the complete astaxanthin pathway, Wang and colleagues cloned several genes from other bacteria into plasmids that would allow their overexpression in *E. coli*. These genes included *crtE* from *Erwinia uredovora*, which encodes an enzyme that catalyzes step ❸; and *crtB*, *crtI*,


Δ^3-Isopentenyl pyrophosphate (IPP)
IPP isomerase
❶
Dimethylallyl pyrophosphate (DMAPP)
Geranyl pyrophosphate (C_{10}) + PP_i
❷
IPP
O—(P)—(P)
Farnesyl pyrophosphate (C_{15}) + PP_i
❸
IPP
O—(P)—(P)
Geranylgeranyl pyrophosphate (C_{20}) + PP_i
❹
Geranylgeranyl pyrophosphate
Phytoene (C_{40}) + $2PP_i$
❺
Lycopene (C_{40})
❻
β-Carotene (C_{40})
❼
❽
Astaxanthin (C_{40})

crtY, *crtZ*, and *crtW* from *Agrobacterium aurantiacum*, which encode enzymes for steps ❹, ❺, ❻, ❼, and ❽, respectively.

The investigators also cloned the gene *gps* from *Archaeoglobus fulgidus*, overexpressed this gene in *E. coli*, and extracted the gene product. When this extract was reacted with [^{14}C]IPP and DMAPP or geranyl pyrophosphate or farnesyl pyrophosphate, only ^{14}C-labeled geranylgeranyl pyrophosphate was produced in all cases.

(e) Based on these data, which step(s) in the pathway are catalyzed by the enzyme encoded by *gps*? Explain your reasoning.

Wang and coworkers then constructed several *E. coli* strains overexpressing different genes; they measured the orange color of the colonies (wild-type *E. coli* colonies are off-white) and the amount of astaxanthin produced (as measured by its orange color). Their results are shown below (ND indicates not determined).

Strain	Gene(s) overexpressed	Orange color	Astaxanthin yield (μg/g dry weight)
1	*crtBIZYW*	–	ND
2	*crtBIZYW, ispA*	–	ND
3	*crtBIZYW, idi*	–	ND
4	*crtBIZYW, idi, ispA*	–	ND
5	*crtBIZYW, crtE*	+	32.8
6	*crtBIZYW, crtE, ispA*	+	35.3
7	*crtBIZYW, crtE, idi*	++	234.1
8	*crtBIZYW, crtE, idi, ispA*	+++	390.3
9	*crtBIZYW, gps*	+	35.6
10	*crtBIZYW, gps, idi*	+++	1,418.8

(f) Comparing the results for strains 1 through 4 with those for strains 5 through 8, what can you conclude about the expression level of an enzyme capable of catalyzing step 3 of the astaxanthin synthetic pathway in wild-type *E. coli*? Explain your reasoning.

(g) Based on the data above, which enzyme is rate-limiting in this pathway, IPP isomerase or the enzyme encoded by *idi*? Explain your reasoning.

Reference

Wang, C.-W., M.-K. Oh, and J.C. Liao. 1999. Engineered isoprenoid pathway enhances astaxanthin production in *Escherichia coli*. *Biotechnol. Bioeng.* 62:235–241.

Chapter 22

BIOSYNTHESIS OF AMINO ACIDS, NUCLEOTIDES, AND RELATED MOLECULES

22.1 Overview of Nitrogen Metabolism *795*

22.2 Biosynthesis of Amino Acids *805*

22.3 Molecules Derived from Amino Acids *817*

22.4 Biosynthesis and Degradation of Nucleotides *823*

Nitrogen ranks behind only carbon, hydrogen, and oxygen in its contribution to the mass of living systems. Most of this nitrogen is bound up in amino acids and nucleotides. In this chapter we address all aspects of the metabolism of these nitrogen-containing compounds except amino acid catabolism, which is the subject of Chapter 18.

In this final chapter covering anabolic processes, some of the underlying principles are unique, while others will be familiar from Chapter 21 and the consideration of gluconeogenesis in Chapter 14:

P1 **Amino acids and nucleotides are the precursors to proteins and nucleic acids, respectively.** They also give rise to numerous neurotransmitters, metabolic cofactors, and other molecules of biological importance.

P2 **The supply of biologically available nitrogen can be limiting in many environments.** Atmospheric N_2 is relatively inert and must be converted to other forms, such as ammonia and nitrate, to accommodate the requirements of life. A complex web of enzymatic processes, based primarily in microorganisms, interconverts the various molecular forms that comprise the global inventory of reactive nitrogen.

P3 **Oxygen and nitrogen metabolism are interlinked.** The oxidation and reduction of the various forms of nitrogen in the biosphere often involve oxygen.

P4 **Regulation again plays an important role.** Many of the processes covered in this chapter are regulated to carefully conserve a critical and limited resource. Tight regulation is needed to maintain balanced supplies of amino acids and nucleotides. The metabolic flux through most of these pathways is far less than for carbohydrate or lipid biosynthetic pathways; most amino acids and nucleotides are not stored, but are synthesized as they are needed.

P5 **The amino acids glutamate and glutamine represent the entry point where reactive forms of nitrogen are incorporated into biological systems.** Reflecting their importance, the concentrations of these amino acids are sufficiently elevated in many tissues that they are major contributors to the electrochemical environment of cells. The prominent role of these two amino acids is a universal feature of animate nitrogen metabolism, yet another molecular manifestation of the shared evolutionary history of all organisms on the planet.

P6 **Like other anabolic pathways, the reaction sequences in amino acid and nucleotide biosynthesis are endergonic and reductive.** They use ATP as a source of metabolic energy and they use a reduced electron carrier (usually NADPH) as a reductant.

Discussing the biosynthetic pathways for amino acids and nucleotides together is a sound approach, not only because both classes of molecules contain nitrogen, but also because the two sets of pathways are extensively intertwined, with several key intermediates in common. Certain amino acids or parts of amino acids are incorporated into the structure of purines and pyrimidines, and in one case, part of a purine ring is incorporated into an amino acid (histidine). The two sets of pathways also share much common chemistry, in particular a preponderance of reactions involving the transfer of nitrogen or one-carbon groups.

The sheer number of steps and variety of intermediates in the pathways described here can be intimidating to the beginning biochemistry student. These pathways are best approached by maintaining a focus on metabolic principles we have already discussed, on key intermediates and precursors, and on common classes of reactions. Even a cursory look at the chemistry can be rewarding, for some of the most unusual chemical transformations in biological systems occur in these pathways; for instance, we find prominent examples of the rare biological use of the metals molybdenum, selenium, and vanadium. The effort also offers a practical dividend, especially for students of human or veterinary medicine. Many genetic diseases of humans and animals have been traced to an absence of one or more enzymes of amino acid and nucleotide metabolism, and many pharmaceuticals in common use to combat infectious diseases are inhibitors of enzymes in these pathways—as are a number of the most important agents in cancer chemotherapy.

22.1 Overview of Nitrogen Metabolism

The biosynthetic pathways leading to amino acids and nucleotides share a requirement for nitrogen. Soluble, biologically useful nitrogen compounds are generally scarce in natural environments; thus, most organisms use ammonia, amino acids, and nucleotides economically. P2 Available amino acids, purines, and pyrimidines formed during metabolic turnover of proteins and nucleic acids are often salvaged and reused. We begin by examining the pathways by which nitrogen from the environment is introduced into biological systems.

A Global Nitrogen Cycling Network Maintains a Pool of Biologically Available Nitrogen

The movement of nitrogen through the biosphere has been viewed historically as a cycle. However, our evolving understanding of the complexity of nitrogenous interconversions makes it clear that nitrogen moves through a complex web, rather than in a neat cycle (**Fig. 22-1**). Earth's atmosphere is four-fifths molecular nitrogen (N_2). However, N_2 is too unreactive to be of use to living organisms. Conversion of N_2 to forms that can support life (NH_3, NO_2^-, NO_3^-) is called **nitrogen fixation**. The reduction of N_2 to NH_3 plays such a central role in making N_2 available that this one reaction is often considered synonymous with nitrogen fixation. In the biosphere, the metabolic processes of countless species function interdependently to salvage and reuse biologically available nitrogen. Most of the key reactions are carried out by bacteria and archaea.

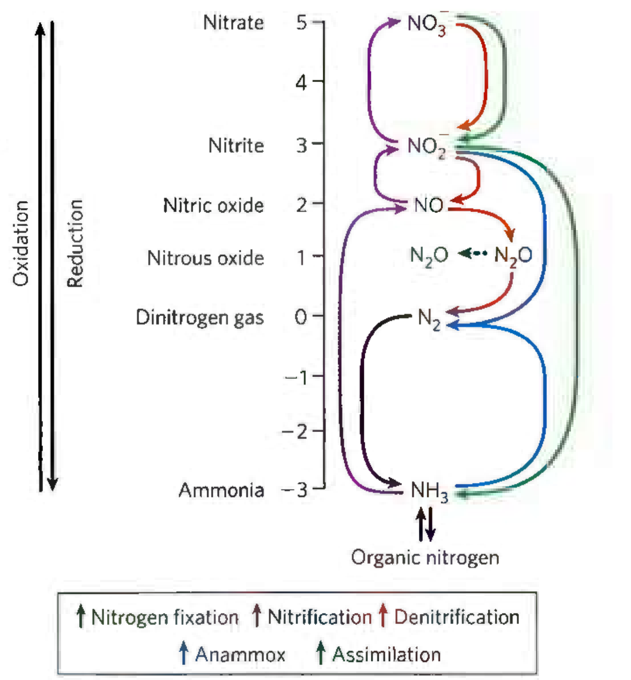


FIGURE 22-1 The global nitrogen web. The total amount of nitrogen fixed annually in the biosphere exceeds 10^{11} kg; industrial sources of fixed nitrogen are now nearly as great. Reactions are identified with the processes they are involved in by colored arrows (see key). The oxidation number of the N atom is indicated on the vertical axis. [Information from M. M. M. Kuypers et al., *Nat. Rev. Microbiol.* 16:263, 2018, Fig. 1.]

The reduction of atmospheric nitrogen (N_2) by nitrogen-fixing bacteria and archaea to yield ammonia (NH_3 or NH_4^+), provides a useful anchor for our discussion. This critical process, described in detail in Section 22.2, provides most of the reduced nitrogen for incorporation into biomolecules.

Free ammonia does not build up, and reduction is balanced by oxidation. P3 Bacteria that derive their energy by oxidizing ammonia to nitrite (NO_2^-) and ultimately nitrate (NO_3^-) are abundant and active in both terrestrial and marine environments. The processes of converting ammonia to nitric oxide, nitrite, and finally nitrate are known as **nitrification** (Fig. 22-1, purple arrows). Individual bacterial or archaeal species may promote one or more of these steps.

Atmospheric N_2 must be replaced in order to maintain levels at a steady-state concentration. Some of the replacement comes from reduction of nitrate and nitrite. The reduction of nitrate and nitrite to N_2 under anaerobic conditions, a process called **denitrification** (Fig. 22-1, red arrows), is carried out by specialized microorganisms in all three domains of life. These organisms use NO_3^- or NO_2^- rather than O_2 as the ultimate electron acceptor in a series of reactions that (like oxidative phosphorylation) generates a transmembrane proton gradient, which is used to synthesize ATP. These microorganisms exist in all anoxic environments where nitrate is present, including soils, marine sediments, and eutrophic marine zones. An alternative path back to atmospheric N_2 is provided by a group of bacteria that promote anaerobic ammonia oxidation, or **anammox** (Fig. 22-1, blue arrows). Anammox converts ammonia and nitrite to N_2. As much as 50% to 70% of the NH_3-to-N_2 conversion in the biosphere may occur through this pathway, which went undetected until the 1980s. The obligate anaerobes that promote anammox are fascinating in their own right and are providing some useful solutions to waste-treatment problems (**Box 22-1**).

Fixation of atmospheric N_2 is not the only source of reduced ammonia for biological systems. Much of it comes from an alternative fate of nitrate that circumvents denitrification. More than 90% of the NH_4^+ generated by vascular plants, algae, and microorganisms comes from **nitrate assimilation**, a two-step reductive process that bypasses atmospheric N_2. First NO_3^- is reduced to NO_2^- by **nitrate reductase**, then the NO_2^- is reduced to NH_4^+ in a six-electron transfer catalyzed by **nitrite reductase** (**Fig. 22-2**). Both reactions involve chains of electron carriers and cofactors we have not yet encountered. Nitrate reductase is a large, soluble protein (M_r 220,000). Within the enzyme, a pair of electrons, donated by NADH, flows through —SH groups of cysteine, FAD, and a cytochrome (cyt b_{557}), then to a novel cofactor containing molybdenum, before reducing the substrate NO_3^- to NO_2^-.

The nitrite reductase of plants is located in the chloroplasts and receives its electrons from ferredoxin (which is reduced in the light-dependent reactions of photosynthesis; see Section 20.2). Six electrons, donated one at a time by ferredoxin, pass through a 4Fe-4S center in the enzyme, then through a novel hemelike molecule (siroheme) before reducing NO_2^- to NH_4^+ (Fig. 22-2). **P6** Nonphotosynthetic microbes possess a distinct nitrite reductase for which NADPH is the electron donor.

Human activity presents an increasing challenge to the global nitrogen balance, and to all life in the biosphere supported by that balance. **P2** Fixed nitrogen is increasingly necessary to boost production in agriculture. Industrial nitrogen-based fertilizers now contribute as much ammonia and other reactive nitrogen species to the biosphere as do natural processes. Nonfarming manufacturing activity releases additional reactive nitrogen into the atmosphere, including nitric oxide, a prominent greenhouse gas. Controlling the damaging effects of agricultural runoff and industrial pollutants will remain an important component of the continuing effort to expand the food supply for a growing human population.

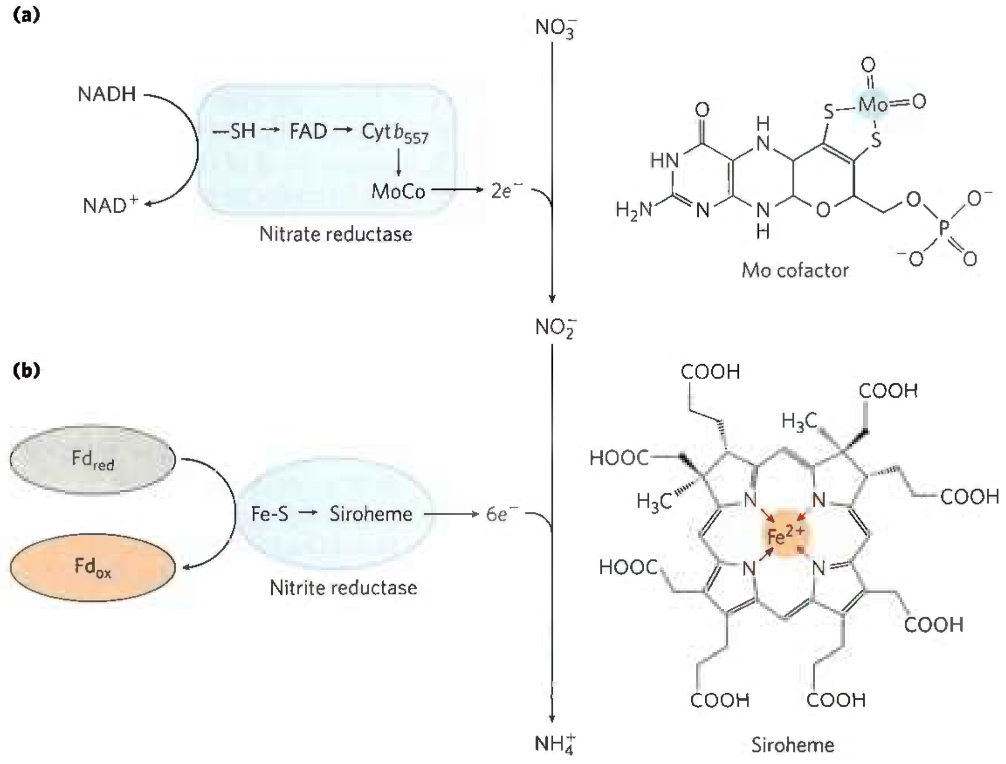


FIGURE 22-2 Nitrate assimilation by nitrate reductase and nitrite reductase. (a) Nitrate reductases of plants and bacteria catalyze the two-electron reduction of NO_3^- to NO_2^-, in which a novel Mo-containing cofactor plays a central role. NADH is the electron donor. (b) Nitrite reductase converts the product of nitrate reductase into NH_4^+ in a six-electron, eight-proton transfer process in which the metallic center in siroheme carries electrons and the carboxyl groups of siroheme may donate protons. The initial source of electrons is reduced ferredoxin.

Nitrogen Is Fixed by Enzymes of the Nitrogenase Complex

The availability of fixed nitrogen, an essential nutrient, may have limited the size of the primordial biosphere. As early cells acquired a capacity to fix atmospheric nitrogen, the biosphere expanded. Evidence for biological nitrogen fixation has been found in sedimentary rocks more than 3 billion years old.

In the biosphere of today, only certain bacteria and archaea can fix atmospheric N_2. These organisms, called diazotrophs, include the cyanobacteria of soils and fresh and salt waters, methanogenic archaea (strict anaerobes that obtain energy and carbon by converting H_2 and CO_2 to methane), other kinds of free-living soil bacteria such as *Azotobacter* species, and the nitrogen-fixing bacteria that live as **symbionts** in the root nodules of leguminous plants. The most important product of nitrogen fixation is ammonia, which can be used by all organisms either directly or after its conversion to other soluble compounds such as nitrites, nitrates, or amino acids.

The reduction of nitrogen to ammonia is an exergonic reaction:

$$N_2 + 3H_2 \longrightarrow 2NH_3 \qquad \Delta G'^{\circ} = -33.5 \text{ kJ/mol}$$

The N≡N triple bond, however, is very stable, with a bond energy of 930 kJ/mol. Nitrogen fixation therefore has an extremely high activation energy, and atmospheric nitrogen is almost chemically inert under normal conditions. Ammonia is produced industrially by the Haber process (named for its inventor, Fritz Haber), which requires temperatures of 400 to 500 °C and nitrogen and hydrogen at pressures of tens of thousands of kilopascals (several hundred atmospheres) to provide the necessary activation energy.

Biological nitrogen fixation must occur at biological temperatures and at 0.8 atm of nitrogen, and the high activation barrier is overcome by other means. This is accomplished, at least in part, by the binding and hydrolysis of ATP. The overall reaction can be written

$$N_2 + 10H^+ + 8e^- + 16ATP \longrightarrow 2NH_4^+ + 16ADP + 16P_i + H_2$$

Biological nitrogen fixation to produce ammonia is carried out by a highly conserved complex of proteins called the **nitrogenase complex**; its central components are **dinitrogenase reductase** and **dinitrogenase** (**Fig. 22-3a**). Dinitrogenase reductase (M_r 60,000) is a dimer of two identical subunits. It contains a single 4Fe-4S redox center (see Fig. 19-5), bound between the subunits, and can be oxidized and reduced by one electron. It also has two binding sites for ATP/ADP (one site on each subunit). Dinitrogenase (M_r 240,000), an $\alpha_2\beta_2$ tetramer, has two Fe-containing cofactors that transfer electrons (Fig. 22-3b). One, the **P cluster**, has a pair of 4Fe-4S centers; these share a sulfur atom, making an

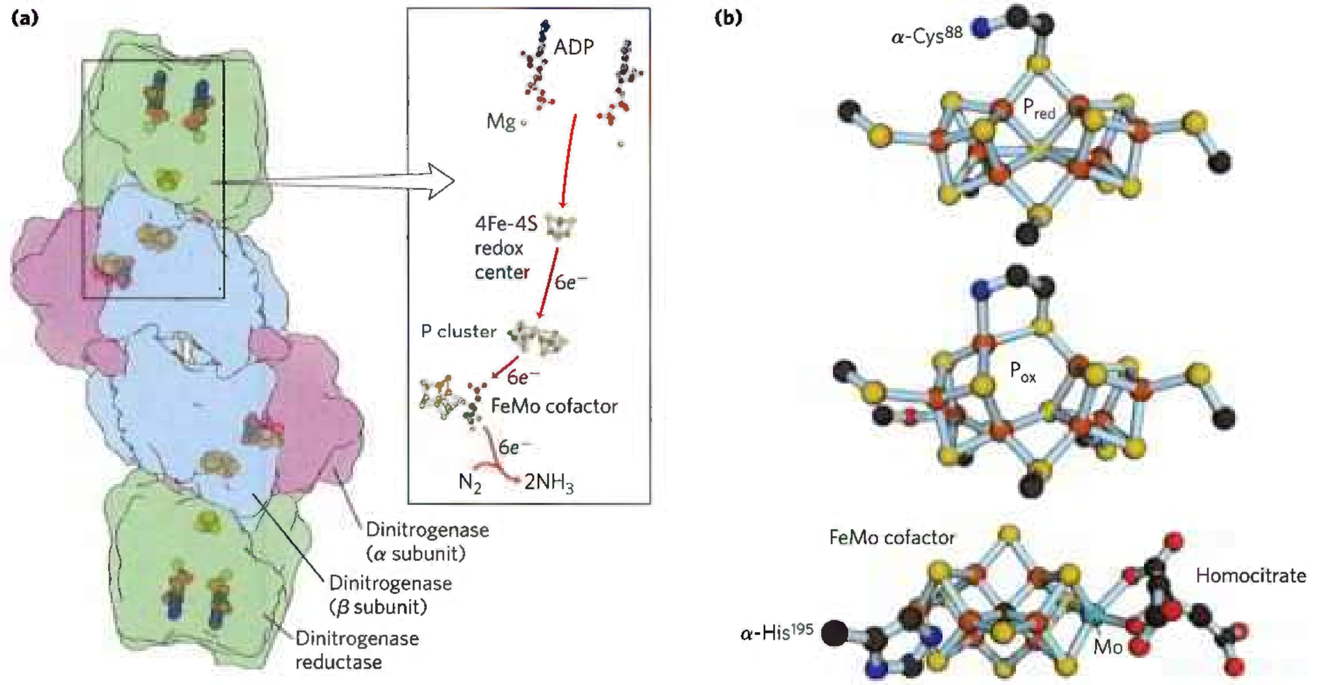


FIGURE 22-3 Enzymes and cofactors of the nitrogenase complex. (a) The holoenzyme consists of two identical dinitrogenase reductase molecules (green), each with a 4Fe-4S redox center and binding sites for two ATP, and two identical dinitrogenase heterodimers (purple and blue), each with a P cluster (Fe-S center) and an FeMo cofactor. In this structure, ADP is bound in the ATP site, to make the crystal more stable. (b) The electron-transfer cofactors. A P cluster is shown here in its reduced (top) and oxidized (middle) forms. The FeMo cofactor (bottom) has a Mo atom with three S ligands, a His ligand, and two oxygen ligands from a molecule of homocitrate. In some organisms, the Mo atom is replaced with a vanadium atom. (Fe is shown in orange, S in yellow.) [Data from (a) PDB ID 1N2C, H. Schindelin et al., *Nature* 387:370, 1997; (b) P_{red}: PDB ID 3MIN, and P_{ox}: PDB ID 2MIN, J. W. Peters et al., *Biochemistry* 36:1181, 1997; FeMo cofactor: PDB ID 1M1N, O. Einsle et al., *Science* 297:1696, 2002.]

BOX 22-1

Unusual Lifestyles of the Obscure but Abundant

Air-breathers that we are, we can easily overlook the bacteria and archaea that thrive in anaerobic environments. Although rarely featured in introductory biochemistry textbooks, these organisms constitute much of the biomass of this planet, and their contributions to the balance of carbon and nitrogen in the biosphere are essential to all forms of life.

As detailed in earlier chapters, the energy used to maintain living systems relies on the generation of proton gradients across membranes. Electrons derived from a reduced substrate are made available to electron carriers in membranes and pass through a series of electron transfers to a final electron acceptor. As a byproduct of this process, protons are released on one side of the membrane, generating the transmembrane proton gradient. The proton gradient is used to synthesize ATP or to drive other energy-requiring processes. For all eukaryotes, the reduced substrate is generally a carbohydrate (glucose or pyruvate) or a fatty acid and the electron acceptor is oxygen.

Many bacteria and archaea are much more versatile. In anaerobic environments such as marine and freshwater sediments, the variety of life strategies is extraordinary. Almost any available redox pair can be an energy source for some specialized organism or group of organisms. For example, a large number of lithotrophic bacteria (a lithotroph is a chemotroph that uses inorganic energy sources) have a hydrogenase that uses molecular hydrogen to reduce NAD^+:

$$H_2 + NAD^+ \xrightarrow{\text{hydrogenase}} NADH + H^+$$

The NADH is a source of electrons for a variety of membrane-bound electron acceptors, generating the proton gradient needed for ATP synthesis. Other lithotrophs oxidize sulfur compounds (H_2S, elemental sulfur, or thiosulfate) or ferrous iron. A widespread group of archaea called methanogens, all strict anaerobes, extract energy from the reduction of CO_2 to methane. And this is just a small sampling of what anaerobic organisms do for a living. Their metabolic pathways are replete with interesting reactions and highly specialized cofactors unknown in our own world of obligate aerobic metabolism. Study of these organisms can yield practical dividends. It can also provide clues about the origins of life on an early Earth, in an atmosphere that lacked molecular oxygen.

The web of reactions that defines nitrogen utilization in the biosphere depends on a wide range of specialized bacteria. Some nitrifying bacteria oxidize ammonia to nitrites, and some oxidize the resulting nitrites to nitrates (see Fig. 22-1, purple arrows). Nitrate is second only to O_2 as a biological electron acceptor, and a great many bacteria and archaea can catalyze the denitrification of nitrates and nitrites to nitrogen, which the nitrogen-fixing bacteria then convert back into ammonia. Ammonia is a major pollutant in sewage and in farm animal waste, and it is a byproduct of fertilizer manufacture and oil refining. Waste-treatment plants have generally made use of communities of nitrifying and denitrifying bacteria to convert ammonia waste to atmospheric nitrogen. The process is expensive, requiring inputs of organic carbon and oxygen.

In the 1960s and 1970s, a few articles appeared in the research literature suggesting that ammonia could be oxidized to nitrogen anaerobically, using nitrite as an electron acceptor; this process was called anammox. The reports received little

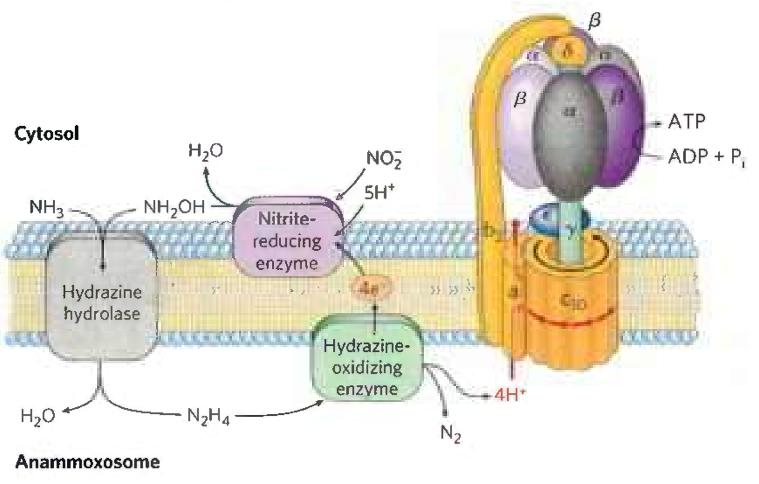


FIGURE 1 The anammox reactions. Ammonia and hydroxylamine are converted to hydrazine and H_2O by hydrazine hydrolase, and the hydrazine is oxidized by hydrazine-oxidizing enzyme, generating N_2 and protons. The protons create a proton gradient for ATP synthesis. On the anammoxosome exterior, protons are used by the nitrite-reducing enzyme, producing hydroxylamine and completing the cycle. All of the anammox enzymes are embedded in the anammoxosome membrane. [Information from L. A. van Niftrik et al., *FEMS Microbiol. Lett.* 233:10, 2004, Fig. 4.]

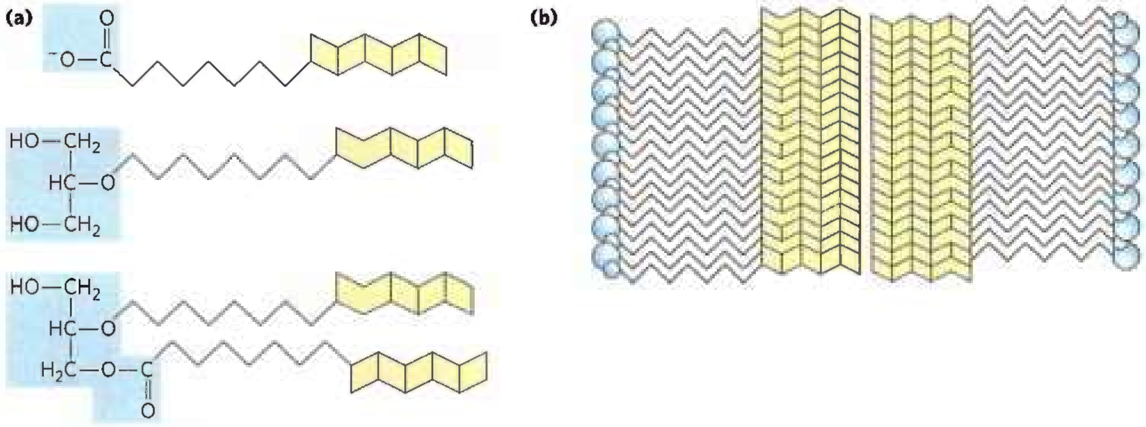


FIGURE 2 (a) Ladderane lipids of the anammoxosome membrane. The mechanism for synthesis of the unstable fused cyclobutane ring structures is unknown. (b) Ladderanes can stack to form a very dense, impermeable, hydrophobic membrane structure, allowing sequestration of the hydrazine produced in the anammox reactions. [Information from L. A. van Niftrik et al., *FEMS Microbiol. Lett.* 233:7, 2004, Fig. 3.]

notice until bacteria promoting anammox were discovered in a waste-treatment system in Delft, the Netherlands, in the mid-1980s. A team of Dutch microbiologists led by Gijs Kuenen and Mike Jetten began to study these bacteria, which were soon identified as belonging to an unusual bacterial phylum, Planctomycetes. Some surprises were to follow.

The biochemistry underlying the anammox process was slowly unraveled (Fig. 1). Hydrazine (N_2H_4), a highly reactive molecule used as a rocket fuel, was an unexpected intermediate. As a small molecule, hydrazine is both highly toxic and difficult to contain. It readily diffuses across typical phospholipid membranes. The anammox bacteria solve this problem by sequestering hydrazine in a specialized organelle, dubbed the **anammoxosome**. The membrane of this organelle is composed of lipids known as **ladderanes** (Fig. 2), never before encountered in biology. The fused cyclobutane rings of ladderanes stack tightly to form a very dense barrier, greatly slowing the release of hydrazine. Cyclobutane rings, with their unusual bond angles, are strained and difficult to synthesize; the bacterial mechanisms for synthesizing these lipids are not yet known.

The anammoxosome was a surprising finding. Bacterial cells generally do not have compartments, and the lack of a membrane-enclosed nucleus is often cited as the primary distinction between eukaryotes and bacteria. One type of organelle in a bacterium was interesting enough, but microbiologists also found that planctomycetes have a nucleus: their chromosomal DNA is contained within a membrane (Fig. 3). Planctomycetes are an ancient bacterial line with multiple genera, three of which are known to carry out the anammox reactions. Discovery of this subcellular organization has prompted further research to trace the origin of the planctomycetes and the evolution of eukaryotic nuclei. Further study of this group may ultimately bring us closer to a key goal of evolutionary biology: a description of the organism affectionately referred to as LUCA—the last universal common ancestor of all life on our planet.

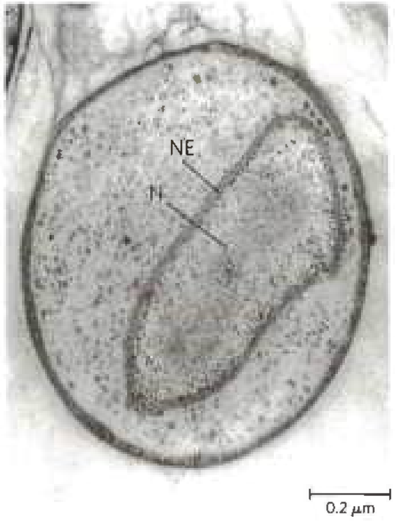


FIGURE 3 Transmission electron micrograph of a cross section through *Gemmata obscuriglobus*, showing the DNA in a nucleus (N) with enclosing nuclear envelope (NE). Bacteria of the *Gemmata* genus (phylum Planctomycetes) do not promote the anammox reactions. [Provided by John Fuerst from R. Lindsay et al., *Arch. Microbiol.* 175:413, 2001, Fig. 6a. © Springer-Verlag, 2001.]

For now, the anammox bacteria offer a major advance in waste treatment, reducing the cost of ammonia removal by as much as 90% (the conventional denitrification steps are eliminated completely, and the aeration costs associated with nitrification are lower) and reducing the release of polluting byproducts. Clearly, a greater familiarity with the bacterial underpinnings of the biosphere can pay big dividends as we deal with the environmental challenges of the twenty-first century.

8Fe-7S center. The second cofactor in dinitrogenase, the **FeMo cofactor**, is a novel structure composed of 7 Fe atoms, 9 inorganic S atoms, a Cys side chain, and a single carbon atom in the center of the FeS cluster. Also part of the cofactor is a molybdenum atom, with ligands that include three inorganic S atoms, a His side chain, and two oxygen atoms from a molecule of homocitrate that is an intrinsic part of the FeMo cofactor.

There are two additional forms of nitrogenase. One includes a dinitrogenase with a vanadium-containing cofactor rather than molybdenum (VFe); the other contains a second Fe atom (FeFe). Each of the nitrogenase complexes is encoded by a separate set of genes. The FeMo nitrogenase complex is the ancestral type, and all nitrogen-fixing bacteria and archaea contain it. Some species can produce one or both of the alternative VFe or FeFe types. Although the alternative enzymes are somewhat less efficient, they may play important roles in environments in which molybdenum is limiting or absent. They may also permit some additional reactions to occur. The vanadium nitrogenase system of *Azotobacter vinelandii* has the remarkable capacity to catalyze the reduction of carbon monoxide (CO) to ethylene (C_2H_4), ethane, and propane.

Nitrogen fixation to produce ammonia is carried out by a highly reduced form of dinitrogenase and requires eight electrons: six for the reduction of N_2 and two to produce one molecule of H_2. Production of H_2 is an obligate part of the reaction mechanism, but the biological role of H_2 in the process is not understood.

Dinitrogenase is reduced by the transfer of electrons from dinitrogenase reductase (**Fig. 22-4**). The dinitrogenase tetramer has two binding sites for the reductase. The required eight electrons are transferred from reductase to dinitrogenase one at a time: a reduced reductase molecule binds to the dinitrogenase and transfers a single electron, then the oxidized reductase dissociates from dinitrogenase, in a repeating cycle. Each turn of the cycle requires the hydrolysis of two ATP molecules by the dimeric reductase. The immediate source of electrons to reduce dinitrogenase reductase varies, with reduced **ferredoxin** (see Section 20.2), reduced flavodoxin, and perhaps other sources playing a role. In at least one species, the ultimate source of electrons to reduce ferredoxin is pyruvate.

In the reaction carried out by dinitrogenase reductase, both ATP binding and ATP hydrolysis bring about protein conformational changes that help overcome the high activation energy of nitrogen fixation. The binding of two ATP molecules to the reductase shifts the reduction potential (E'°) of this protein from –300 to –420 mV, an enhancement of its reducing power that is required to transfer electrons through dinitrogenase to N_2; the standard reduction potential for the half-reaction $N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$ is –0.34 V. The ATP molecules are then hydrolyzed just before the actual transfer of one electron to dinitrogenase.

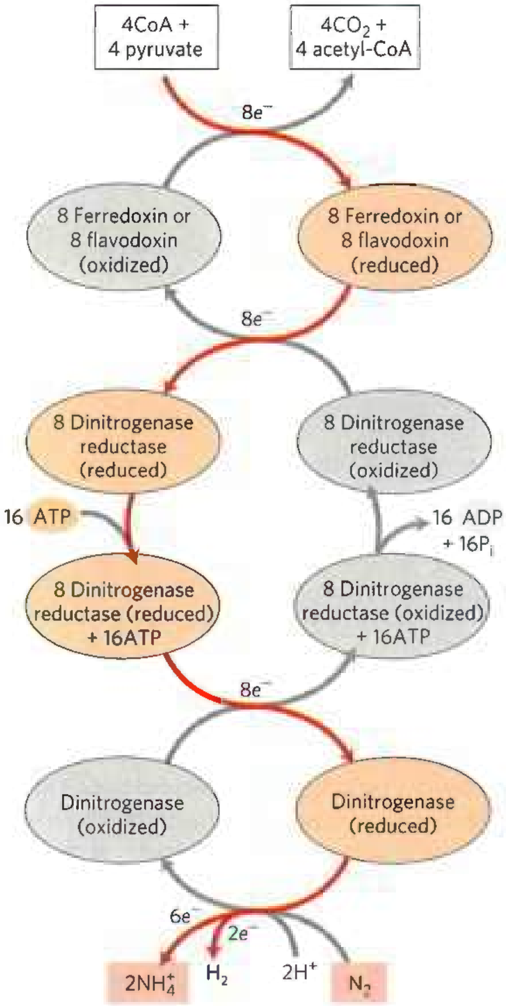

4CoA + 4 pyruvate
4CO$_2$ + 4 acetyl-CoA
8e$^-$
8 Ferredoxin or 8 flavodoxin (oxidized)
8 Ferredoxin or 8 flavodoxin (reduced)
8e$^-$
8 Dinitrogenase reductase (reduced)
8 Dinitrogenase reductase (oxidized)
16 ATP
16 ADP + 16P$_i$
8 Dinitrogenase reductase (reduced) + 16ATP
8 Dinitrogenase reductase (oxidized) + 16ATP
8e$^-$
Dinitrogenase (oxidized)
Dinitrogenase (reduced)
6e$^-$
2e$^-$
2NH$_4^+$
H$_2$
2H$^+$
N$_2$

FIGURE 22-4 Electron path in nitrogen fixation by the nitrogenase complex. Electrons are transferred from pyruvate to dinitrogenase via ferredoxin (or flavodoxin) and dinitrogenase reductase. Dinitrogenase reductase reduces dinitrogenase one electron at a time, with at least six electrons required to fix one molecule of N_2. Two additional electrons are used to reduce $2H^+$ to H_2 in a process that obligatorily accompanies nitrogen fixation in anaerobes, making a total of eight electrons required per N_2 molecule. The subunit structures and metal cofactors of the dinitrogenase reductase and dinitrogenase proteins are described in the text and in Figure 22-3.

ATP binding and hydrolysis change the conformation of nitrogenase reductase in two regions, which are structurally homologous with the switch 1 and switch 2 regions of the GTP-binding proteins involved in biological signaling (see Fig. 12-12). ATP binding produces a conformational change that brings the 4Fe-4S center of the reductase closer to the P cluster of dinitrogenase (from 18 Å to 14 Å away), which facilitates electron transfer between the reductase and dinitrogenase. The details of electron transfer from the P cluster to the FeMo cofactor, and the means by which eight electrons are accumulated by nitrogenase, are not yet known in detail. Two pathways that conform to available data, both involving the Mo atom as a central player, are illustrated in **Figure 22-5**.

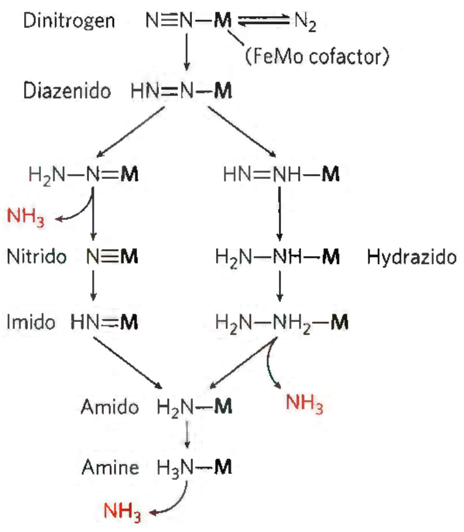


FIGURE 22-5 Two reasonable hypotheses for the intermediates involved in N_2 reduction. In both scenarios, the FeMo cofactor (abbreviated as **M** here) plays a central role, binding directly to one of the nitrogen atoms of N_2 and remaining bound throughout the sequence of reduction steps. [Information from L. C. Seefeldt et al., *Annu. Rev. Biochem.* 78:701, 2009, Fig. 9.]

P3 The nitrogenase complex is remarkably unstable in the presence of oxygen. The reductase is inactivated in air, with a half-life of 30 seconds; dinitrogenase has a half-life of only 10 minutes in air. Free-living bacteria that fix nitrogen cope with this problem in a variety of ways. Some live only anaerobically or repress nitrogenase synthesis when oxygen is present. Some aerobic species, such as *A. vinelandii*, partially uncouple electron transfer from ATP synthesis so that oxygen is burned off as rapidly as it enters the cell (see Box 19-1). When fixing nitrogen, cultures of these bacteria increase in temperature as a result of their efforts to rid themselves of oxygen.

The symbiotic relationship between leguminous plants and the nitrogen-fixing bacteria in their root nodules (**Fig. 22-6**) takes care of both the energy requirements and the oxygen lability of the nitrogenase complex. The energy required for nitrogen fixation was probably the evolutionary driving force for this plant-bacteria association. The bacteria in root nodules have access to a large reservoir of energy in the form of abundant carbohydrate and citric acid cycle intermediates made available by the plant. This may allow the bacteria to fix hundreds of times more nitrogen than do their free-living cousins under conditions generally encountered in soils. To solve the oxygen-toxicity problem, the bacteria in root nodules are bathed in a solution of the oxygen-binding heme protein **leghemoglobin**, produced by the plant (although the heme may be contributed by the bacteria). Leghemoglobin binds all available oxygen so that it cannot interfere with nitrogen fixation, and it efficiently delivers the oxygen to the bacterial electron-transfer system. The benefit to the plant, of course, is a ready supply of reduced nitrogen. In fact, the bacterial symbionts typically produce far more NH_3 than is needed by their symbiotic partner; the excess is released into the soil. The efficiency of the symbiosis between plants and bacteria is evident in the enrichment of soil nitrogen brought about by leguminous plants. This enrichment of NH_3 in the soil is the basis of crop rotation methods, in which plantings of nonleguminous plants (such as maize) that extract fixed nitrogen from the soil are alternated with plantings of legumes such as alfalfa, peas, or clover.

Nitrogen fixation is energetically costly: 16 ATP and 8 electrons yield only 2 NH_3. It is therefore not surprising that the process is tightly regulated so that NH_3 is produced only when needed. High [ADP], an indicator of low [ATP], is a strong inhibitor of nitrogenase. NH_4^+ represses the expression of the ~20 nitrogen fixation (*nif*) genes,

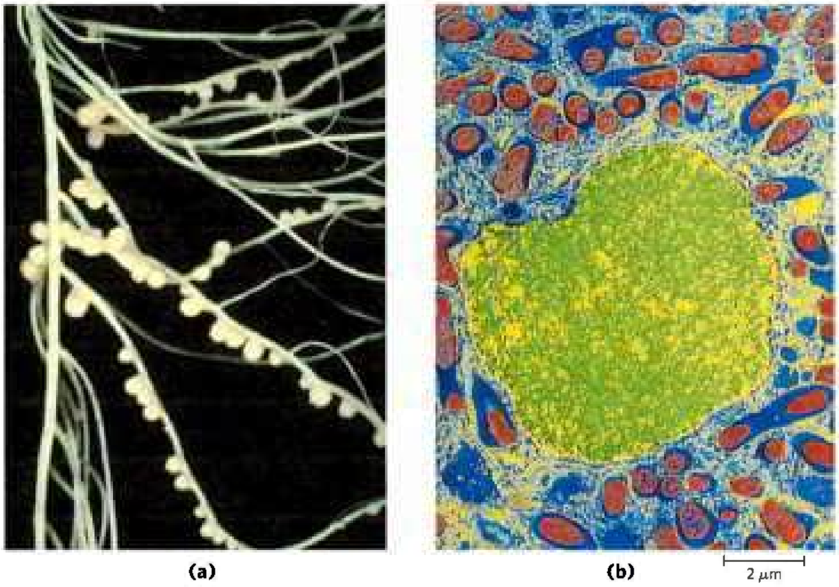


FIGURE 22-6 Nitrogen-fixing nodules. (a) Pea plant (*Pisum sativum*) root nodules containing the nitrogen-fixing bacterium *Rhizobium leguminosarum*. The nodules are pink due to the presence of leghemoglobin; this heme protein has a very high binding affinity for oxygen, which strongly inhibits nitrogenase. (b) Artificially colorized electron micrograph of a thin section through a pea root nodule. Symbiotic nitrogen-fixing bacteria, or bacteroids (red), live inside the nodule cell, surrounded by the peribacteroid membrane (blue). Bacteroids produce the nitrogenase complex that converts atmospheric nitrogen (N_2) to ammonium (NH_4^+); without the bacteroids, the plant is unable to utilize N_2. (The cell nucleus is shown in yellow/green. Not visible in this micrograph are other organelles of the infected root cell that are normally found in plant cells.) [(a, b) Jeremy Burgess/Science Source.]

effectively shutting down the pathway. Covalent alteration of nitrogenase is also used in some diazotrophs to control nitrogen fixation in response to the availability of NH_4^+ in the surroundings. Transfer of an ADP-ribosyl group from NADH to a specific Arg residue in the nitrogenase reductase shuts down N_2 fixation in *Rhodospirillum*, for example. This is the same covalent modification that we saw in the case of G protein inhibition by the toxins of cholera and pertussis (see Fig. 12-14).

Nitrogen fixation is the subject of intense study because of its immense practical importance. Industrial production of ammonia for use in fertilizers requires a large and expensive input of energy, and this has spurred a drive to develop recombinant or transgenic organisms that can fix nitrogen. In principle, recombinant DNA techniques (Chapter 9) might be used to transfer the DNA that encodes the enzymes of nitrogen fixation into non-nitrogen-fixing bacteria and plants. However, those genes alone will not suffice. About 20 genes are essential to nitrogenase activity in bacteria, many of them needed for the synthesis, assembly, and insertion of the cofactors. There is also the problem of protecting the enzyme in its new setting from destruction by oxygen. In all, there are formidable challenges in engineering new nitrogen-fixing plants. Success in these efforts will depend on overcoming the problem of oxygen toxicity in any cell that produces nitrogenase.

Ammonia Is Incorporated into Biomolecules through Glutamate and Glutamine

Reduced nitrogen in the form of NH_4^+ is assimilated into amino acids and then into other nitrogen-containing biomolecules. P5 Two amino acids, **glutamate** and **glutamine**, provide the critical entry point. Recall that these same two amino acids play central roles in the catabolism of ammonia and amino groups in amino acid oxidation (Chapter 18). Glutamate is the source of amino groups for most other amino acids, through transamination reactions (the reverse of the reaction shown in Fig. 18-4). The amide nitrogen of glutamine is a source of amino groups in a wide range of biosynthetic processes. In most types of cells, and in extracellular fluids in higher organisms, one or both of these amino acids are present at higher concentrations—sometimes an order of magnitude or more higher—than other amino acids. An *Escherichia coli* cell requires so much glutamate that this amino acid is one of the primary solutes in the cytosol. Its concentration is regulated not only in response to the cell's nitrogen requirements but also to maintain an osmotic balance between the cytosol and the external medium.

The biosynthetic pathways to glutamate and glutamine are simple, and all or some of the steps occur in most organisms. The most important pathway for the assimilation of NH_4^+ into glutamate requires two reactions. The net effect is to convert glutamate, α-ketoglutarate, and ammonia into two molecules of glutamate.

First, NH_4^+ is reacted with glutamate to produce glutamine, using the enzyme **glutamine synthetase**. This reaction takes place in two steps, with enzyme-bound γ-glutamyl phosphate as an intermediate (see Fig. 18-8):

$$\text{(1)}\quad \text{Glutamate} + \text{ATP} \longrightarrow \gamma\text{-glutamyl phosphate} + \text{ADP}$$

$$\text{(2)}\quad \gamma\text{-Glutamyl phosphate} + NH_4^+ \longrightarrow \text{glutamine} + P_i + H^+$$

$$\textit{Sum:}\ \text{Glutamate} + NH_4^+ + \text{ATP} \longrightarrow \text{glutamine} + \text{ADP} + P_i + H^+ \qquad (22\text{-}1)$$

Glutamine synthetase is found in all organisms. In addition to its importance for NH_4^+ assimilation in bacteria, it has a central role in amino acid metabolism in mammals, converting free NH_4^+, which is toxic, to glutamine for transport in the blood (Chapter 18).

In the second reaction needed for NH_4^+ assimilation, the glutamine reacts with α-ketoglutarate to generate two molecules of glutamate. In bacteria and plants, this reaction is catalyzed by **glutamate synthase**. (An alternative name for this enzyme, glutamate:oxoglutarate aminotransferase, yields the acronym GOGAT, by which the enzyme also is known.) α-Ketoglutarate, an intermediate of the citric acid cycle, undergoes reductive amination with glutamine as nitrogen donor:

$$\alpha\text{-Ketoglutarate} + \text{glutamine} + \text{NAD(P)H} + H^+ \longrightarrow 2\ \text{glutamate} + \text{NAD(P)}^+ \qquad (22\text{-}2)$$

The net reaction of glutamine synthetase and glutamate synthase (Eqns 22-1 and 22-2) is

$$\alpha\text{-Ketoglutarate} + NH_4^+ + \text{NAD(P)H} + \text{ATP} \longrightarrow \text{glutamate} + \text{NAD(P)}^+ + \text{ADP} + P_i$$

Glutamate synthase is not present in animals, which instead maintain high levels of glutamate by processes such as the transamination of α-ketoglutarate during amino acid catabolism. Plants possess a second alternative form of glutamate synthase that uses reduced ferredoxin rather than NADPH as a source of reducing electrons.

Glutamate can also be formed in yet another, albeit minor, pathway: the reaction of α-ketoglutarate and NH_4^+ to form glutamate in one step. P6 This is catalyzed by glutamate dehydrogenase, an enzyme present in all organisms. Reducing power is furnished by NADPH:

$$\alpha\text{-Ketoglutarate} + NH_4^+ + \text{NAD(P)H} + H^+ \longrightarrow \text{glutamate} + \text{NAD(P)}^+ + H_2O$$

We encountered this reaction in the catabolism of amino acids (see Fig. 18-7). In eukaryotic cells, glutamate dehydrogenase is located in the mitochondrial matrix. The reaction equilibrium favors the reactants, and the K_m for NH_4^+ (~1 mM) is so high that the reaction is not important for NH_4^+ assimilation in mammals. (Recall that the glutamate dehydrogenase reaction, in reverse (see Fig. 18-10), is one source of NH_4^+ destined for the urea cycle.) In microorganisms and plants, concentrations of NH_4^+ high enough for the glutamate dehydrogenase reaction to make a significant contribution to glutamate levels generally occur only when NH_3 is added artificially to the growth environment. In general, soil bacteria and plants rely on the two-enzyme pathway outlined above (Eqns 22-1, 22-2).

Glutamine Synthetase Is a Primary Regulatory Point in Nitrogen Metabolism

There are three known classes of glutamine synthetases. The class I enzyme (GSI, found in bacteria) has 12 identical subunits of M_r 50,000 (**Fig. 22-7**) and is regulated both allosterically and by covalent modification. The class II enzyme (GSII, found in eukaryotes and some bacteria) has 10 identical subunits. The third class of glutamine synthetases (GSIII), so far found only in two bacterial species, are much larger enzymes, consisting of a double-ringed dodecamer of identical chains.

P4 Befitting their central metabolic role as an entry point for reduced nitrogen, GSI glutamine synthetases are highly regulated. In enteric bacteria such as *E. coli*, the regulation is unusually complex. Alanine, glycine, and at least six end products of glutamine metabolism are allosteric inhibitors (**Fig. 22-8**). Each inhibitor alone produces only partial inhibition, but the effects of multiple inhibitors are more than additive, and all eight together virtually shut down the enzyme. This is an example of cumulative feedback inhibition. This control mechanism provides a constant adjustment of glutamine levels to match immediate metabolic requirements.

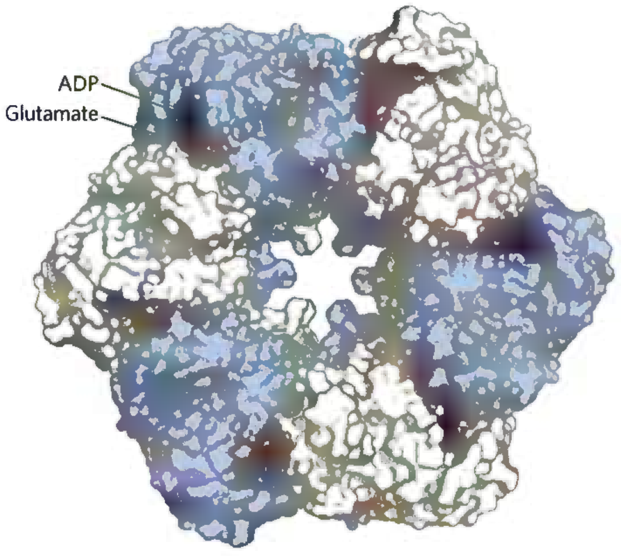


FIGURE 22-7 Subunit structure of bacterial type I glutamine synthetase. This view shows 6 of the 12 identical subunits; a second layer of 6 subunits lies directly beneath those shown. Each of the 12 subunits has an active site, where ATP and glutamate are bound in orientations that favor transfer of a phosphoryl group from ATP to the side-chain carboxyl of glutamate. In this crystal structure, ADP occupies the ATP site. [Data from PDB ID 2GLS, M. M. Yamashita et al., *J. Biol. Chem.* 264:17,681, 1989.]

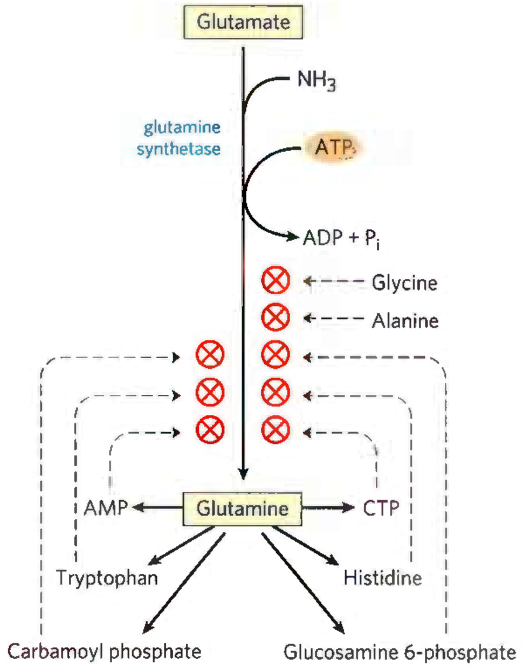


FIGURE 22-8 Allosteric regulation of glutamine synthetase. The enzyme undergoes cumulative regulation by six end products of glutamine metabolism. Alanine and glycine probably serve as indicators of the general status of amino acid metabolism in the cell.

Superimposed on the allosteric regulation is inhibition by adenylylation of (addition of AMP to) Tyr^{397}, located near the enzyme's active site (**Fig. 22-9**). This covalent modification increases sensitivity to the allosteric inhibitors, and activity decreases as more subunits are adenylylated. Both adenylylation and de-adenylylation are promoted by **adenylyltransferase** (AT in Fig. 22-9), part of a complex enzymatic cascade that responds to levels of glutamine, α-ketoglutarate, ATP, and P_i. The activity of adenylyltransferase is modulated by binding to a regulatory protein called P_{II}, and the activity of P_{II}, in turn, is regulated by covalent modification (uridylylation), again at a Tyr residue. The adenylyltransferase complex with uridylylated P_{II} (P_{II}-UMP) stimulates de-adenylylation, whereas the same complex with deuridylylated P_{II} stimulates adenylylation of glutamine synthetase. Both uridylylation and deuridylylation of P_{II} are brought about by a single enzyme, **uridylyltransferase**. Uridylylation is inhibited by binding of glutamine and P_i to uridylyltransferase and is stimulated by binding of α-ketoglutarate and ATP to P_{II}.

The regulation does not stop there. The uridylylated P_{II} also mediates the activation of transcription of the gene encoding glutamine synthetase, thus increasing the cellular concentration of the enzyme; the deuridylylated P_{II} brings about a decrease in transcription of the same gene. This mechanism involves an interaction of P_{II} with additional proteins involved in gene regulation, of a type described in Chapter 28. The net result of this elaborate system of controls is a decrease in glutamine synthetase

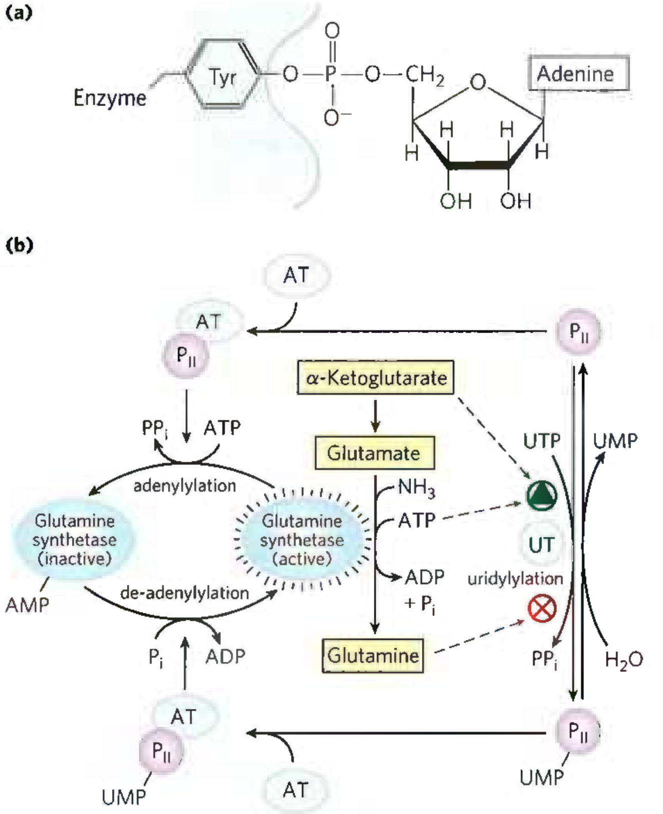

(a)
Enzyme
Tyr
O
P
O−
CH2
Adenine
H
OH
OH
(b)
AT
PII
α-Ketoglutarate
Glutamate
Glutamine
PPi
ATP
adenylylation
de-adenylylation
Glutamine synthetase (inactive)
Glutamine synthetase (active)
AMP
Pi
ADP
NH3
ATP
ADP + Pi
UTP
UMP
UT
uridylylation
PPi
H2O
UMP

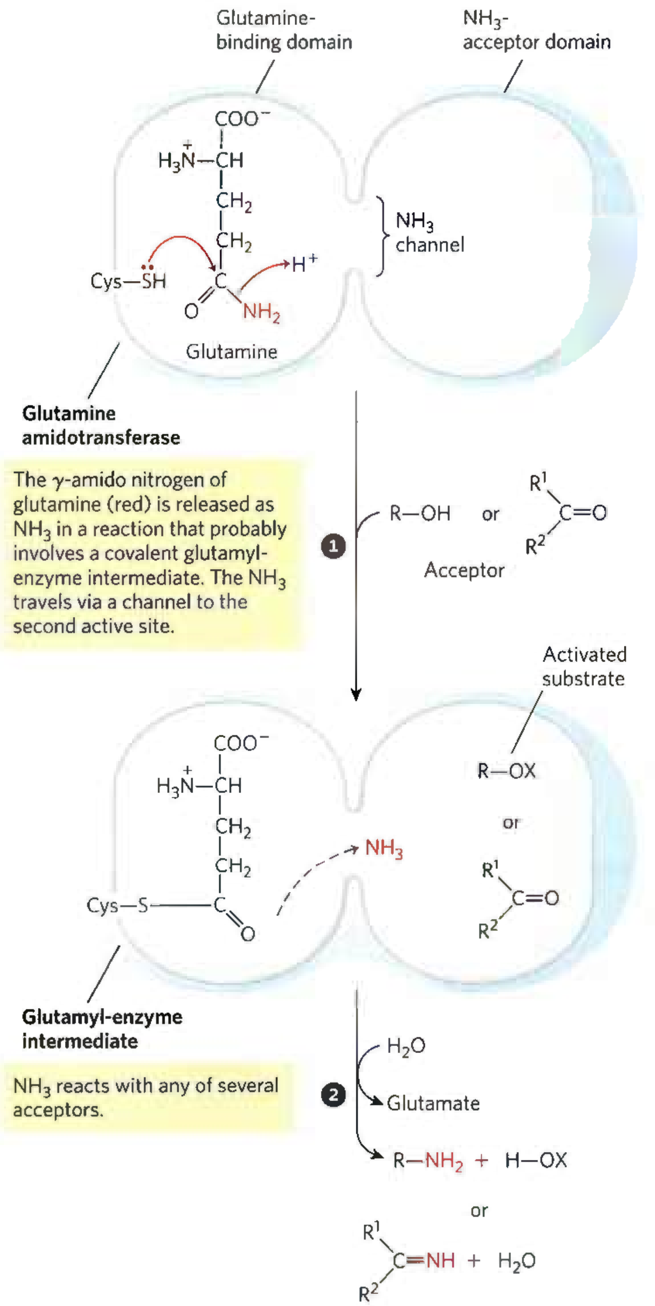
FIGURE 22-9 Second level of regulation of glutamine synthetase: covalent modifications. (a) An adenylylated Tyr residue. (b) Cascade leading to adenylylation (inactivation) of glutamine synthetase. AT represents adenylyltransferase; UT, uridylyltransferase. P_{II} is a regulatory protein, itself regulated by uridylylation.

activity when glutamine levels are high, and an increase in activity when glutamine levels are low and α-ketoglutarate and ATP (substrates for the synthetase reaction) are available. The multiple layers of regulation permit a sensitive response in which glutamine synthesis is tailored to cellular needs.

Several Classes of Reactions Play Special Roles in the Biosynthesis of Amino Acids and Nucleotides

The pathways described in this chapter include a variety of interesting chemical rearrangements. Several of these recur and deserve special note before we progress to the pathways themselves. These are (1) transamination reactions and other rearrangements promoted by enzymes containing pyridoxal phosphate; (2) transfer of one-carbon groups, with either tetrahydrofolate (usually at the —CHO and —CH_2OH oxidation levels) or *S*-adenosylmethionine (at the —CH_3 oxidation level) as cofactor; and (3) transfer of amino groups derived from the amide nitrogen of glutamine. Pyridoxal phosphate (PLP), tetrahydrofolate (H_4 folate), and *S*-adenosylmethionine (adoMet) are described in some detail in Chapter 18 (see Figs. 18-6, 18-17, and 18-18). Here we focus on amino group transfer involving the amide nitrogen of glutamine.

More than a dozen known biosynthetic reactions use glutamine as the major physiological source of amino groups, and most of these occur in the pathways outlined in this chapter. As a class, the enzymes catalyzing these reactions are called **glutamine amidotransferases**. All have two structural domains: one binding glutamine, the other binding the second substrate, which serves as amino group acceptor (**Fig. 22-10**). A conserved Cys residue in the


Glutamine-binding domain
NH3-acceptor domain
COO−
H3N+—CH
CH2
CH2
C
O
NH2
Cys—SH
H+
NH3 channel
Glutamine
Glutamine amidotransferase
1
R—OH or R1 C=O R2
Acceptor
Activated substrate
Cys—S—C
NH3
R—OX
or
R1 C=O R2
Glutamyl-enzyme intermediate
2
H2O
Glutamate
R—NH2 + H—OX
or
R1 C=NH + H2O R2

The γ-amido nitrogen of glutamine (red) is released as NH_3 in a reaction that probably involves a covalent glutamyl-enzyme intermediate. The NH_3 travels via a channel to the second active site.

NH_3 reacts with any of several acceptors.

MECHANISM FIGURE 22-10 Proposed mechanism for glutamine amidotransferases. Each enzyme has two domains. The glutamine-binding domain contains structural elements conserved among many of these enzymes, including a Cys residue required for activity. The NH_3-acceptor (second-substrate) domain varies. Two types of amino acceptors are shown. X represents an activating group, typically a phosphoryl group derived from ATP, that facilitates displacement of a hydroxyl group from R—OH by NH_3.

glutamine-binding domain is believed to act as a nucleophile, cleaving the amide bond of glutamine and forming a covalent glutamyl-enzyme intermediate. The NH_3 produced in this reaction is not released, but instead is transferred through an "ammonia channel" to a second active site, where it reacts with the second substrate to form the aminated product. The covalent intermediate is hydrolyzed to the free enzyme and glutamate. If the second substrate must be activated, the usual method is the use of ATP to generate an acyl phosphate intermediate (R—OX in Fig. 22-10, where X is a phosphoryl group). The enzyme glutaminase acts similarly but uses H_2O as the second substrate, yielding NH_4^+ and glutamate (see Fig. 18-8).

SUMMARY 22.1 *Overview of Nitrogen Metabolism*

- The molecular nitrogen that makes up 80% of Earth's atmosphere is unavailable to most living organisms until it is reduced. A complex web of reactions converts atmospheric N_2 to biologically useful forms and maintains a global balance between them. Prominent species that are interconverted within this web include ammonia (NH_3 or NH_4^+; most reduced), nitrite (NO_2^-), and nitrate (NO_3^-; most oxidized). Conversion of N_2 to ammonia is fixation. Nitrification constitutes the steps converting ammonia to nitrate. Conversion of nitrate to N_2 constitutes denitrification. The alternative conversion of nitrate to ammonia is nitrate assimilation.
- Fixation of N_2 as NH_3 is carried out by the nitrogenase complex, in a reaction that requires large investments of ATP and of reducing power. The nitrogenase complex is highly labile in the presence of O_2, and it is subject to regulation by the supply of NH_3.
- In living systems, reduced nitrogen is incorporated first into amino acids and then into a variety of other biomolecules, including nucleotides. The key entry point is the amino acid glutamate. Glutamate and glutamine are the nitrogen donors in a wide range of biosynthetic reactions.
- Glutamine synthetase, which catalyzes the formation of glutamine from glutamate, is a main regulatory enzyme of nitrogen metabolism.
- The amino acid and nucleotide biosynthetic pathways make repeated use of the biological cofactors pyridoxal phosphate, tetrahydrofolate, and *S*-adenosylmethionine. Pyridoxal phosphate is required for transamination reactions involving glutamate and for other amino acid transformations. One-carbon transfers require *S*-adenosylmethionine and tetrahydrofolate. Glutamine amidotransferases catalyze reactions that incorporate nitrogen derived from the amide group of glutamine.

22.2 Biosynthesis of Amino Acids

All amino acids are derived from intermediates in glycolysis, the citric acid cycle, or the pentose phosphate pathway (**Fig. 22-11**). Nitrogen enters these biosynthetic pathways by way of glutamate and glutamine. Some pathways are simple, others are not. Ten of the amino acids are just one or several steps removed from the common metabolite from which they are derived. The biosynthetic pathways for others, such as the aromatic amino acids, are more complex.

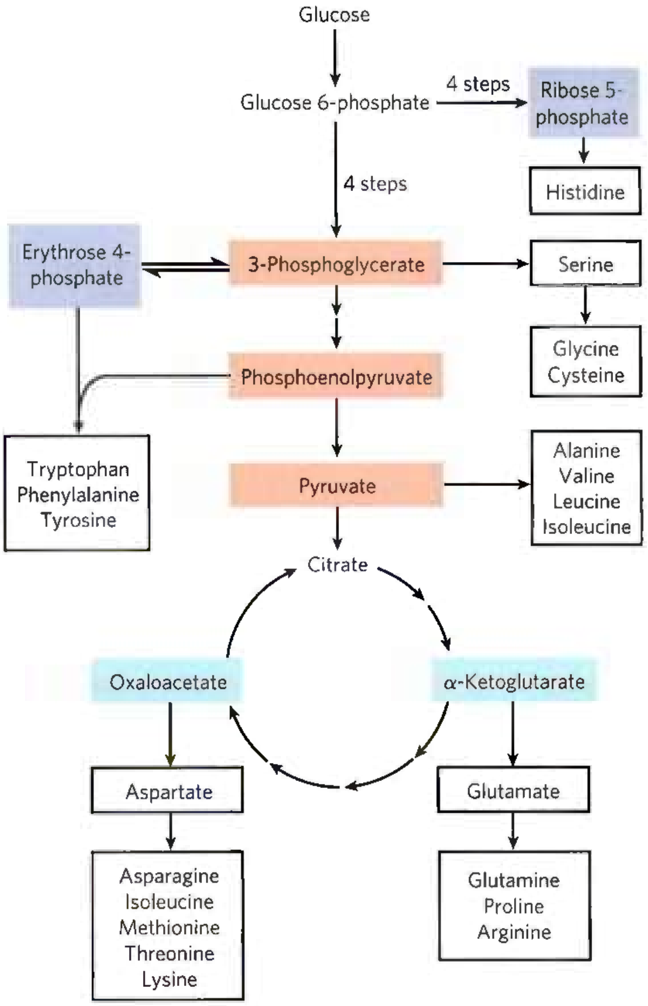


FIGURE 22-11 Overview of amino acid biosynthesis. The carbon skeleton precursors derive from three sources: glycolysis (light red), the citric acid cycle (blue), and the pentose phosphate pathway (purple).

Organisms Vary Greatly in Their Ability to Synthesize the 20 Common Amino Acids

Whereas most bacteria and plants can synthesize all 20 amino acids, mammals can synthesize only about half of them—generally those with simple pathways. These are often called **nonessential amino acids** (see Table 18-1). The label is somewhat misleading, however, because innate biosynthetic pathways often do not provide enough of these amino acids to support optimal growth and health. The remaining amino acids, the **essential amino acids**, cannot be synthesized by most mammals and must be obtained from food. A few amino acids are conditionally essential in mammals, required at particular stages of development. Unless otherwise indicated, the pathways for the 20 common amino acids presented below are those operative in bacteria.

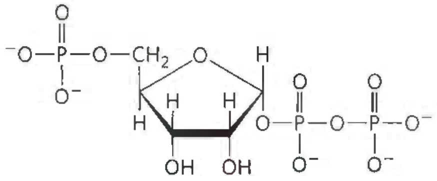
TABLE 22-1 Amino Acid Biosynthetic Families, Grouped by Metabolic Precursor

α-Ketoglutarate Glutamate Glutamine Proline Arginine	**Pyruvate** Alanine Valine[a] Leucine[a] Isoleucine[a]
3-Phosphoglycerate Serine Glycine Cysteine	**Phosphoenolpyruvate and erythrose 4-phosphate** Tryptophan[a] Phenylalanine[a] Tyrosine[b]
Oxaloacetate Aspartate Asparagine Isoleucine Methionine[a] Threonine[a] Lysine[a]	**Ribose 5-phosphate** Histidine[a]

[a] Essential amino acids in mammals.

[b] Derived from phenylalanine in mammals.

A useful way to organize these biosynthetic pathways is to group them into six families corresponding to their metabolic precursors (**Table 22-1**), and we use this approach to structure the detailed descriptions that follow. In addition to these six precursors, there is a notable intermediate in several pathways of amino acid and nucleotide synthesis: **5-phosphoribosyl-1-pyrophosphate (PRPP)**:

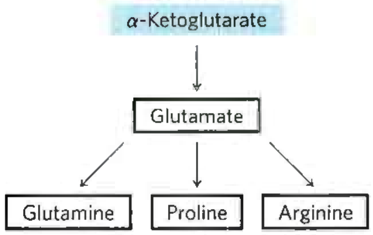


PRPP is synthesized from ribose 5-phosphate derived from the pentose phosphate pathway (see Fig. 14-31), in a reaction catalyzed by **ribose phosphate pyrophosphokinase**:

$$\text{Ribose 5-phosphate} + \text{ATP} \longrightarrow \text{5-phosphoribosyl-1-pyrophosphate} + \text{AMP}$$

This enzyme is allosterically regulated by many of the biomolecules for which PRPP is a precursor.

α-Ketoglutarate Gives Rise to Glutamate, Glutamine, Proline, and Arginine

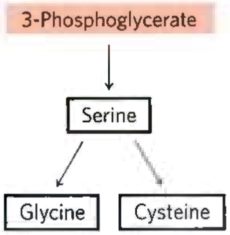


We have already described the biosynthesis of **glutamate** and **glutamine**. **Proline** is a cyclized derivative of glutamate (**Fig. 22-12**). P6 In the first step of proline synthesis, ATP reacts with the γ-carboxyl group of glutamate to form an acyl phosphate, which is reduced by NADPH or NADH to glutamate γ-semialdehyde. This intermediate undergoes rapid spontaneous cyclization and is then reduced further to yield proline.

Arginine is synthesized from glutamate via ornithine and the urea cycle in animals (Chapter 18). In principle, ornithine could also be synthesized from glutamate γ-semialdehyde by transamination, but the spontaneous cyclization of the semialdehyde in the proline pathway precludes a sufficient supply of this intermediate for ornithine synthesis. Bacteria have a de novo biosynthetic pathway for ornithine (and thus arginine) that parallels some steps of the proline pathway but includes two additional steps that avoid the problem of the spontaneous cyclization of glutamate γ-semialdehyde (Fig. 22-12). In the first step, the α-amino group of glutamate is blocked by an acetylation requiring acetyl-CoA; then, after the transamination step, the acetyl group is removed to yield ornithine.

The pathways to proline and arginine are somewhat different in mammals. Proline can be synthesized by the pathway shown in Figure 22-12, but it is also formed from arginine obtained from dietary or tissue protein. Arginase, a urea cycle enzyme, converts arginine to ornithine and urea (see Figs. 18-10, 18-26). The ornithine is converted to glutamate γ-semialdehyde by the enzyme **ornithine δ-aminotransferase** (**Fig. 22-13**). The semialdehyde cyclizes to Δ^1-pyrroline-5-carboxylate, which is then converted to proline (Fig. 22-12). The pathway for arginine synthesis shown in Figure 22-12 is absent in mammals. P1 When arginine from dietary intake or protein turnover is insufficient for protein synthesis, the ornithine δ-aminotransferase reaction operates in the direction of ornithine formation. Ornithine is then converted to citrulline and arginine in the urea cycle.

Serine, Glycine, and Cysteine Are Derived from 3-Phosphoglycerate



The major pathway for the formation of **serine** is the same in all organisms (**Fig. 22-14**). In the first step, the hydroxyl group of 3-phosphoglycerate is oxidized by a dehydrogenase (using NAD$^+$) to yield 3-phosphohydroxypyruvate. Transamination from glutamate yields

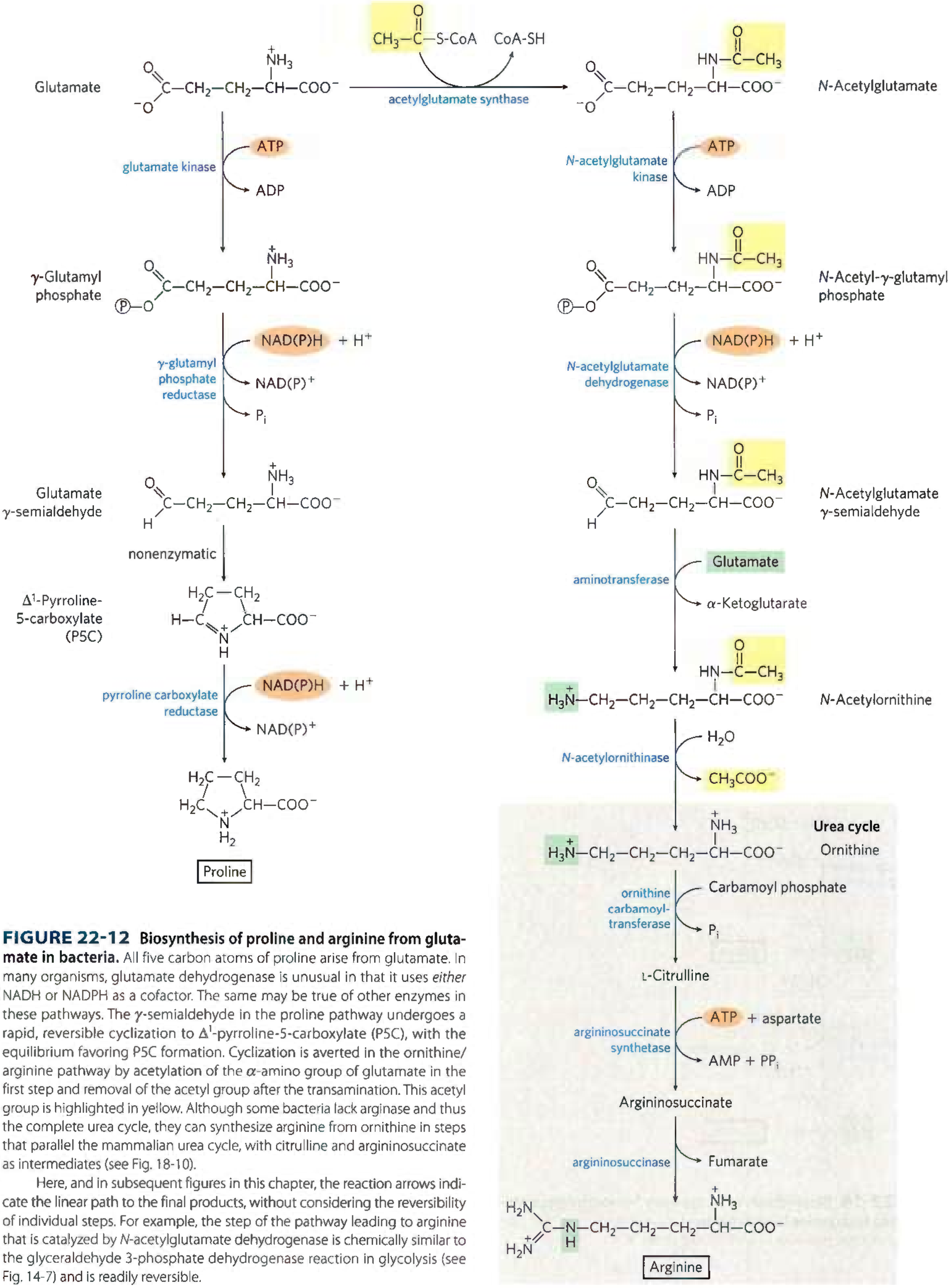

Glutamate
CH3–C(=O)–S-CoA
CoA-SH
acetylglutamate synthase
N-Acetylglutamate
glutamate kinase
ATP
ADP
N-acetylglutamate kinase
ATP
ADP
γ-Glutamyl phosphate
N-Acetyl-γ-glutamyl phosphate
γ-glutamyl phosphate reductase
NAD(P)H + H+
NAD(P)+
Pi
N-acetylglutamate dehydrogenase
NAD(P)H + H+
NAD(P)+
Pi
Glutamate γ-semialdehyde
N-Acetylglutamate γ-semialdehyde
nonenzymatic
aminotransferase
Glutamate
α-Ketoglutarate
Δ1-Pyrroline-5-carboxylate (P5C)
N-Acetylornithine
pyrroline carboxylate reductase
NAD(P)H + H+
NAD(P)+
N-acetylornithinase
H2O
CH3COO−
Proline
Urea cycle
Ornithine
ornithine carbamoyl-transferase
Carbamoyl phosphate
Pi
L-Citrulline
argininosuccinate synthetase
ATP + aspartate
AMP + PPi
Argininosuccinate
argininosuccinase
Fumarate
Arginine

FIGURE 22-12 Biosynthesis of proline and arginine from glutamate in bacteria. All five carbon atoms of proline arise from glutamate. In many organisms, glutamate dehydrogenase is unusual in that it uses *either* NADH or NADPH as a cofactor. The same may be true of other enzymes in these pathways. The γ-semialdehyde in the proline pathway undergoes a rapid, reversible cyclization to Δ^1-pyrroline-5-carboxylate (P5C), with the equilibrium favoring P5C formation. Cyclization is averted in the ornithine/arginine pathway by acetylation of the α-amino group of glutamate in the first step and removal of the acetyl group after the transamination. This acetyl group is highlighted in yellow. Although some bacteria lack arginase and thus the complete urea cycle, they can synthesize arginine from ornithine in steps that parallel the mammalian urea cycle, with citrulline and argininosuccinate as intermediates (see Fig. 18-10).

Here, and in subsequent figures in this chapter, the reaction arrows indicate the linear path to the final products, without considering the reversibility of individual steps. For example, the step of the pathway leading to arginine that is catalyzed by *N*-acetylglutamate dehydrogenase is chemically similar to the glyceraldehyde 3-phosphate dehydrogenase reaction in glycolysis (see Fig. 14-7) and is readily reversible.

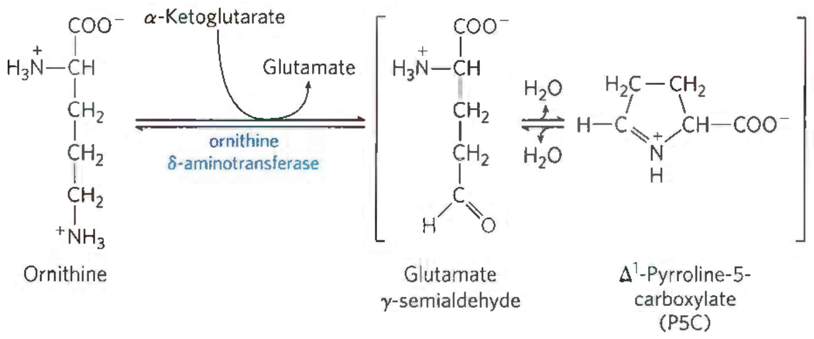

α-Ketoglutarate
Glutamate
ornithine δ-aminotransferase
H_2O
H_2O
Ornithine
Glutamate γ-semialdehyde
Δ¹-Pyrroline-5-carboxylate (P5C)

FIGURE 22-13 Ornithine δ-aminotransferase reaction: a step in the mammalian pathway to proline. This enzyme is found in the mitochondrial matrix of most tissues. Although the equilibrium favors P5C formation, the reverse reaction is the only mammalian pathway for synthesis of ornithine (and thus arginine) when arginine levels are insufficient for protein synthesis.

3-phosphoserine, which is hydrolyzed to free serine by phosphoserine phosphatase.

Serine (three carbons) is the precursor of **glycine** (two carbons) through removal of a carbon atom by **serine hydroxymethyltransferase** (Fig. 22-14). Tetrahydrofolate accepts the β carbon (C-3) of serine, which forms a methylene bridge between N-5 and N-10 to yield N^5,N^{10}-methylenetetrahydrofolate (see Fig. 18-17). The overall reaction, which is reversible, also requires pyridoxal phosphate. In the liver of vertebrates, glycine can be made by another route: the reverse of the reaction shown in Figure 18-20c, catalyzed by **glycine synthase** (also called **glycine cleavage enzyme**):

$$\begin{aligned}&CO_2 + NH_4^+ + N^5,N^{10}\text{-methylenetetrahydrofolate} + \\ &\quad NADH + H^+ \longrightarrow \text{glycine} + \text{tetrahydrofolate} + NAD^+\end{aligned}$$

Plants and bacteria produce the reduced sulfur required for the synthesis of **cysteine** (and methionine, described later) from environmental sulfates; the pathway is shown on the right side of **Figure 22-15**. Sulfate is activated in two steps to produce 3′-phosphoadenosine 5′-phosphosulfate (PAPS), which undergoes an eight-electron reduction to sulfide. The sulfide is then used in the formation of cysteine from serine in a two-step pathway. Mammals synthesize cysteine from two amino acids: methionine furnishes the sulfur atom, and serine furnishes the carbon skeleton. Methionine is first converted to S-adenosylmethionine (see Fig. 18-18), which can lose its methyl group to any of a number of acceptors to form S-adenosylhomocysteine (adoHcy). This demethylated product is hydrolyzed to free homocysteine, which undergoes a reaction with serine, catalyzed by **cystathionine β-synthase**, to yield cystathionine (**Fig. 22-16**). Finally, **cystathionine γ-lyase**, a PLP-requiring enzyme, catalyzes removal of ammonia and cleavage of cystathionine to yield free cysteine.

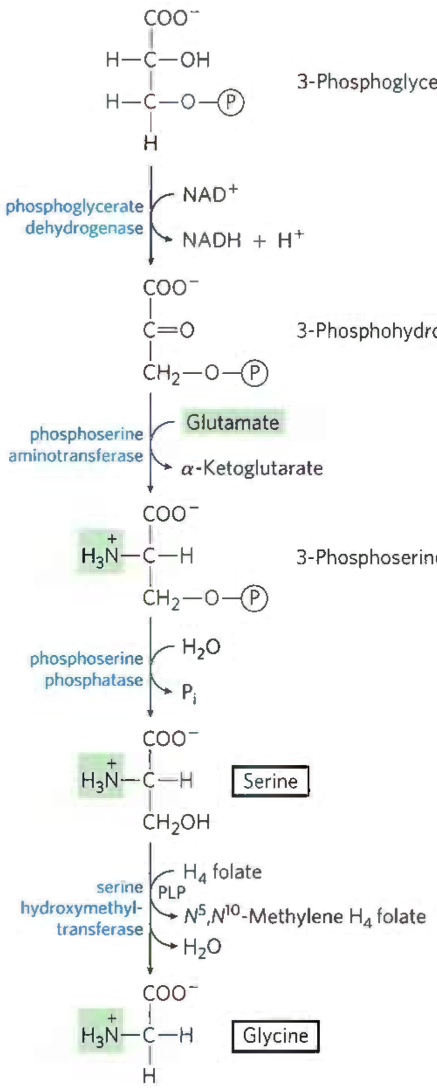

3-Phosphoglycerate
phosphoglycerate dehydrogenase
NAD^+
$NADH + H^+$
3-Phosphohydroxypyruvate
phosphoserine aminotransferase
Glutamate
α-Ketoglutarate
3-Phosphoserine
phosphoserine phosphatase
H_2O
P_i
Serine
serine hydroxymethyl-transferase
PLP
H_4 folate
N^5,N^{10}-Methylene H_4 folate
H_2O
Glycine

FIGURE 22-14 Biosynthesis of serine from 3-phosphoglycerate and of glycine from serine in all organisms. As indicated in the text, this is only one of multiple pathways to synthesize glycine.

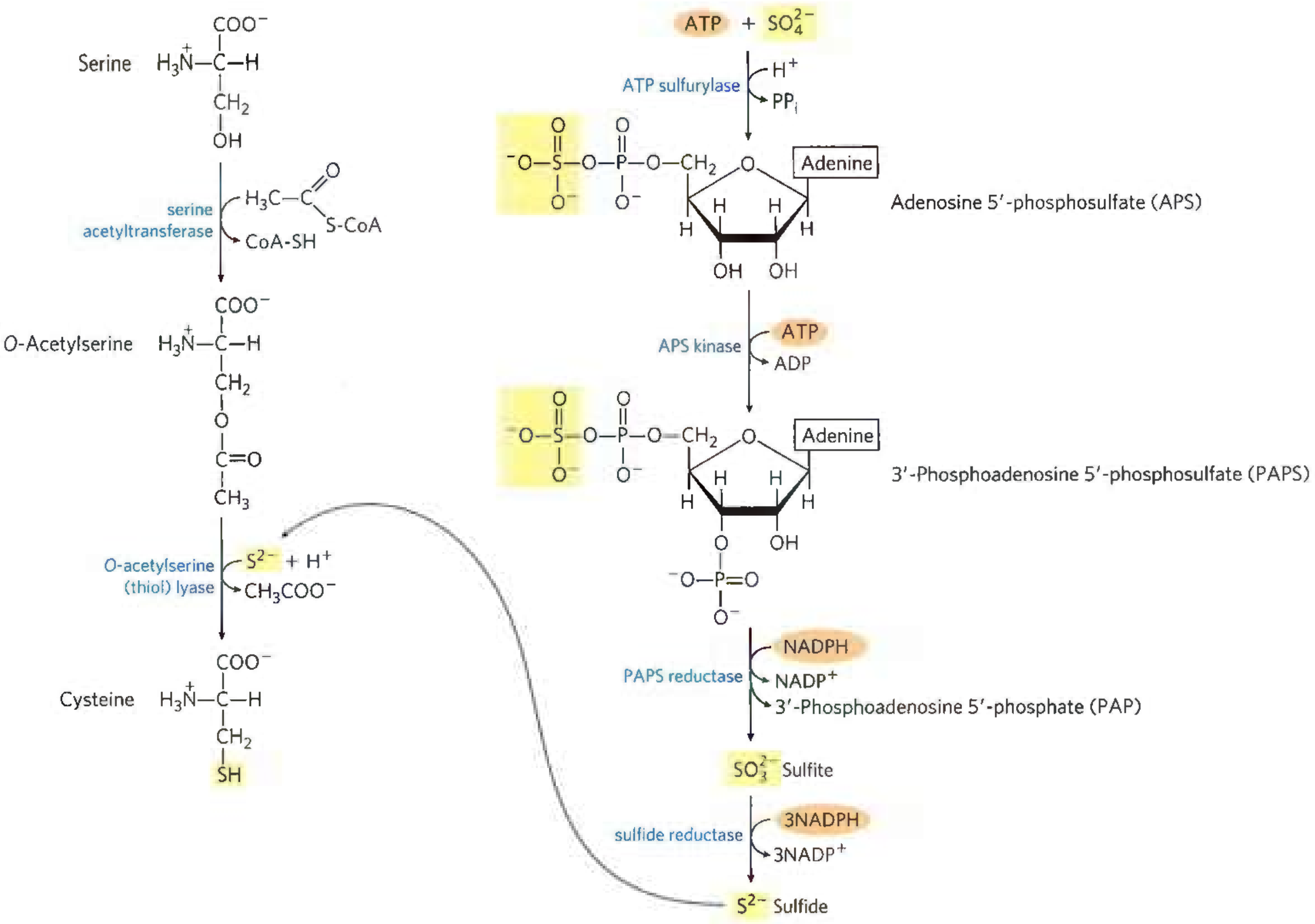

Serine
serine acetyltransferase
CoA-SH
S-CoA
O-Acetylserine
O-acetylserine (thiol) lyase
S^{2-} + H^+
CH_3COO^-
Cysteine
ATP + SO_4^{2-}
ATP sulfurylase
H^+
PP_i
Adenine
Adenosine 5′-phosphosulfate (APS)
APS kinase
ATP
ADP
3′-Phosphoadenosine 5′-phosphosulfate (PAPS)
PAPS reductase
NADPH
$NADP^+$
3′-Phosphoadenosine 5′-phosphate (PAP)
SO_3^{2-} Sulfite
sulfide reductase
3NADPH
$3NADP^+$
S^{2-} Sulfide

FIGURE 22-15 **Biosynthesis of cysteine from serine in bacteria and plants.** The origin of reduced sulfur is shown in the pathway on the right.

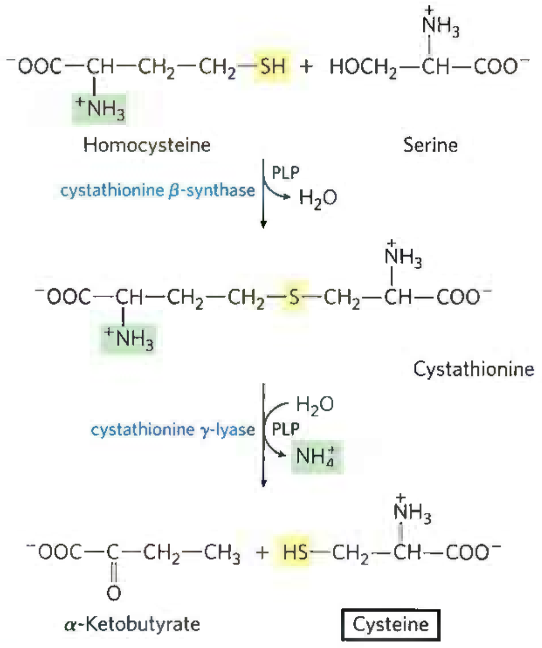
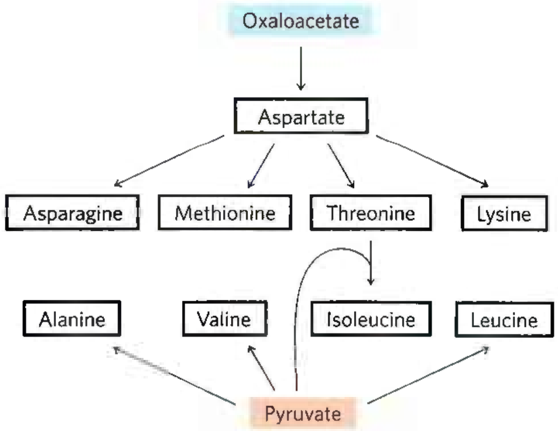

Homocysteine
Serine
cystathionine β-synthase
PLP
H_2O
Cystathionine
cystathionine γ-lyase
H_2O
PLP
NH_4^+
α-Ketobutyrate
Cysteine

FIGURE 22-16 **Biosynthesis of cysteine from homocysteine and serine in mammals.** The homocysteine is formed from methionine.

Three Nonessential and Six Essential Amino Acids Are Synthesized from Oxaloacetate and Pyruvate


Oxaloacetate
Aspartate
Asparagine
Methionine
Threonine
Lysine
Alanine
Valine
Isoleucine
Leucine
Pyruvate

Alanine and **aspartate** are synthesized from pyruvate and oxaloacetate, respectively, by transamination from glutamate. **Asparagine** is synthesized by amidation of aspartate, catalyzed by the enzyme asparagine synthetase. The NH_4^+ is donated by glutamine. These are nonessential amino acids, and their simple biosynthetic pathways occur in all organisms.

The malignant lymphocytes present in childhood acute lymphoblastic leukemia (ALL) produce little or no asparagine synthetase, and they are thus sensitive to asparagine depletion. The chemotherapy for ALL is administered together with an L-asparaginase derived from bacteria, with the enzyme functioning to reduce serum asparagine. The combined treatment results in a greater than 95% remission rate in cases of childhood ALL (L-asparaginase treatment alone produces remission in 40% to 60% of cases). However, the asparaginase treatment has some deleterious side effects, and about 10% of patients who achieve remission eventually suffer relapse, with tumors resistant to drug therapy. Researchers are now developing inhibitors of human asparagine synthetase to augment these therapies for childhood ALL. ■

Methionine, threonine, lysine, isoleucine, valine, and leucine are essential amino acids; humans cannot synthesize them. Their biosynthetic pathways in bacteria are complex and interconnected (**Fig. 22-17**). In some cases, the pathways in bacteria, fungi, and plants differ significantly.

Aspartate gives rise to **methionine**, **threonine**, and **lysine**. Branch points occur at aspartate β-semialdehyde, an intermediate in all three pathways, and at homoserine, a precursor of threonine and methionine. Threonine, in turn, is one of the precursors of isoleucine. The **valine** and **isoleucine** pathways share four enzymes (Fig. 22-17). Pyruvate gives rise to valine and isoleucine in pathways that begin with condensation of two carbons of pyruvate (in the form of hydroxyethyl thiamine pyrophosphate; see Fig. 14-13b) with another molecule of pyruvate (the valine path) or with α-ketobutyrate (the isoleucine path). The α-ketobutyrate is derived from threonine in a reaction

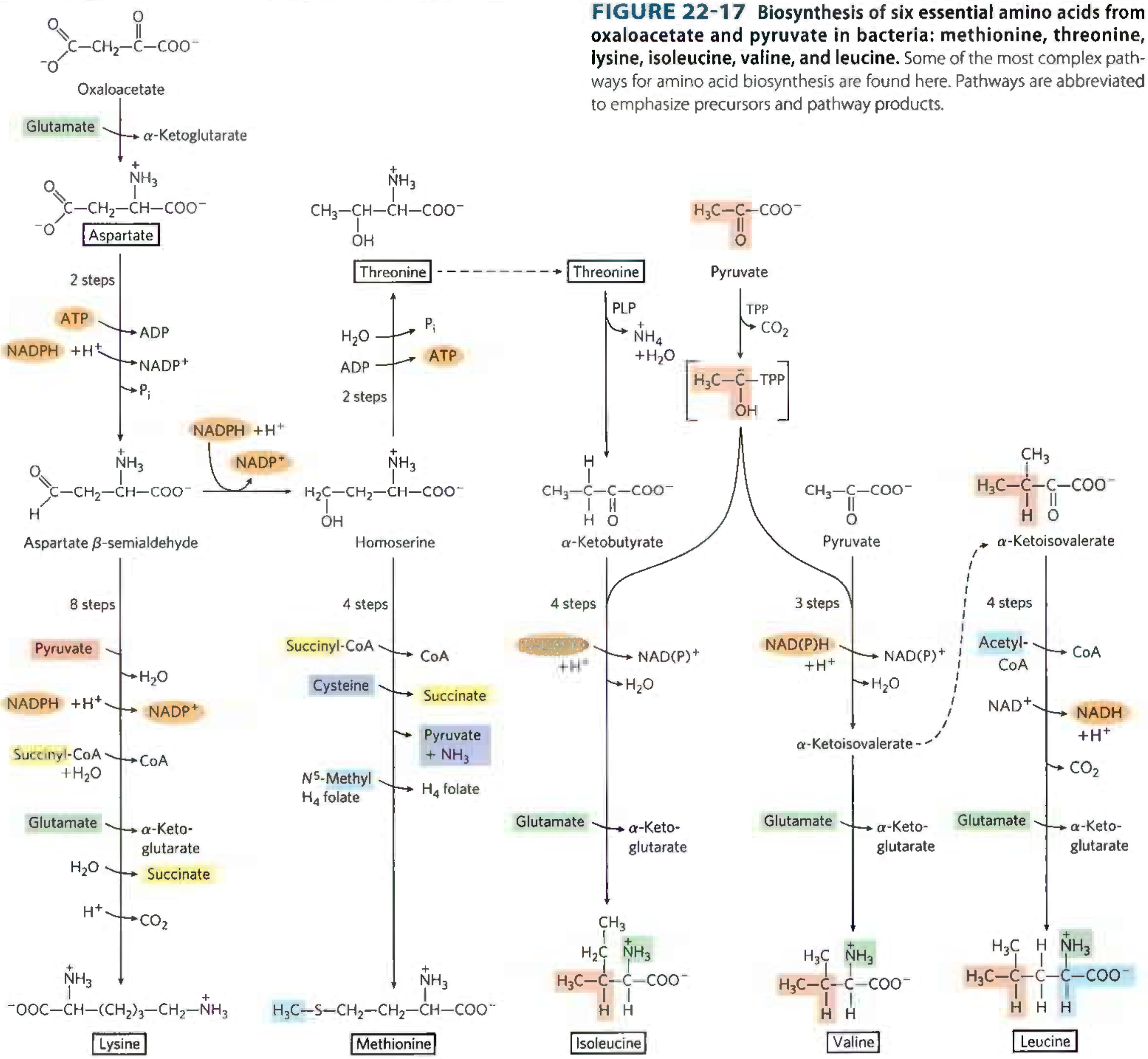
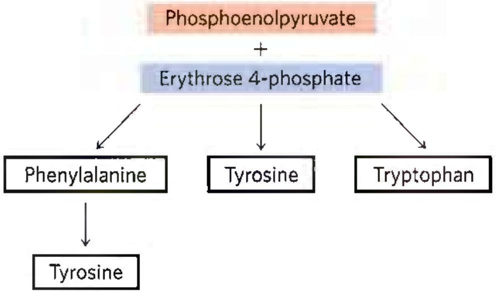

Oxaloacetate
Glutamate
α-Ketoglutarate
Aspartate
2 steps
ATP
ADP
NADPH +H⁺
NADP⁺
Pᵢ
NADPH +H⁺
NADP⁺
Aspartate β-semialdehyde
Homoserine
Threonine
H₂O
Pᵢ
ADP
ATP
2 steps
8 steps
Pyruvate
H₂O
NADPH +H⁺
NADP⁺
Succinyl-CoA +H₂O
CoA
Glutamate
α-Keto-glutarate
H₂O
Succinate
H⁺
CO₂
Lysine
4 steps
Succinyl-CoA
CoA
Cysteine
Succinate
Pyruvate + NH₃
N⁵-Methyl H₄ folate
H₄ folate
Methionine
Threonine
PLP
NH₄ +H₂O
α-Ketobutyrate
4 steps
+H⁺
NAD(P)⁺
H₂O
Glutamate
α-Keto-glutarate
Isoleucine
Pyruvate
TPP
CO₂
Pyruvate
3 steps
NAD(P)H +H⁺
NAD(P)⁺
H₂O
α-Ketoisovalerate
Glutamate
α-Keto-glutarate
Valine
α-Ketoisovalerate
4 steps
Acetyl-CoA
CoA
NAD⁺
NADH +H⁺
CO₂
Glutamate
α-Keto-glutarate
Leucine

FIGURE 22-17 Biosynthesis of six essential amino acids from oxaloacetate and pyruvate in bacteria: methionine, threonine, lysine, isoleucine, valine, and leucine. Some of the most complex pathways for amino acid biosynthesis are found here. Pathways are abbreviated to emphasize precursors and pathway products.

that requires pyridoxal phosphate. An intermediate in the valine pathway, α-ketoisovalerate, is the starting point for a four-step branch pathway leading to **leucine**.

Chorismate Is a Key Intermediate in the Synthesis of Tryptophan, Phenylalanine, and Tyrosine


Phosphoenolpyruvate
+
Erythrose 4-phosphate
Phenylalanine
Tyrosine
Tryptophan
Tyrosine

The three amino acid side chains that contain aromatic rings, tryptophan, phenylalanine, and tyrosine, present a special chemical problem for biosynthesis. Aromatic rings are not readily available in the environment, even though the benzene ring is very stable. The branched pathway to tryptophan, phenylalanine, and tyrosine, occurring in bacteria, fungi, and plants, is the main biological route of aromatic ring formation. It proceeds through ring closure of an aliphatic precursor followed by stepwise addition of double bonds. The first four steps produce shikimate, a seven-carbon molecule derived from erythrose 4-phosphate and phosphoenolpyruvate (**Fig. 22-18**). Shikimate is converted to chorismate in three steps that include the addition of three more carbons from another molecule of phosphoenolpyruvate. Chorismate is the first branch point of the pathway, with one branch leading to tryptophan, the other to phenylalanine and tyrosine.

In the **tryptophan** branch (**Fig. 22-19**), chorismate is converted to anthranilate in a reaction in which glutamine donates the nitrogen that will become part of the

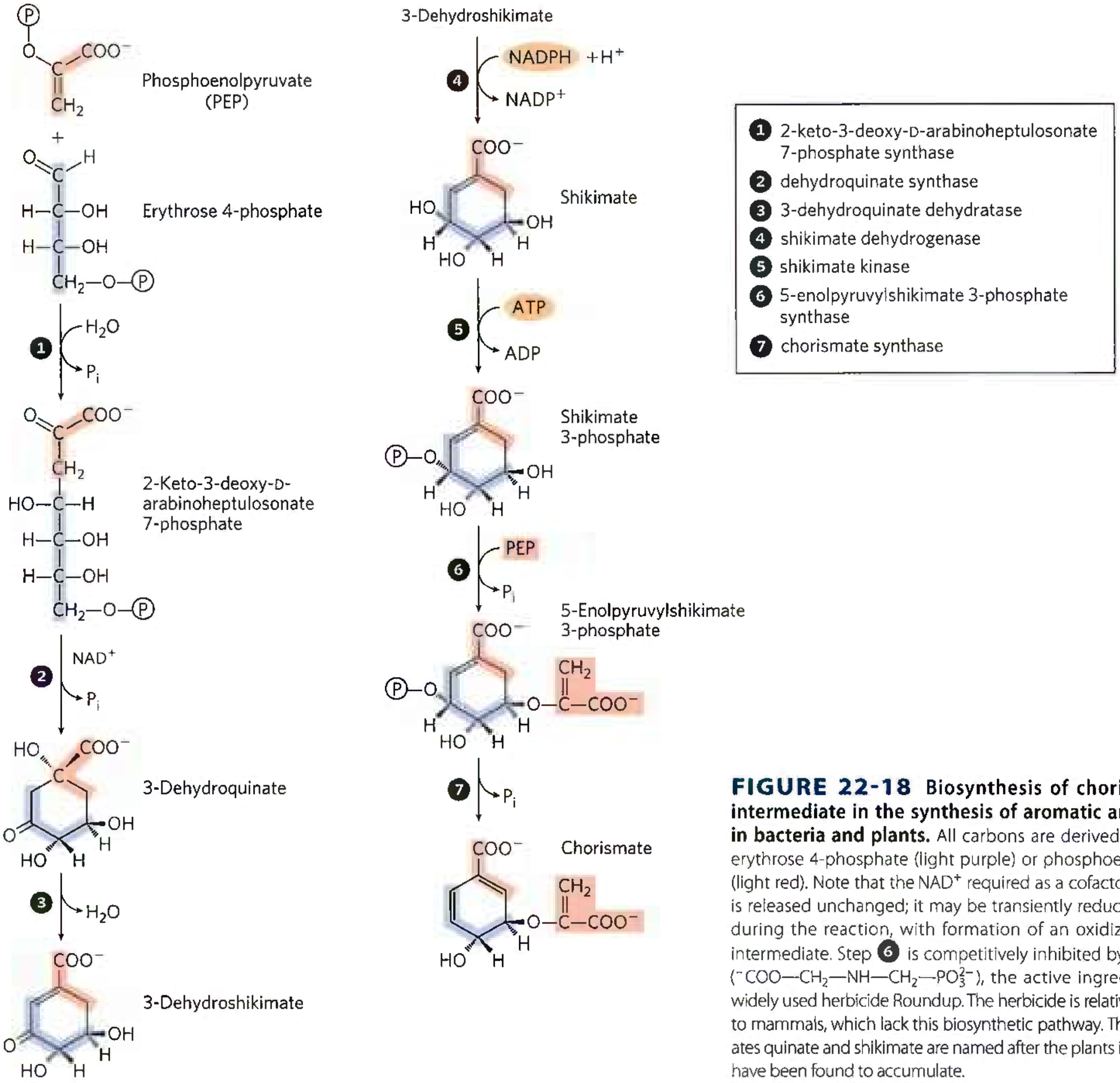

Phosphoenolpyruvate (PEP)
Erythrose 4-phosphate
H₂O
Pᵢ
2-Keto-3-deoxy-D-arabinoheptulosonate 7-phosphate
NAD⁺
Pᵢ
3-Dehydroquinate
H₂O
3-Dehydroshikimate
3-Dehydroshikimate
NADPH +H⁺
NADP⁺
Shikimate
ATP
ADP
Shikimate 3-phosphate
PEP
Pᵢ
5-Enolpyruvylshikimate 3-phosphate
Pᵢ
Chorismate
① 2-keto-3-deoxy-D-arabinoheptulosonate 7-phosphate synthase
② dehydroquinate synthase
③ 3-dehydroquinate dehydratase
④ shikimate dehydrogenase
⑤ shikimate kinase
⑥ 5-enolpyruvylshikimate 3-phosphate synthase
⑦ chorismate synthase

FIGURE 22-18 Biosynthesis of chorismate, an intermediate in the synthesis of aromatic amino acids in bacteria and plants. All carbons are derived from either erythrose 4-phosphate (light purple) or phosphoenolpyruvate (light red). Note that the NAD$^+$ required as a cofactor in step ② is released unchanged; it may be transiently reduced to NADH during the reaction, with formation of an oxidized reaction intermediate. Step ⑥ is competitively inhibited by glyphosate ($^-COO—CH_2—NH—CH_2—PO_3^{2-}$), the active ingredient in the widely used herbicide Roundup. The herbicide is relatively nontoxic to mammals, which lack this biosynthetic pathway. The intermediates quinate and shikimate are named after the plants in which they have been found to accumulate.

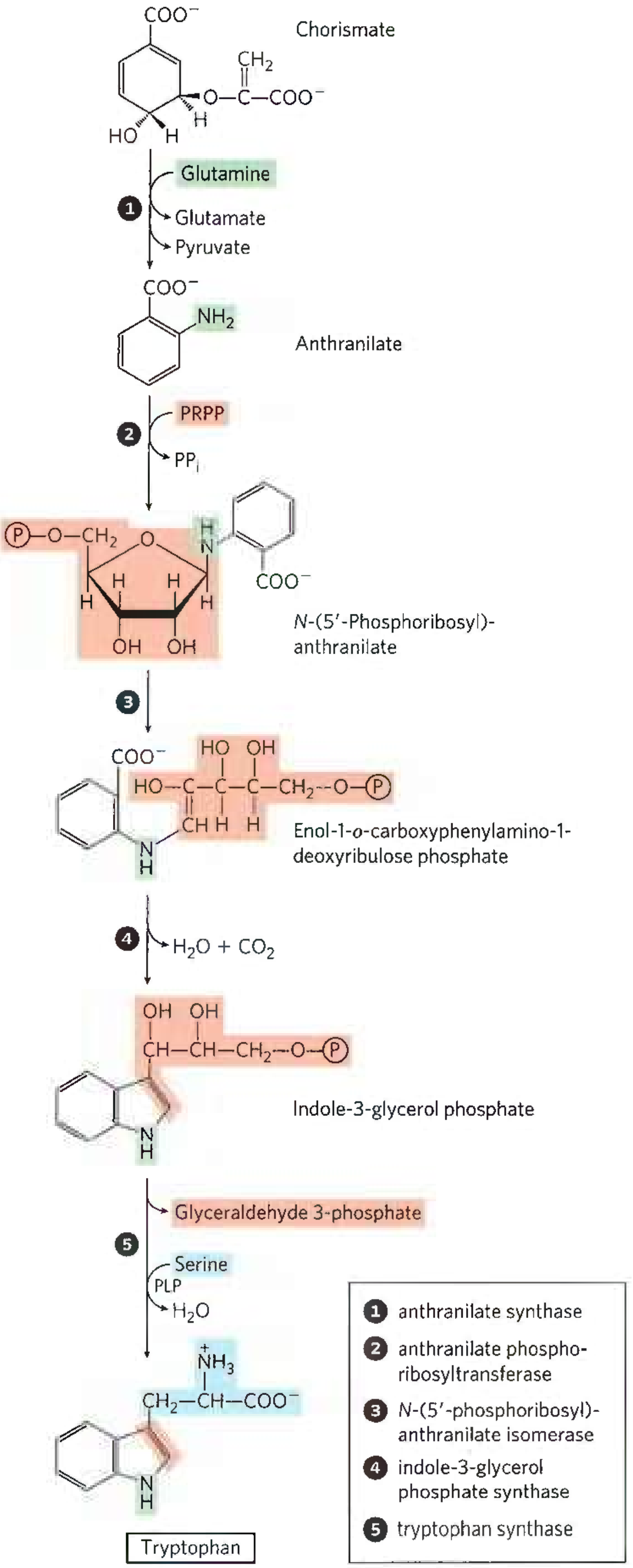

Chorismate
Glutamine
Glutamate
Pyruvate
Anthranilate
PRPP
PP_i
N-(5′-Phosphoribosyl)-anthranilate
Enol-1-o-carboxyphenylamino-1-deoxyribulose phosphate
$H_2O + CO_2$
Indole-3-glycerol phosphate
Glyceraldehyde 3-phosphate
Serine
PLP
H_2O
Tryptophan
① anthranilate synthase
② anthranilate phosphoribosyltransferase
③ N-(5′-phosphoribosyl)-anthranilate isomerase
④ indole-3-glycerol phosphate synthase
⑤ tryptophan synthase

FIGURE 22-19 Biosynthesis of tryptophan from chorismate in bacteria and plants. In *E. coli*, enzymes catalyzing steps ① and ② are subunits of a single complex.

indole ring. Anthranilate then condenses with PRPP. The indole ring of tryptophan is derived from the ring carbons and amino group of anthranilate plus two carbons derived from PRPP. The final reaction in the sequence is catalyzed by **tryptophan synthase**. This enzyme has an $\alpha_2\beta_2$ subunit structure and can be dissociated into two α subunits and a β_2 unit that catalyze different parts of the overall reaction:

$$\text{Indole-3-glycerol phosphate} \xrightarrow[\alpha\ \text{subunit}]{} \text{indole} + \text{glyceraldehyde 3-phosphate}$$

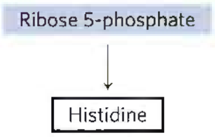
$$\text{Indole} + \text{serine} \xrightarrow[\beta_2\ \text{subunit}]{} \text{tryptophan} + H_2O$$

The second part of the reaction requires pyridoxal phosphate (**Fig. 22-20**). Indole formed in the first part is not released by the enzyme, but instead moves through a channel from the α-subunit active site to one of the β-subunit active sites, where it condenses with a Schiff base intermediate derived from serine and PLP. Intermediate channeling of this type may be a feature of the entire pathway from chorismate to tryptophan. Enzyme active sites catalyzing different steps (sometimes not sequential steps) of the pathway to tryptophan are found on single polypeptides in some species of fungi and bacteria, but they are separate proteins in other species. In addition, the activity of some of these enzymes requires a noncovalent association with other enzymes of the pathway. These observations suggest that all the pathway enzymes are components of a large, multienzyme complex, a metabolon, in both bacteria and eukaryotes. Such complexes are generally not preserved intact when the enzymes are isolated using traditional biochemical methods (see Section 16.4).

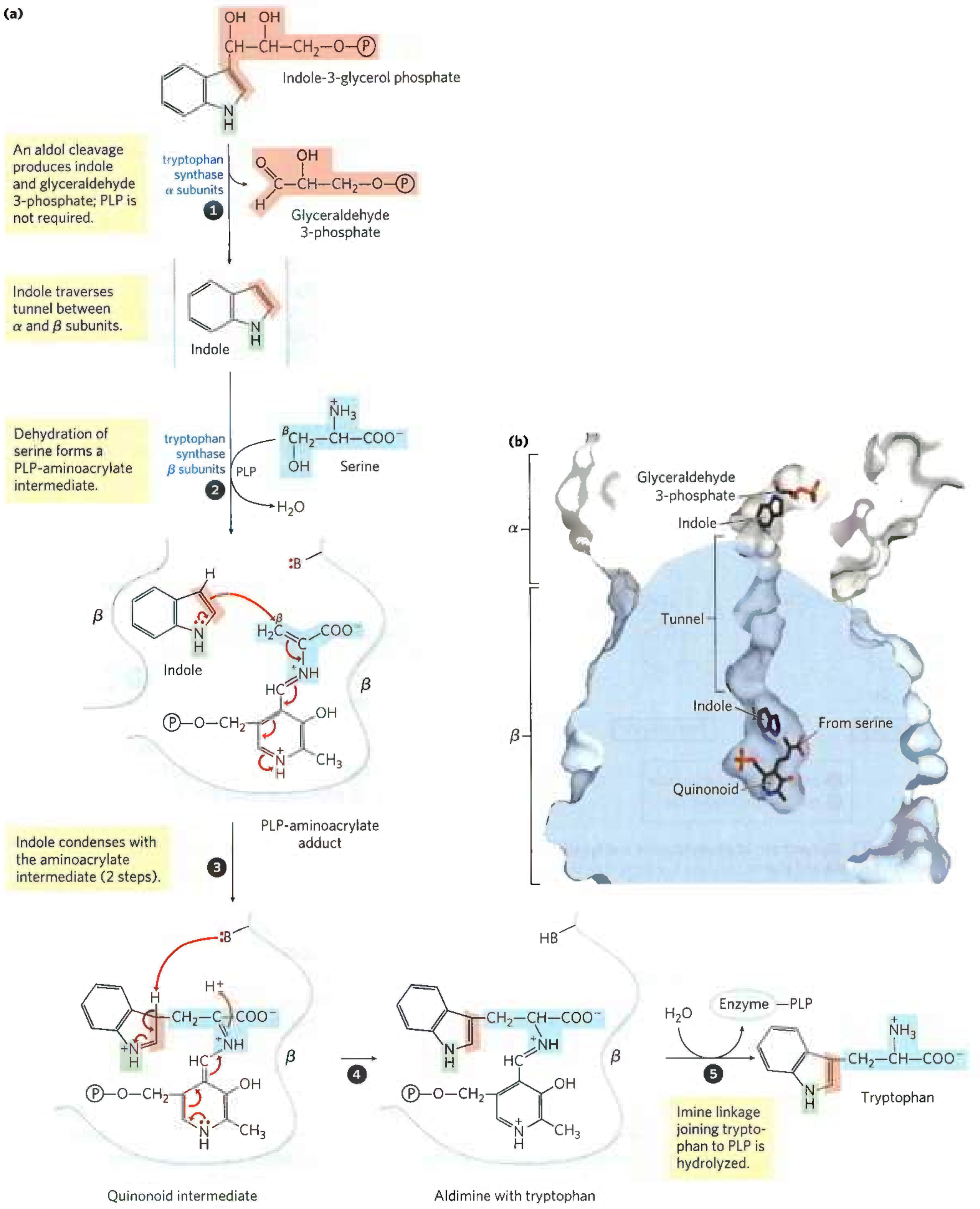
In plants and bacteria, **phenylalanine** and **tyrosine** are synthesized from chorismate in pathways much less complex than the tryptophan pathway. The common intermediate is prephenate (**Fig. 22-21**). The final step in both cases is transamination with glutamate.

Animals can produce tyrosine directly from phenylalanine through hydroxylation at C-4 of the phenyl group by **phenylalanine hydroxylase**; this enzyme also participates in the degradation of phenylalanine (see Figs. 18-23, 18-24). Tyrosine is considered a conditionally essential amino acid, or as nonessential insofar as it can be synthesized from the essential amino acid phenylalanine.

Histidine Biosynthesis Uses Precursors of Purine Biosynthesis


Ribose 5-phosphate
Histidine

The pathway to **histidine** in all plants and bacteria differs in several respects from other amino acid


(a)
OH OH
CH—CH—CH2—O—Ⓟ
Indole-3-glycerol phosphate
N H
tryptophan synthase α subunits
1
OH
O
C—CH—CH2—O—Ⓟ
H
Glyceraldehyde 3-phosphate
Indole
tryptophan synthase β subunits
2
PLP
NH3
βCH2—CH—COO−
OH
Serine
H2O
:B
H
β
Indole
H2C
COO−
NH
HC
OH
Ⓟ—O—CH2
N
CH3
β
PLP-aminoacrylate adduct
3
:B
H+
CH2—C—COO−
NH
HC
OH
Ⓟ—O—CH2
CH3
β
Quinonoid intermediate
4
HB
CH2—CH—COO−
NH
HC
OH
Ⓟ—O—CH2
CH3
β
Aldimine with tryptophan
H2O
Enzyme —PLP
5
NH3
CH2—CH—COO−
Tryptophan
(b)
α
β
Glyceraldehyde 3-phosphate
Indole
Tunnel
Indole
From serine
Quinonoid

An aldol cleavage produces indole and glyceraldehyde 3-phosphate; PLP is not required.

Indole traverses tunnel between α and β subunits.

Dehydration of serine forms a PLP-aminoacrylate intermediate.

Indole condenses with the aminoacrylate intermediate (2 steps).

Imine linkage joining tryptophan to PLP is hydrolyzed.

MECHANISM FIGURE 22-20 Tryptophan synthase reaction. (a) This enzyme catalyzes a multistep reaction with several types of chemical rearrangements. The PLP-facilitated transformations occur at the β carbon (C-3) of the amino acid, as opposed to the α-carbon reactions described in Figure 18-6. The β carbon of serine is attached to the indole ring system. (b) Indole generated on the α subunit (white) moves through a tunnel to the β subunit (blue), where it condenses with the serine moiety. [(b) Data from PDB ID 1KFJ, V. Kulik et al., *J. Mol. Biol.* 324:677, 2002.]

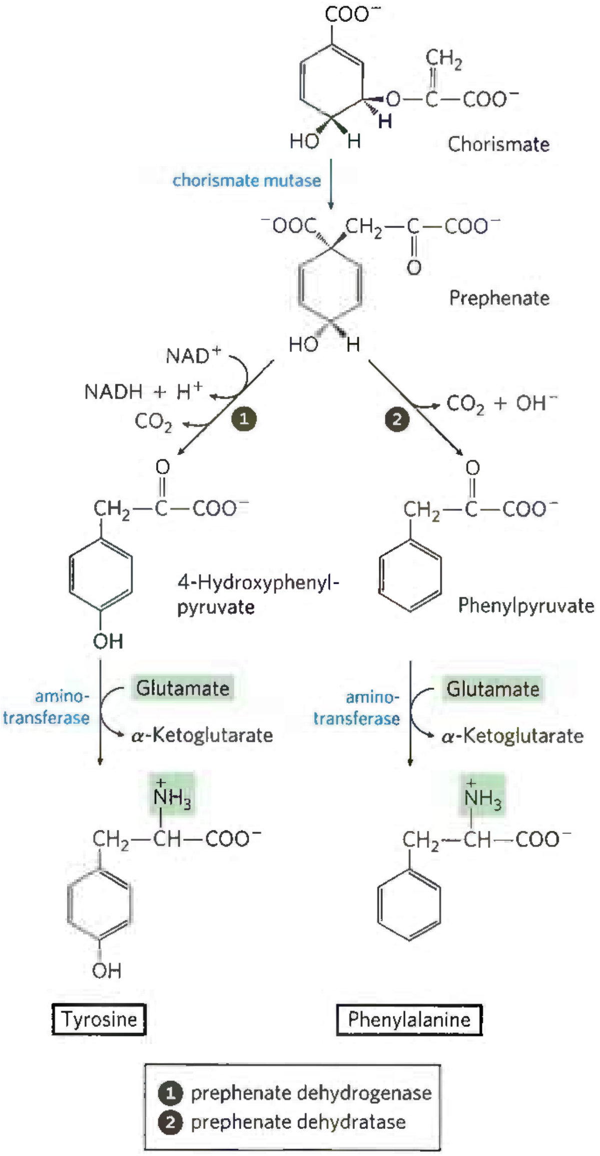


FIGURE 22-21 Biosynthesis of phenylalanine and tyrosine from chorismate in bacteria and plants. Conversion of chorismate to prephenate is a rare biological example of a Claisen rearrangement.

biosynthetic pathways. Histidine is derived from three precursors (**Fig. 22-22**): PRPP contributes five carbons, the purine ring of ATP contributes a nitrogen and a carbon, and glutamine supplies the second ring nitrogen. The key steps are condensation of ATP and PRPP, in which N-1 of the purine ring is linked to the activated C-1 of the ribose of PRPP (step ① in Fig. 22-22); purine ring opening that ultimately leaves N-1 and C-2 of adenine linked to the ribose (step ③); and formation of the imidazole ring, a reaction in which glutamine donates a nitrogen (step ⑤). The use of ATP as a metabolite rather than a high-energy cofactor is unusual—but not wasteful, because it dovetails with the purine biosynthetic pathway. The remnant of ATP that is released after the transfer of N-1 and C-2 is 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an intermediate of purine biosynthesis (see Fig. 22-35) that is rapidly recycled to ATP.

Amino Acid Biosynthesis Is under Allosteric Regulation

As detailed in Chapter 13, the control of flux through a metabolic pathway often reflects the activity of multiple enzymes in that pathway. **P4** In the case of amino acid synthesis, regulation often takes place in part through feedback inhibition of the first reaction by the end product of the pathway. This first reaction is often catalyzed by an allosteric enzyme that plays an important role in the overall control of flux through that pathway. As an example, **Figure 22-23** shows the allosteric regulation of isoleucine synthesis from threonine. The end product, isoleucine, is an allosteric inhibitor of the first reaction in the sequence. In bacteria, such allosteric modulation of amino acid synthesis contributes to the minute-to-minute adjustment of pathway activity to cellular needs.

Allosteric regulation of an individual enzyme can be considerably more complex. An example is the remarkable set of allosteric controls exerted on glutamine synthetase of *E. coli* (Fig. 22-8). Six products derived from glutamine serve as negative feedback modulators of the enzyme, and the overall effects of these and other modulators are more than additive. Such regulation is called **concerted inhibition**.

Additional mechanisms contribute to the regulation of the amino acid biosynthetic pathways. **P1** **P4** Because the 20 common amino acids must be made in the correct proportions for protein synthesis, cells have developed ways not only of controlling the rate of synthesis of individual amino acids but also of coordinating their formation. Such coordination is especially well developed in fast-growing bacterial cells. **Figure 22-24** shows how *E. coli* cells coordinate the synthesis of lysine, methionine, threonine, and isoleucine, all made from aspartate. Several important types of inhibition patterns are evident. The step from aspartate to aspartyl-β-phosphate is catalyzed by three isozymes, each independently controlled by different modulators. This **enzyme multiplicity** prevents one biosynthetic end product from shutting down key steps in a pathway when other products of the same pathway are required. The steps from aspartate β-semialdehyde to homoserine and from threonine to α-ketobutyrate (detailed in Fig. 22-17) are also catalyzed by dual, independently controlled isozymes. One isozyme for the conversion of aspartate to aspartyl-β-phosphate is allosterically inhibited by two different modulators, lysine and isoleucine, whose action is more than additive—another example of concerted inhibition. The sequence from aspartate to isoleucine undergoes multiple, overlapping negative feedback inhibitions; for example, isoleucine inhibits the conversion of threonine to α-ketobutyrate (as described above), and threonine inhibits its own formation at three points: from homoserine,

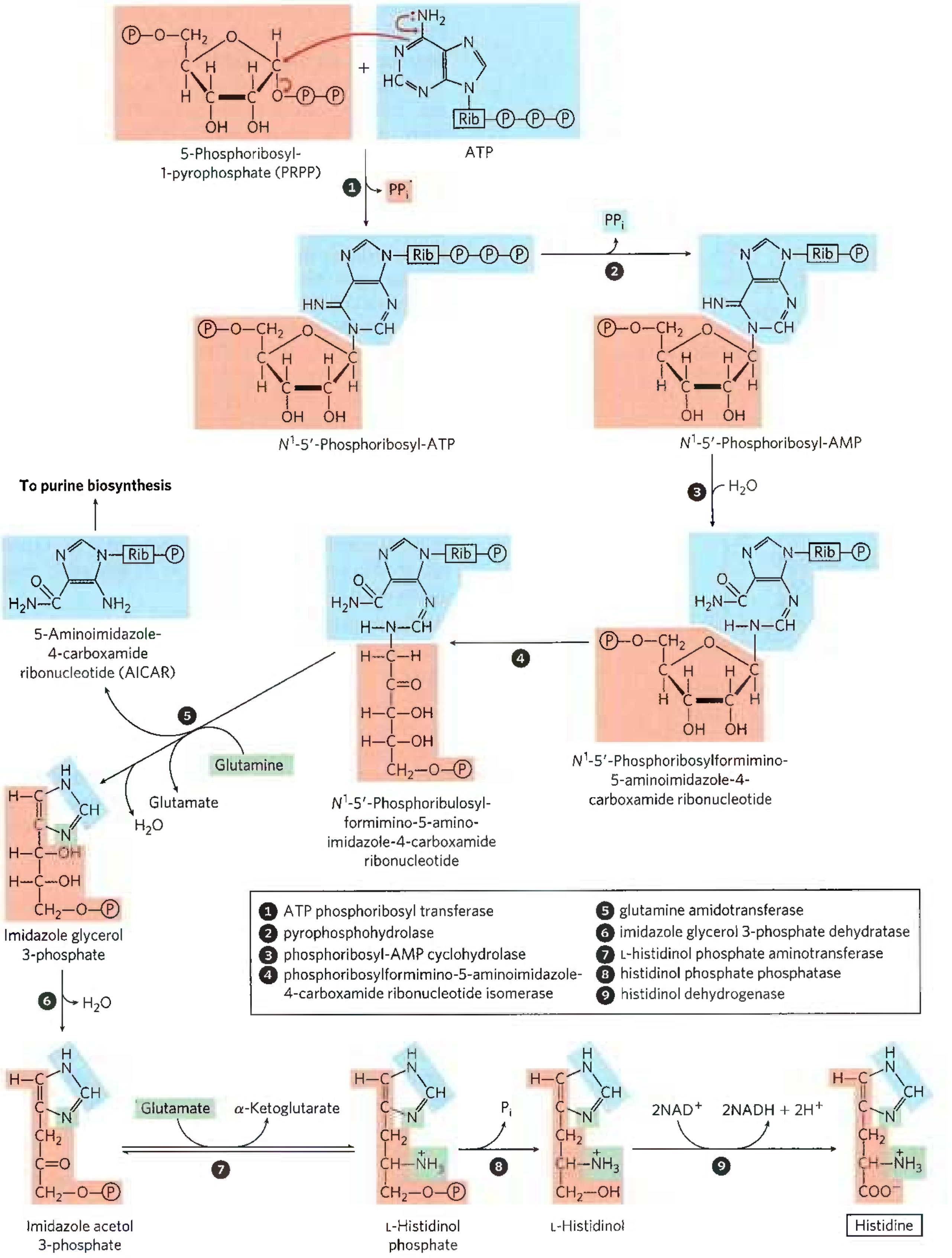

5-Phosphoribosyl-1-pyrophosphate (PRPP)
ATP
PP$_i$
N^1-5′-Phosphoribosyl-ATP
PP$_i$
N^1-5′-Phosphoribosyl-AMP
H$_2$O
To purine biosynthesis
5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR)
N^1-5′-Phosphoribulosyl-formimino-5-amino-imidazole-4-carboxamide ribonucleotide
N^1-5′-Phosphoribosylformimino-5-aminoimidazole-4-carboxamide ribonucleotide
Glutamine
Glutamate
H$_2$O
Imidazole glycerol 3-phosphate
H$_2$O
Imidazole acetol 3-phosphate
Glutamate
α-Ketoglutarate
L-Histidinol phosphate
P$_i$
L-Histidinol
2NAD$^+$
2NADH + 2H$^+$
Histidine
❶ ATP phosphoribosyl transferase
❷ pyrophosphohydrolase
❸ phosphoribosyl-AMP cyclohydrolase
❹ phosphoribosylformimino-5-aminoimidazole-4-carboxamide ribonucleotide isomerase
❺ glutamine amidotransferase
❻ imidazole glycerol 3-phosphate dehydratase
❼ L-histidinol phosphate aminotransferase
❽ histidinol phosphate phosphatase
❾ histidinol dehydrogenase

FIGURE 22-22 Biosynthesis of histidine in bacteria and plants. Atoms derived from PRPP and ATP are shaded light red and blue, respectively. Two of the histidine nitrogens are derived from glutamine and glutamate (green). Note that the derivative of ATP remaining after step ❺ (AICAR) is an intermediate in purine biosynthesis (see Fig. 22-35, step ❾), so ATP is rapidly regenerated.

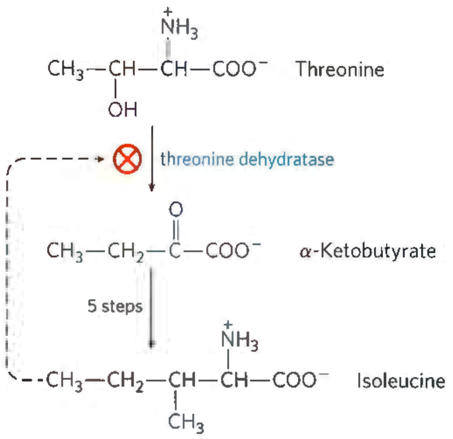


FIGURE 22-23 Allosteric regulation of isoleucine biosynthesis. The first reaction in the pathway from threonine to isoleucine is inhibited by the end product, isoleucine. This was one of the first examples of allosteric feedback inhibition to be discovered.

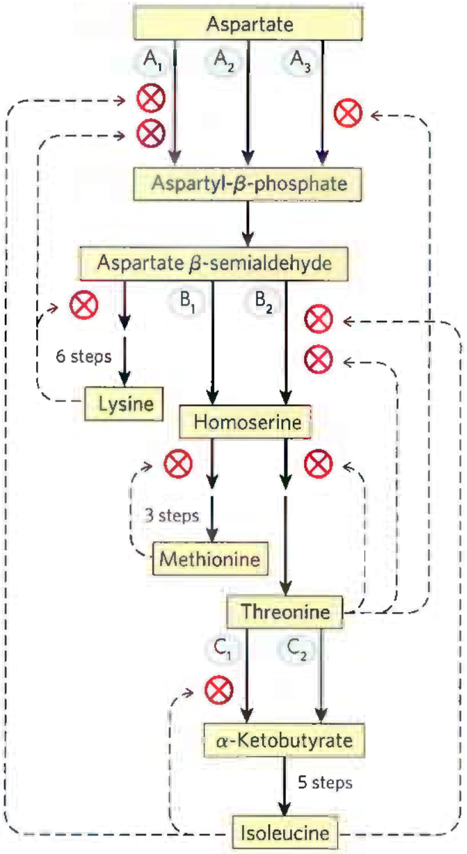


FIGURE 22-24 Interlocking regulatory mechanisms in the biosynthesis of several amino acids derived from aspartate in *E. coli*. Three enzymes (A, B, C) have either two or three isozyme forms, indicated by numerical subscripts. In each case, one isozyme (A_2, B_1, and C_2) has no allosteric regulation; these isozymes are regulated by changes in the amount of enzyme synthesized. Synthesis of isozymes A_2 and B_1 is repressed when methionine levels are high, and synthesis of isozyme C_2 is repressed when isoleucine levels are high. Enzyme A is aspartokinase; B, homoserine dehydrogenase; C, threonine dehydratase.

from aspartate β-semialdehyde, and from aspartate (see Fig. 22-17). This overall regulatory mechanism is called **sequential feedback inhibition**.

Similar patterns are evident in the pathways leading to the aromatic amino acids. The first step of the early pathway to the common intermediate chorismate is catalyzed by the enzyme 2-keto-3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase (❶ in Fig. 22-18). Most microorganisms and plants have three DAHP synthase isozymes. One is allosterically inhibited (feedback inhibition) by phenylalanine, another by tyrosine, and the third by tryptophan. This scheme helps the overall pathway to respond to cellular requirements for one or more of the aromatic amino acids. Additional regulation takes place after the pathway branches at chorismate. For example, the enzymes catalyzing the first two steps of the tryptophan branch are subject to allosteric inhibition by tryptophan.

SUMMARY 22.2 *Biosynthesis of Amino Acids*

- Plants and bacteria synthesize all 20 common amino acids. Mammals can synthesize about half; the others are required in the diet (essential or conditionally essential amino acids).
- Glutamate is formed by reductive amination of α-ketoglutarate and serves as the precursor of glutamine, proline, and arginine.
- The carbon chain of serine is derived from 3-phosphoglycerate. Serine is a precursor of glycine; the β-carbon atom of serine is transferred to tetrahydrofolate. In microorganisms, cysteine is produced from serine and from sulfide produced by the reduction of environmental sulfate. Mammals produce cysteine from methionine and serine by a series of reactions requiring *S*-adenosylmethionine and cystathionine.
- Alanine and aspartate (and thus asparagine) are formed from pyruvate and oxaloacetate, respectively, by transamination. Pyruvate and oxaloacetate also give rise to methionine, threonine, lysine, valine, isoleucine, and leucine via longer pathways.
- The aromatic amino acids (phenylalanine, tyrosine, and tryptophan) form by a pathway in which chorismate occupies a key branch point. Tyrosine can also be formed by hydroxylation of phenylalanine (and thus is considered conditionally essential). Phosphoribosyl pyrophosphate is a precursor of tryptophan and histidine.
- The pathway to histidine is interconnected with the purine synthetic pathway.
- The amino acid biosynthetic pathways are subject to allosteric end-product inhibition; the regulatory enzyme is usually the first in the sequence. Regulation of the various synthetic pathways is coordinated.

22.3 Molecules Derived from Amino Acids

In addition to their role as the building blocks of proteins, amino acids are precursors of many specialized biomolecules, including hormones, coenzymes, nucleotides, alkaloids, cell wall polymers, porphyrins, antibiotics, pigments, and neurotransmitters. We describe here the pathways to a number of these amino acid derivatives.

Glycine Is a Precursor of Porphyrins

The biosynthesis of **porphyrins**, for which glycine is a major precursor, is our first example because of the central importance of the porphyrin nucleus in heme proteins such as hemoglobin and the cytochromes. The porphyrins are constructed from four molecules of the monopyrrole derivative **porphobilinogen**, which itself is derived from two molecules of δ-aminolevulinate. There are two major pathways to δ-aminolevulinate. In higher eukaryotes (**Fig. 22-25a**), glycine reacts with succinyl-CoA in the first step to yield α-amino-β-ketoadipate, which is then decarboxylated to δ-aminolevulinate. In plants, algae, and most bacteria, δ-aminolevulinate is formed from glutamate (Fig. 22-25b). The glutamate is first esterified to glutamyl-$tRNA^{Glu}$; **P6** reduction by NADPH converts the glutamate to glutamate 1-semialdehyde, which is cleaved from the tRNA. An aminotransferase converts the glutamate 1-semialdehyde to δ-aminolevulinate.

In all organisms, two molecules of δ-aminolevulinate condense to form porphobilinogen and, through a series of complex enzymatic reactions, four molecules of porphobilinogen come together to form **protoporphyrin** (**Fig. 22-26**). The iron atom is incorporated after the protoporphyrin has been assembled, in a step catalyzed by ferrochelatase. Porphyrin biosynthesis is regulated in higher eukaryotes by heme, which serves as a feedback inhibitor of early steps in the synthetic pathway. Genetic defects in the biosynthesis of porphyrins can lead to the accumulation of pathway intermediates, causing a variety of human diseases known collectively as **porphyrias** (**Box 22-2**).

Heme Degradation Has Multiple Functions

The iron-porphyrin (heme) group of hemoglobin, released from dying erythrocytes in the spleen, is degraded to yield free Fe^{2+} and, ultimately, **bilirubin**. The pathway also contributes the pigment present in mixtures of the bile salts derived from cholesterol.

The first step in the two-step pathway to bilirubin, catalyzed by heme oxygenase, converts heme to biliverdin, a linear (open) tetrapyrrole derivative (**Fig. 22-27** on p. 820). The other products of the reaction are free Fe^{2+} and CO. The Fe^{2+} is quickly bound by ferritin. Carbon monoxide is a poison that binds to hemoglobin (see Box 5-1), and the production of CO by heme oxygenase ensures that, even in the absence of environmental exposure, about 1% of an individual's heme is complexed with CO.

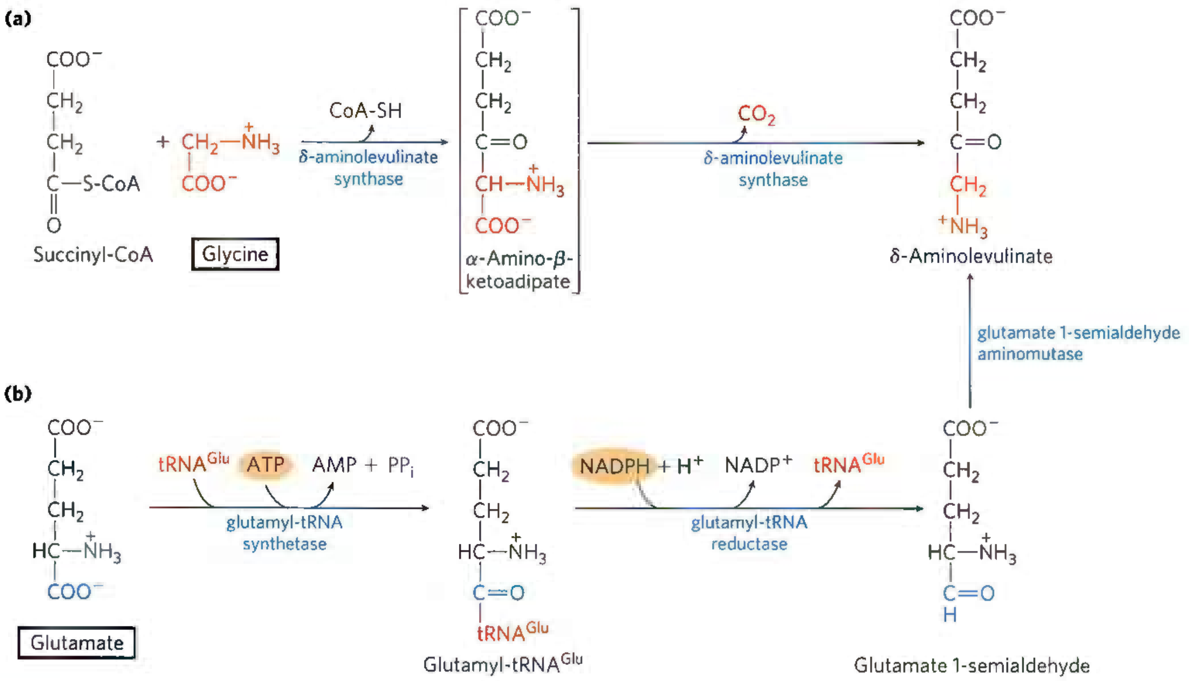
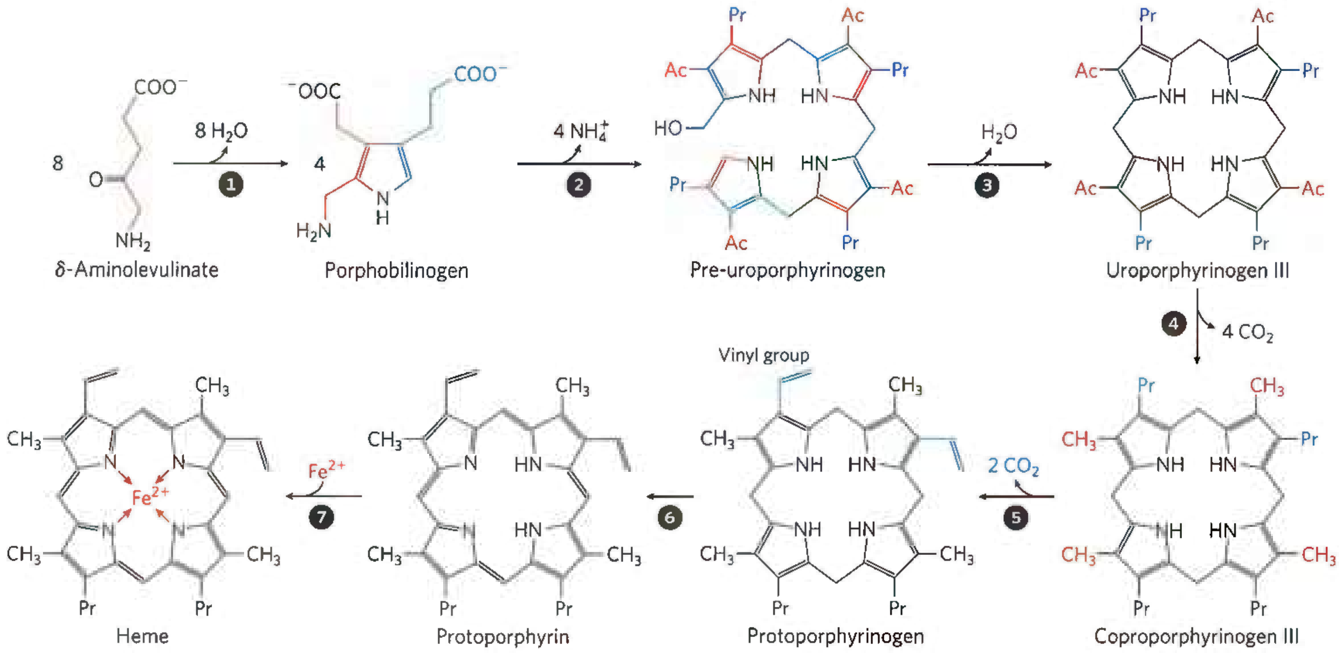


FIGURE 22-25 **Biosynthesis of δ-aminolevulinate.** (a) In most animals, including mammals, δ-aminolevulinate is synthesized from glycine and succinyl-CoA. The atoms furnished by glycine are shown in red. (b) In bacteria and plants, the precursor of δ-aminolevulinate is glutamate.



① porphobilinogen synthase
② uroporphyrinogen synthase
③ uroporphyrinogen III cosynthase
④ uroporphyrinogen decarboxylase
⑤ coproporphyrinogen oxidase
⑥ protoporphyrinogen oxidase
⑦ ferrochelatase

FIGURE 22-26 Biosynthesis of heme from δ-aminolevulinate. Ac represents acetyl (—CH_2COO^-); Pr, propionyl (—$CH_2CH_2COO^-$).

Biliverdin is converted to bilirubin in the second step, catalyzed by biliverdin reductase. You can monitor the reactions in the breakdown of heme (Fig. 22-27) colorimetrically in a familiar in situ experiment. When you are bruised, the black and/or purple color results from hemoglobin released from damaged erythrocytes. Over time, the color changes to the green of biliverdin, and then to the yellow of bilirubin. Bilirubin is largely insoluble, and it travels in the bloodstream as one of many metabolites, fatty acids and others, complexed with serum albumin (see Fig. 17-3). In the liver, bilirubin is transformed to the bile pigment bilirubin diglucuronide. This product is sufficiently water-soluble to be secreted with other components of bile into the small intestine, where microbial enzymes convert it to several products, predominantly urobilinogen. Some urobilinogen is reabsorbed into the blood and transported to the kidney, where it is converted to urobilin, the compound that gives urine its yellow color. Urobilinogen remaining in the intestine is converted (in another microbe-dependent reaction) to stercobilin, which imparts the red-brown color to feces.

Impaired liver function or blocked bile secretion causes bilirubin to leak from the liver into the blood, resulting in a yellowing of the skin and eyeballs, a condition called jaundice. In cases of jaundice, determination of the concentration of bilirubin in the blood may be useful in the diagnosis of underlying liver disease. Newborn infants sometimes develop jaundice because they have not yet produced enough glucuronyl bilirubin transferase to process their bilirubin. A traditional treatment to reduce excess bilirubin, exposure to a fluorescent lamp, causes a photochemical conversion of bilirubin to compounds that are more soluble and easily excreted.

These pathways of heme breakdown play significant roles in protecting cells from oxidative damage and in regulating certain cellular functions. The CO produced by heme oxygenase is toxic at high concentrations, but at the very low concentrations generated during heme degradation it seems to have some regulatory and/or signaling functions. It acts as a vasodilator, much the same as (but less potent than) nitric oxide (discussed below). Low levels of CO also have some regulatory effects on neurotransmission. Bilirubin is the most abundant antioxidant in mammalian tissues and is responsible for most of the antioxidant activity in serum. Its protective effects seem to be especially important in the developing brain

BOX 22-2 MEDICINE

On Kings and Vampires

Porphyrias are a group of genetic diseases that result from defects in enzymes of the biosynthetic pathway from glycine to porphyrins (Fig. 1); specific porphyrin precursors accumulate in erythrocytes, body fluids, and the liver. The most common form is acute intermittent porphyria. Most individuals inheriting this condition are heterozygotes and are usually asymptomatic, because the single copy of the normal gene provides a sufficient level of enzyme function. However, certain nutritional or environmental factors (as yet poorly understood) can cause a buildup of δ-aminolevulinate and porphobilinogen, leading to attacks of acute abdominal pain and neurological dysfunction. King George III, British monarch during the American Revolution, suffered several episodes of apparent madness that tarnished the record of this otherwise accomplished man. The symptoms of his condition suggest that George III suffered from acute intermittent porphyria.

About 5,000 to 10,000 humans worldwide suffer from a genetic condition in which the activity of ferrochelatase (step ⑦) is reduced, leading to abnormally high concentrations of protoporphyrin in tissues. When exposed to light, the excess protoporphyrin releases free radicals that damage cellular macromolecules, including proteins and lipids in cell membranes. The resulting cell damage and inflammation in the endothelial (skin) cells can be very painful. Patients are forced to adopt a lifestyle in which they avoid sunlight and even bright indoor light. A new drug called afamelanotide, slowly released over several months from a small implant under the skin, has shown success in alleviating the symptoms of sunlight exposure. The drug interacts with the melanocortin-1 receptor, stimulating melanin production and modulating the expression of antioxidants that eliminate the free radical species. Afamelanotide may also prove useful in treating vitiligo, a more common condition in which melanin production is not uniform and skin color is lost in blotches.

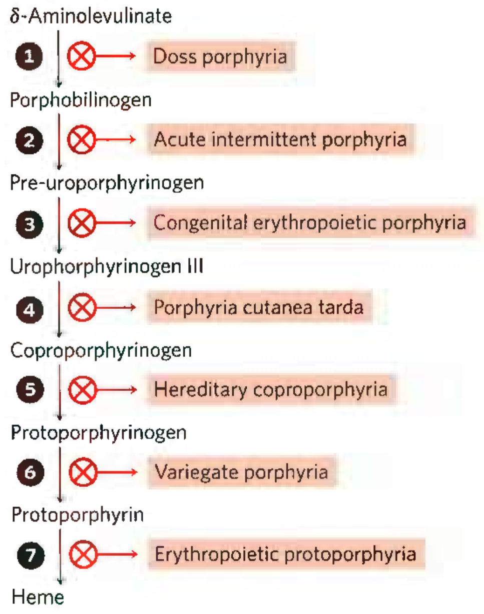


FIGURE 1 The key to Figure 22-26 identifies the defective enzyme at each step.

One of the rarer porphyrias results in an accumulation of uroporphyrinogen I, an abnormal isomer of a protoporphyrin precursor. This compound stains the urine red, causes the teeth to fluoresce strongly in ultraviolet light, and makes the skin abnormally sensitive to sunlight. Many individuals with this porphyria are anemic because insufficient heme is synthesized. This genetic condition may have given rise to the vampire stories of folk legend.

The symptoms of many porphyrias are now readily controlled with dietary changes or the administration of heme or heme derivatives.

of newborn infants. The cell toxicity associated with jaundice may be due to bilirubin levels in excess of the serum albumin needed to solubilize it.

P4 Given these varied roles of heme degradation products, the degradative pathway is subject to regulation, mainly at the first step. Humans have at least three isozymes of heme oxygenase (HO). HO-1 is highly regulated; the expression of its gene is induced by a wide range of stress conditions, including shear stress, uncontrolled angiogenesis (development of blood vessels), hypoxia, hyperoxia, heat shock, exposure to ultraviolet light, hydrogen peroxide, and many other metabolic insults. HO-2 is found mainly in the brain and the testes, where it is continuously expressed. The third isozyme, HO-3, is not catalytically active, but may play a role in oxygen sensing. ■

Amino Acids Are Precursors of Creatine and Glutathione

Phosphocreatine, derived from **creatine**, is an important energy buffer in skeletal muscle (see Box 23-1). Creatine is synthesized from glycine and arginine (**Fig. 22-28**); methionine, in the form of *S*-adenosylmethionine, acts as methyl group donor.

Glutathione (GSH), present in plants, animals, and some bacteria, often at high levels, can be thought of as a redox buffer. It is derived from glutamate, cysteine, and glycine (**Fig. 22-29**). P6 The γ-carboxyl group of glutamate is activated by ATP to form an acyl phosphate intermediate, which is then attacked by the α-amino group of cysteine. A second condensation reaction follows, with the α-carboxyl group of cysteine activated to an acyl phosphate to permit reaction with glycine.

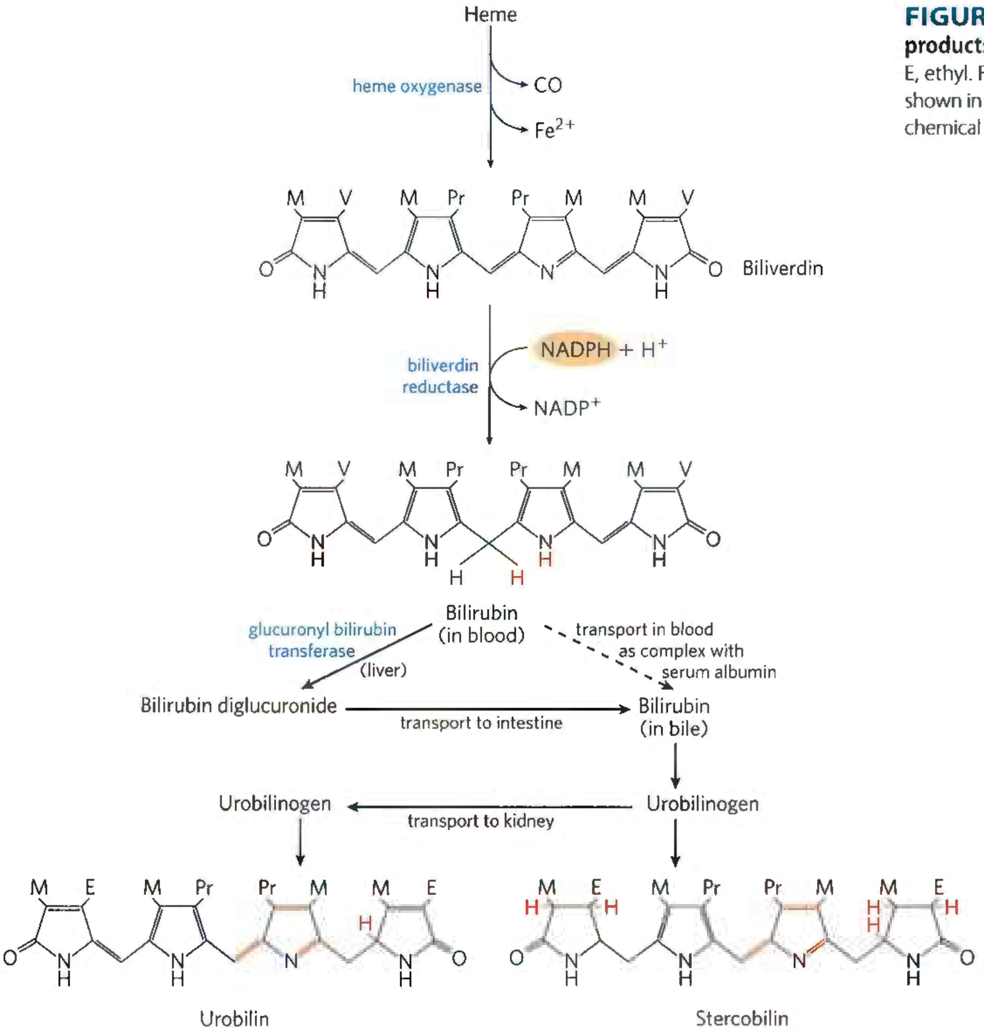

Heme
heme oxygenase
CO
Fe2+
M V M Pr Pr M M V
Biliverdin
biliverdin reductase
NADPH + H+
NADP+
M V M Pr Pr M M V
H H
Bilirubin (in blood)
glucuronyl bilirubin transferase
(liver)
transport in blood as complex with serum albumin
Bilirubin diglucuronide
transport to intestine
Bilirubin (in bile)
Urobilinogen
transport to kidney
Urobilinogen
M E M Pr Pr M M E
Urobilin
M E M Pr Pr M M E
Stercobilin

FIGURE 22-27 Bilirubin and its breakdown products. M represents methyl; V, vinyl; Pr, propionyl; E, ethyl. For ease of comparison, these structures are shown in linear form, rather than in their correct stereochemical conformations.

The oxidized form of glutathione (glutathione disulfide, or GSSG), produced in the course of its redox activities, contains two glutathione molecules linked by a disulfide bond.

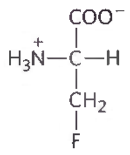
Glutathione helps maintain the sulfhydryl groups of proteins in the reduced state and the iron of heme in the ferrous (Fe^{2+}) state, and it serves as a reducing agent for glutaredoxin in deoxyribonucleotide synthesis (see Fig. 22-41). Its redox function is also used to remove toxic peroxides formed in the normal course of growth and metabolism under aerobic conditions:

$$2\,\mathrm{GSH} + \mathrm{R{-}O{-}O{-}H} \longrightarrow \mathrm{GSSG} + \mathrm{H_2O} + \mathrm{R{-}OH}$$

This reaction is catalyzed by **glutathione peroxidase**, a remarkable enzyme in that it contains a covalently bound selenium (Se) atom in the form of selenocysteine (see Fig. 3-8a), which is essential for its activity.

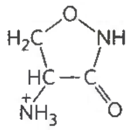
D-Amino Acids Are Found Primarily in Bacteria

Although D-amino acids do not generally occur in proteins, they do serve some special functions in the structure of bacterial cell walls and peptide antibiotics. Bacterial peptidoglycans (see Fig. 6-32) contain both D-alanine and D-glutamate. D-Amino acids arise directly from the L isomers by the action of amino acid racemases, which have pyridoxal phosphate as cofactor (see Fig. 18-6). Amino acid racemization is uniquely important to bacterial metabolism, and enzymes such as alanine racemase are prime targets for pharmaceutical agents. One such agent, **L-fluoroalanine**, is being tested as an antibacterial drug. Another, **cycloserine**, is used to treat tuberculosis. Because these inhibitors also affect some PLP-requiring human enzymes, however, they have potentially undesirable side effects.



$\mathrm{H_3\overset{+}{N}{-}\underset{\underset{F}{|}}{\underset{CH_2}{|}}{\overset{\overset{COO^-}{|}}{C}}{-}H}$

L-Fluoroalanine



Cycloserine

Aromatic Amino Acids Are Precursors of Many Plant Substances

Phenylalanine, tyrosine, and tryptophan are converted to a variety of important compounds in plants. The rigid polymer **lignin**, derived from phenylalanine and tyrosine,

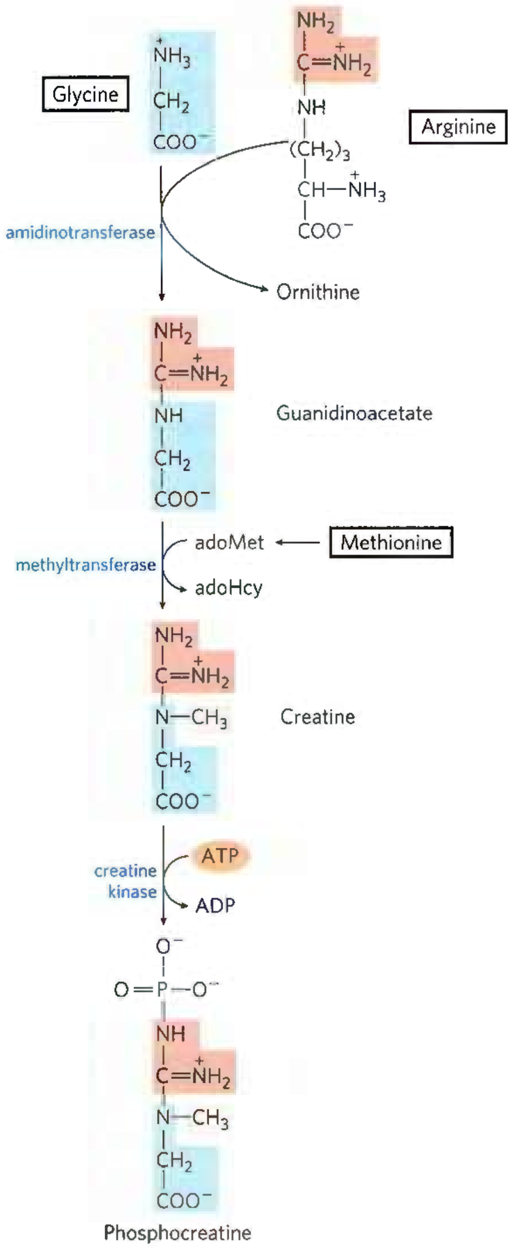

Glycine
Arginine
amidinotransferase
Ornithine
Guanidinoacetate
methyltransferase
adoMet
Methionine
adoHcy
Creatine
creatine kinase
ATP
ADP
Phosphocreatine

FIGURE 22-28 Biosynthesis of creatine and phosphocreatine. Creatine is made from three amino acids: glycine, arginine, and methionine. This pathway shows the versatility of amino acids as precursors of other nitrogenous biomolecules.

is second only to cellulose in abundance in plant tissues. The structure of the lignin polymer is complex and not well understood. Tryptophan is also the precursor of the plant growth hormone indole-3-acetate, or **auxin** (Fig. 22-30a), which is important in the regulation of a wide range of biological processes in plants.

Phenylalanine and tyrosine also give rise to many commercially significant natural products, including the tannins that inhibit oxidation in wines; alkaloids such as morphine, which have potent physiological effects; and the flavoring of cinnamon oil (Fig. 22-30b), nutmeg, cloves, vanilla, cayenne pepper, and other products.

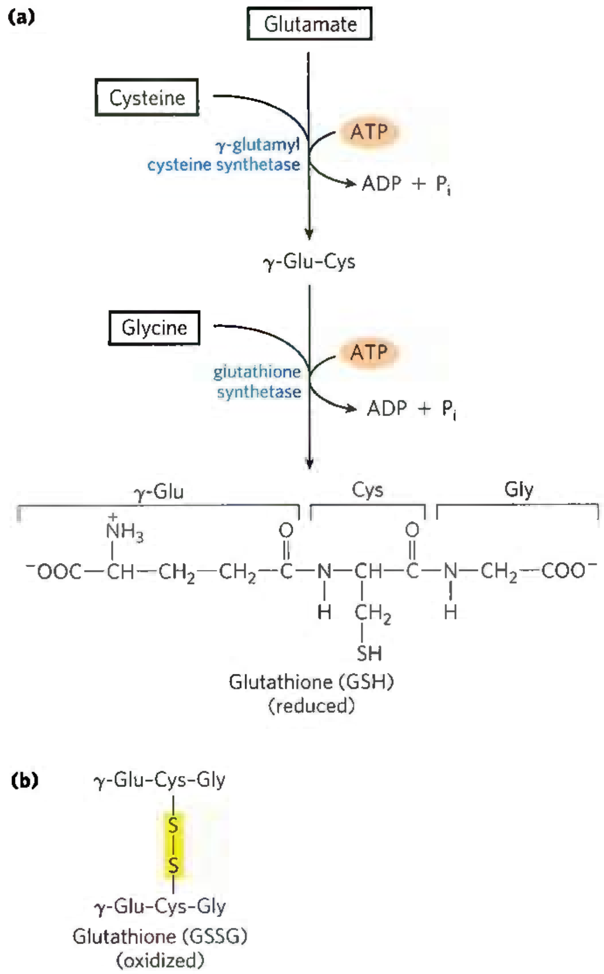

(a)
Glutamate
Cysteine
γ-glutamyl cysteine synthetase
ATP
ADP + P_i
γ-Glu-Cys
Glycine
glutathione synthetase
ATP
ADP + P_i
γ-Glu
Cys
Gly
Glutathione (GSH) (reduced)
(b)
γ-Glu-Cys-Gly
S
S
γ-Glu-Cys-Gly
Glutathione (GSSG) (oxidized)

FIGURE 22-29 Glutathione metabolism. (a) Biosynthesis of glutathione. (b) Oxidized form of glutathione.

Biological Amines Are Products of Amino Acid Decarboxylation

Many important neurotransmitters are primary or secondary amines, derived from amino acids in simple pathways. In addition, some polyamines that form complexes with DNA are derived from the amino acid ornithine, a component of the urea cycle. A common denominator of many of these pathways is amino acid decarboxylation, another PLP-requiring reaction (see Fig. 18-6).

The synthesis of some neurotransmitters is illustrated in Figure 22-31. Tyrosine gives rise to a family of catecholamines that includes **dopamine**, **norepinephrine**, and **epinephrine**. Levels of catecholamines are correlated with, among other things, changes in blood pressure. The neurological disorder Parkinson disease is associated with an underproduction of dopamine, and it has traditionally been treated by administering L-dopa. Overproduction of dopamine in the brain may be linked to psychological disorders such as schizophrenia.

Glutamate decarboxylation gives rise to **γ-aminobutyrate (GABA)**, an inhibitory neurotransmitter. Its

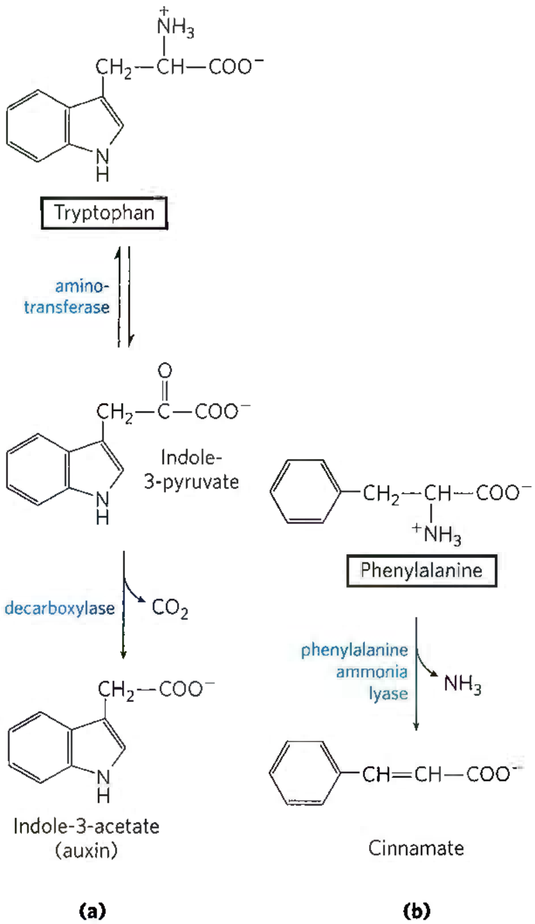
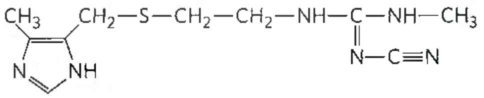


FIGURE 22-30 Biosynthesis of two plant substances from amino acids. (a) Indole-3-acetate (auxin) and (b) cinnamate (cinnamon flavor).

underproduction is associated with epileptic seizures. GABA analogs are used in the treatment of epilepsy and hypertension. Levels of GABA can also be increased by administering inhibitors of the GABA-degrading enzyme GABA aminotransferase. Another important neurotransmitter, **serotonin**, is derived from tryptophan in a two-step pathway.

Histidine undergoes decarboxylation to **histamine**, a powerful vasodilator in animal tissues. Histamine is released in large amounts as part of the allergic response, and it also stimulates acid secretion in the stomach. A growing array of pharmaceutical agents are being designed to interfere with either the synthesis or the action of histamine. A prominent example is the histamine receptor antagonist **cimetidine** (Tagamet), a structural analog of histamine:



It promotes the healing of duodenal ulcers by inhibiting secretion of gastric acid.

Polyamines such as **spermine** and **spermidine**, involved in DNA packaging, are derived from methionine and ornithine by the pathway shown in **Figure 22-32**. The first step is decarboxylation of ornithine, a precursor of arginine (Fig. 22-12). **Ornithine decarboxylase**, a PLP-requiring enzyme, is the target of several powerful inhibitors used as pharmaceutical agents (see Box 6-1).

Arginine Is the Precursor for Biological Synthesis of Nitric Oxide

A surprise finding in the mid-1980s was the role of nitric oxide (NO)—previously known mainly as a component of smog—as an important biological messenger. This simple gaseous substance diffuses readily through membranes, although its high reactivity limits its range of diffusion to about a 1 mm radius from the site of synthesis. In humans NO plays a role in a range of physiological processes, including neurotransmission, blood clotting, and the control of blood pressure. Its mode of action is described in Box 12-2.

P6 Nitric oxide is synthesized from arginine in an NADPH-dependent reaction catalyzed by nitric oxide synthase (**Fig. 22-33**), a dimeric enzyme structurally related to NADPH cytochrome P-450 reductase (see Box 21-1). The reaction is a five-electron oxidation. Each subunit of the enzyme contains one bound molecule of each of four different cofactors: FMN, FAD, tetrahydrobiopterin, and Fe^{3+} heme. NO is an unstable molecule and cannot be stored. Its synthesis is stimulated by interaction of nitric oxide synthase with Ca^{2+}-calmodulin (see Fig. 12-17).

SUMMARY 22.3 *Molecules Derived from Amino Acids*

- Many important biomolecules are derived from amino acids. Glycine is a precursor of porphyrins.
- Degradation of iron-porphyrin (heme) generates bilirubin, which is converted to bile pigments with several physiological functions.
- Glycine and arginine give rise to creatine and phosphocreatine, an energy buffer. Glutathione, formed from three amino acids, is an important cellular reducing agent.
- Bacteria synthesize D-amino acids from L-amino acids in racemization reactions requiring pyridoxal phosphate. D-Amino acids are commonly found in certain bacterial walls and certain antibiotics.
- Plants make many substances from aromatic amino acids.
- The PLP-dependent decarboxylation of some amino acids yields important biological amines, including neurotransmitters and polyamines.
- Arginine is the precursor of nitric oxide, a biological messenger.

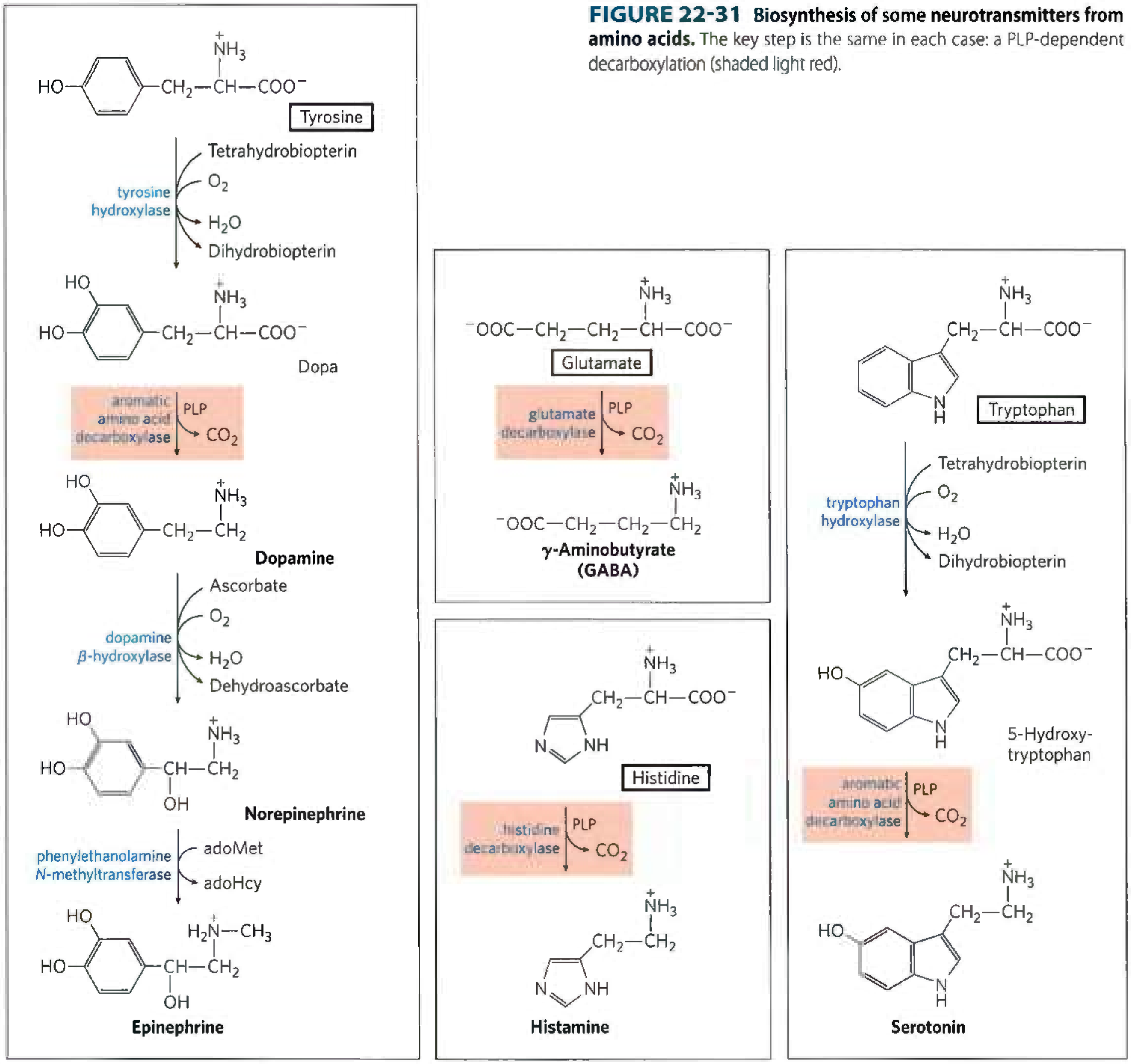

Tyrosine
Tetrahydrobiopterin
O_2
tyrosine hydroxylase
H_2O
Dihydrobiopterin
Dopa
aromatic amino acid decarboxylase
PLP
CO_2
Dopamine
Ascorbate
O_2
dopamine β-hydroxylase
H_2O
Dehydroascorbate
Norepinephrine
phenylethanolamine N-methyltransferase
adoMet
adoHcy
Epinephrine
Glutamate
glutamate decarboxylase
PLP
CO_2
γ-Aminobutyrate (GABA)
Histidine
histidine decarboxylase
PLP
CO_2
Histamine
Tryptophan
Tetrahydrobiopterin
O_2
tryptophan hydroxylase
H_2O
Dihydrobiopterin
5-Hydroxy-tryptophan
aromatic amino acid decarboxylase
PLP
CO_2
Serotonin

FIGURE 22-31 Biosynthesis of some neurotransmitters from amino acids. The key step is the same in each case: a PLP-dependent decarboxylation (shaded light red).

22.4 Biosynthesis and Degradation of Nucleotides

As discussed in Chapter 8, nucleotides have a variety of important functions in all cells. They are the precursors of DNA and RNA. They are essential carriers of chemical energy—a role primarily of ATP and to some extent GTP. They are components of the cofactors NAD, FAD, *S*-adenosylmethionine, and coenzyme A, as well as of activated biosynthetic intermediates such as UDP-glucose and CDP-diacylglycerol. Some, such as cAMP and cGMP, are also cellular second messengers.

Two types of pathways lead to nucleotides: the **de novo pathways** and the **salvage pathways**. De novo synthesis of nucleotides begins with their metabolic precursors: amino acids, ribose 5-phosphate, CO_2, and NH_3. Salvage pathways recycle the free bases and nucleosides released from nucleic acid breakdown. Both types of pathways are important in cellular metabolism, and both are discussed in this section.

The de novo pathways for purine and pyrimidine biosynthesis seem to be nearly identical in all living organisms. Notably, the free bases guanine, adenine, thymine, cytidine, and uracil are *not* intermediates in these pathways; that is, the bases are not synthesized and then attached to ribose, as might be expected. The purine ring structure is built up one or a few atoms at a time, attached to ribose throughout the process. The

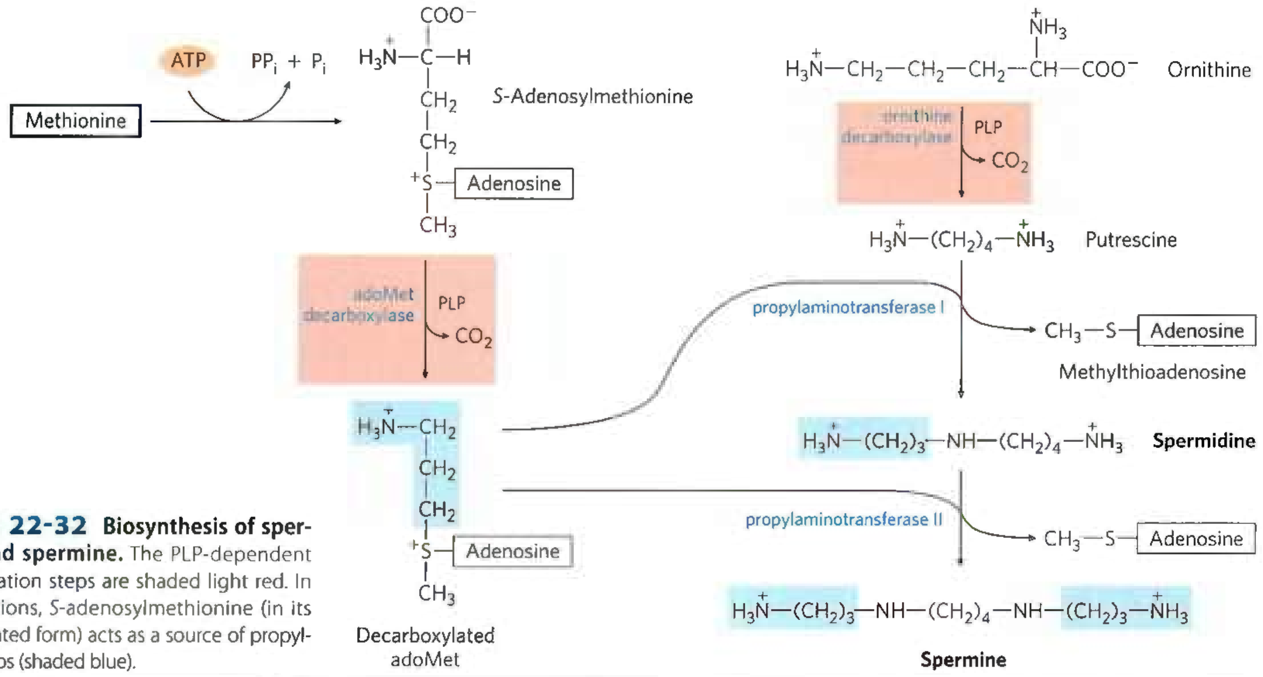

Methionine
ATP
$PP_i + P_i$
S-Adenosylmethionine
Adenosine
adoMet decarboxylase
PLP
CO_2
Decarboxylated adoMet
Ornithine
ornithine decarboxylase
PLP
CO_2
Putrescine
propylaminotransferase I
Methylthioadenosine
Spermidine
propylaminotransferase II
Adenosine
Spermine

FIGURE 22-32 Biosynthesis of spermidine and spermine. The PLP-dependent decarboxylation steps are shaded light red. In these reactions, S-adenosylmethionine (in its decarboxylated form) acts as a source of propylamino groups (shaded blue).

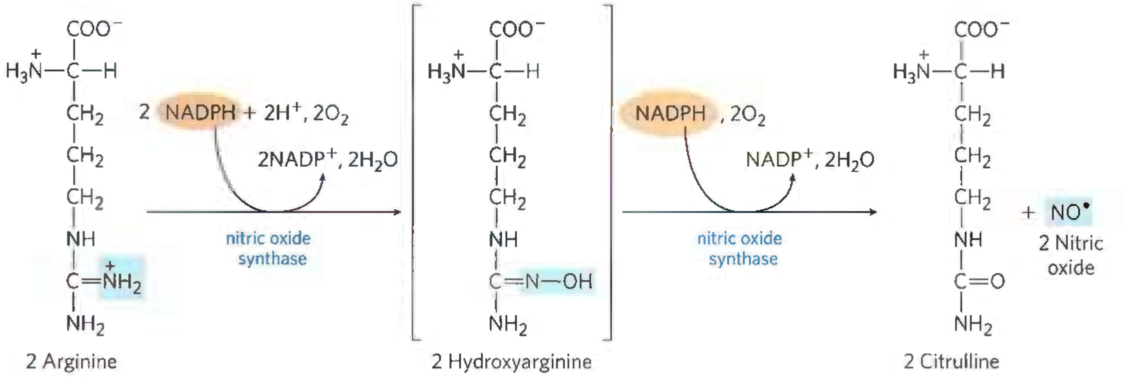

2 Arginine
2 NADPH + 2H$^+$, 2O$_2$
2NADP$^+$, 2H$_2$O
nitric oxide synthase
2 Hydroxyarginine
NADPH, 2O$_2$
NADP$^+$, 2H$_2$O
nitric oxide synthase
2 Citrulline
+ NO•
2 Nitric oxide

FIGURE 22-33 Biosynthesis of nitric oxide. The nitrogen of the NO is derived from the guanidinium group of arginine.

pyrimidine ring is synthesized as **orotate**, attached to ribose phosphate, and then converted to the common pyrimidine nucleotides required in nucleic acid synthesis. Although the free bases are not intermediates in the de novo pathways, they are intermediates in some of the salvage pathways.

Several important precursors are shared by the de novo pathways for synthesis of pyrimidines and purines. Phosphoribosyl pyrophosphate (PRPP) is important in both, and in these pathways the structure of ribose is retained in the product nucleotide, in contrast to its fate in the tryptophan and histidine biosynthetic pathways discussed earlier. An amino acid is an important precursor in each type of pathway: glycine for purines and aspartate for pyrimidines. Glutamine again is the most important source of amino groups—in five different steps in the de novo pathways. Aspartate is also used as the source of an amino group in the purine pathways, in two steps.

Two other features deserve mention. First, there is evidence, especially in the de novo purine pathway, that the enzymes are present as large, multienzyme complexes or metabolons in the cell, a recurring theme in our discussion of metabolism. Second, the cellular pools of nucleotides (other than ATP) are quite small, perhaps 1% or less of the amounts required to synthesize the cell's DNA. Therefore, cells must continue to synthesize nucleotides during nucleic acid synthesis, and in some cases, nucleotide synthesis may limit the rates of DNA replication and transcription. Because of the importance of these processes in dividing cells, agents that inhibit nucleotide synthesis have become particularly important in medicine.

We examine here the biosynthetic pathways of purine and pyrimidine nucleotides and their regulation, the formation of the deoxynucleotides, and the degradation of purines and pyrimidines to uric acid and urea. We end with a discussion of chemotherapeutic agents that affect nucleotide synthesis.

De Novo Purine Nucleotide Synthesis Begins with PRPP

The two parent purine nucleotides of nucleic acids are adenosine 5′-monophosphate (AMP; adenylate) and guanosine 5′-monophosphate (GMP; guanylate), containing the purine bases adenine and guanine. **Figure 22-34** shows the origin of the carbon and nitrogen atoms of the purine ring system, as determined by John M. Buchanan using isotopic tracer experiments in birds (who conveniently excrete excess nitrogen as insoluble uric acid, a purine analog). The detailed pathway of purine biosynthesis was worked out primarily by Buchanan and G. Robert Greenberg in the 1950s.

In the first committed step of the pathway, an amino group donated by glutamine is attached at C-1 of PRPP (**Fig. 22-35**). The resulting **5-phosphoribosylamine** is highly unstable, with a half-life of 30 seconds at pH 7.5. This intermediate is rapidly funneled into the next biosynthetic step, and the purine ring is subsequently built up on this structure. The pathway described here is identical in all organisms, with the exception of one step that differs in higher eukaryotes, as noted below.

The second step is the addition of three atoms from glycine (Fig. 22-35, step ②). An ATP is consumed to activate the glycine carboxyl group (in the form of an acyl phosphate) for this condensation reaction. The added glycine amino group is then formylated by N^{10}-formyltetrahydrofolate (step ③), and a nitrogen is contributed by glutamine (step ④), before dehydration and ring closure yield the five-membered imidazole ring of the purine nucleus, as 5-aminoimidazole ribonucleotide (AIR; step ⑤).

At this point, three of the six atoms needed for the second ring in the purine structure are in place. To complete the process, a carboxyl group is first added (step ⑥). This carboxylation is unusual in that it does not require biotin, but instead uses the bicarbonate generally present in aqueous solutions. A rearrangement transfers the carboxylate from the exocyclic amino group to position 4 of the imidazole ring (step ⑦). Steps ⑥ and ⑦ are found only in bacteria and fungi. In higher eukaryotes, including humans, the 5-aminoimidazole ribonucleotide product of step ⑤ is carboxylated directly to carboxyaminoimidazole ribonucleotide in one step instead of two (step (6a)). The enzyme catalyzing this reaction is AIR carboxylase.

Aspartate now donates its amino group in two steps (⑧ and ⑨): formation of an amide bond, followed by elimination of the carbon skeleton of aspartate (as fumarate). (Recall that aspartate plays an analogous role in two steps of the urea cycle; see Fig. 18-10.) The final carbon is contributed by N^{10}-formyltetrahydrofolate (step ⑩), and a second ring closure takes place to yield the second fused ring of the purine nucleus (step ⑪). The first intermediate with a complete purine ring is **inosinate (IMP)**.

As in the tryptophan and histidine biosynthetic pathways, the enzymes of IMP synthesis seem to be organized as large metabolons in the cell. Once again, evidence comes from the existence of single polypeptides with several functions, some catalyzing nonsequential steps in the pathway. In eukaryotic cells ranging from yeast to fruit flies to chickens, steps ①, ③, and ⑤ in Figure 22-35 are catalyzed by a multifunctional protein. An additional multifunctional protein catalyzes steps ⑩ and ⑪. In humans, a multifunctional enzyme combines the activities of AIR carboxylase and SAICAR synthetase (steps (6a) and ⑧). In bacteria, these activities are found on separate proteins, but the proteins may form a metabolon. The channeling of reaction intermediates from one enzyme to the next permitted by these complexes is probably especially important for unstable intermediates such as 5-phosphoribosylamine.

Conversion of inosinate to adenylate requires the insertion of an amino group derived from aspartate (**Fig. 22-36**); this takes place in two reactions similar to those used to introduce N-1 of the purine ring (Fig. 22-35, steps ⑧ and ⑨). A crucial difference is that GTP rather than ATP is the source of the high-energy phosphate in synthesizing adenylosuccinate. Guanylate is formed by the NAD$^+$-requiring oxidation of inosinate at C-2, followed by addition of an amino group derived from glutamine. ATP is cleaved to AMP and PP$_i$ in the final step (Fig. 22-36).

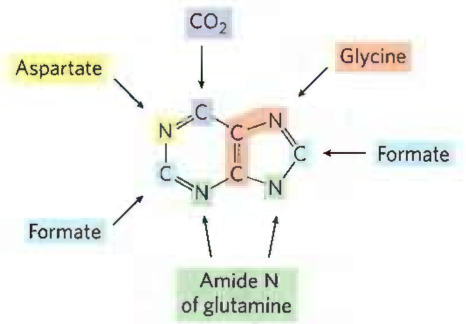


FIGURE 22-34 **Origin of the ring atoms of purines.** This information was obtained from isotopic experiments with ^{14}C- or ^{15}N-labeled precursors. Formate is supplied in the form of N^{10}-formyltetrahydrofolate.

Purine Nucleotide Biosynthesis Is Regulated by Feedback Inhibition

P4 Four major feedback mechanisms cooperate in regulating the overall rate of de novo purine nucleotide synthesis and the relative rates of formation of the two

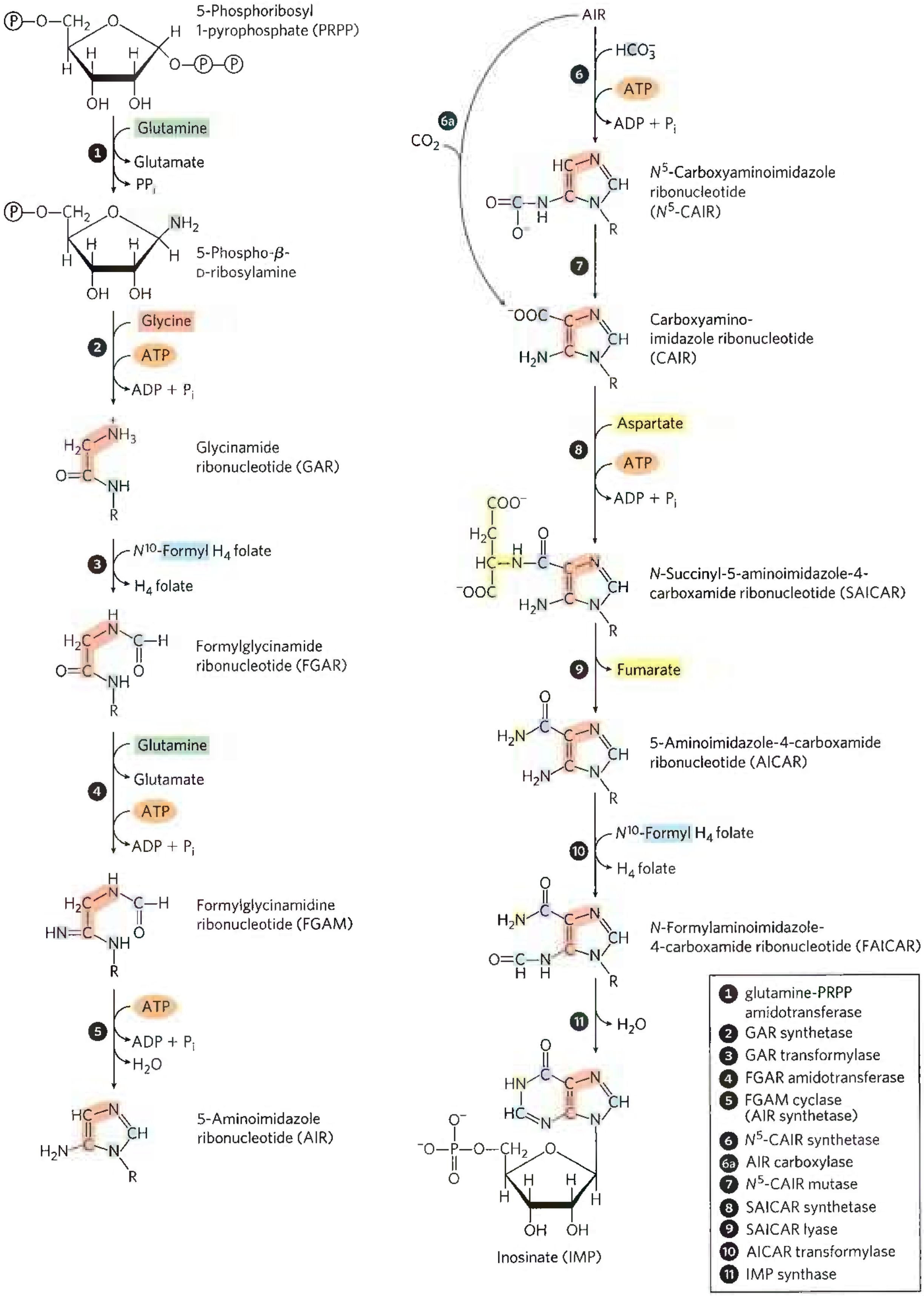
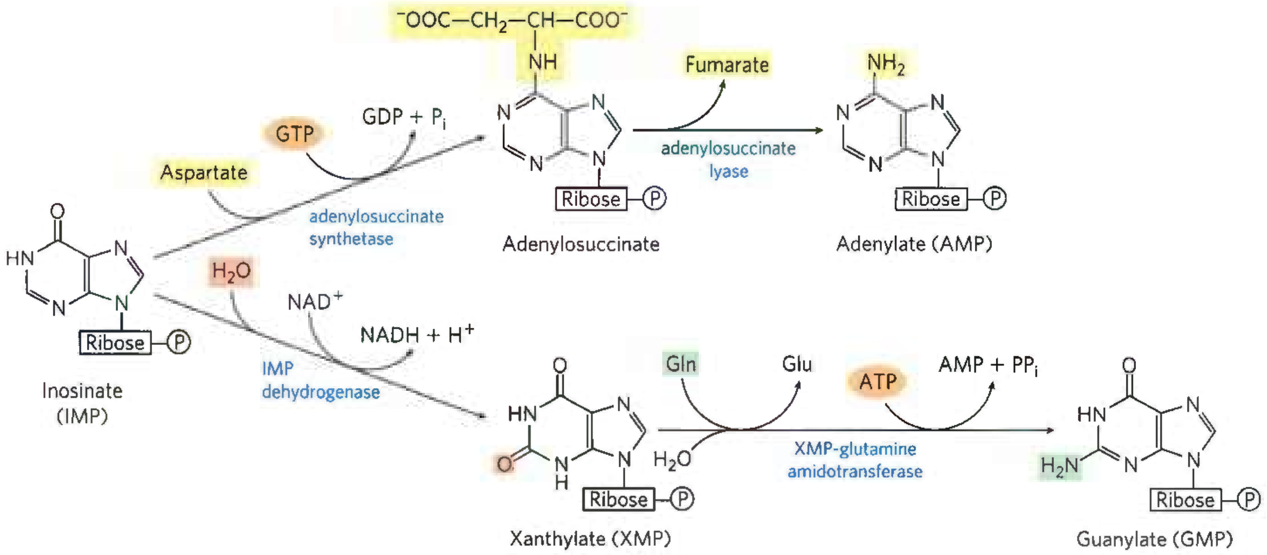

5-Phosphoribosyl 1-pyrophosphate (PRPP)
Glutamine
❶
Glutamate
PP_i
5-Phospho-β-D-ribosylamine
Glycine
❷
ATP
ADP + P_i
Glycinamide ribonucleotide (GAR)
N10-Formyl H4 folate
❸
H4 folate
Formylglycinamide ribonucleotide (FGAR)
Glutamine
❹
Glutamate
ATP
ADP + P_i
Formylglycinamidine ribonucleotide (FGAM)
ATP
❺
ADP + P_i
H2O
5-Aminoimidazole ribonucleotide (AIR)
AIR
HCO3−
❻
ATP
ADP + P_i
6a
CO2
N5-Carboxyaminoimidazole ribonucleotide (N5-CAIR)
❼
Carboxyamino-imidazole ribonucleotide (CAIR)
Aspartate
❽
ATP
ADP + P_i
N-Succinyl-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAR)
❾
Fumarate
5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR)
N10-Formyl H4 folate
❿
H4 folate
N-Formylaminoimidazole-4-carboxamide ribonucleotide (FAICAR)
⓫
H2O
Inosinate (IMP)
❶ glutamine-PRPP amidotransferase
❷ GAR synthetase
❸ GAR transformylase
❹ FGAR amidotransferase
❺ FGAM cyclase (AIR synthetase)
❻ N5-CAIR synthetase
6a AIR carboxylase
❼ N5-CAIR mutase
❽ SAICAR synthetase
❾ SAICAR lyase
❿ AICAR transformylase
⓫ IMP synthase

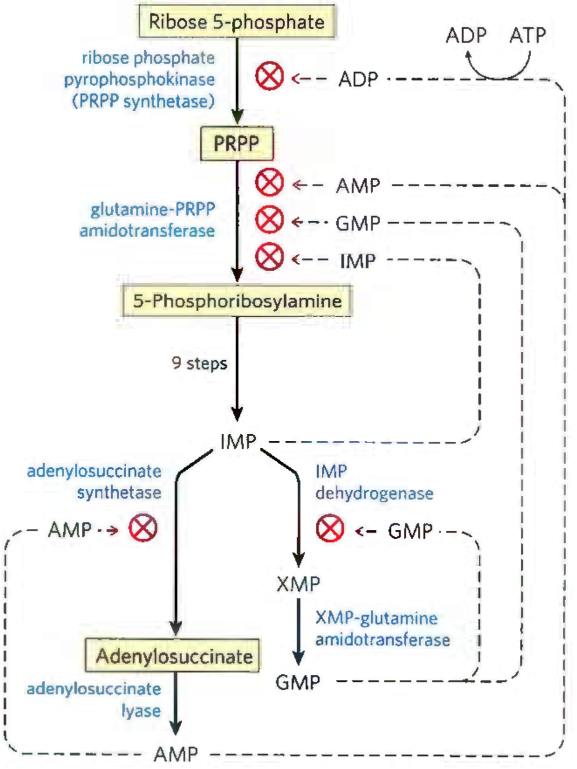
FIGURE 22-35 De novo synthesis of purine nucleotides: construction of the purine ring of inosinate (IMP). Each addition to the purine ring is shaded to match Figure 22-34. After step ❷, R symbolizes the 5-phospho-D-ribosyl group on which the purine ring is built. Formation of 5-phosphoribosylamine (step ❶) is the first committed step in purine synthesis. Note that the product of step ❾, AICAR, is the remnant of ATP released during histidine biosynthesis (see Fig. 22-22, step ❺). Abbreviations are given for most intermediates to simplify the naming of the enzymes. Step 6a is the alternative path from AIR to CAIR occurring in higher eukaryotes.



⁻OOC—CH₂—CH—COO⁻
NH
Fumarate
NH₂
GDP + Pᵢ
GTP
Aspartate
adenylosuccinate synthetase
adenylosuccinate lyase
Ribose—P
Adenylosuccinate
Adenylate (AMP)
HN
Inosinate (IMP)
H₂O
NAD⁺
NADH + H⁺
IMP dehydrogenase
Gln
Glu
ATP
AMP + PPᵢ
H₂O
XMP-glutamine amidotransferase
Xanthylate (XMP)
Guanylate (GMP)
H₂N

FIGURE 22-36 **Biosynthesis of AMP and GMP from IMP.**

end products, adenylate and guanylate (**Fig. 22-37**). The first mechanism is exerted on the first reaction that is unique to purine synthesis: transfer of an amino group to PRPP to form 5-phosphoribosylamine. This reaction is catalyzed by the allosteric enzyme glutamine-PRPP amidotransferase, which is inhibited by the end products IMP, AMP, and GMP. AMP and GMP act synergistically in this concerted inhibition. Thus, whenever either AMP or GMP accumulates to excess, the first step in its biosynthesis from PRPP is partially inhibited.



Ribose 5-phosphate
ribose phosphate pyrophosphokinase (PRPP synthetase)
ADP
ATP
ADP
PRPP
AMP
GMP
IMP
glutamine-PRPP amidotransferase
5-Phosphoribosylamine
9 steps
IMP
adenylosuccinate synthetase
IMP dehydrogenase
AMP
GMP
XMP
XMP-glutamine amidotransferase
Adenylosuccinate
GMP
adenylosuccinate lyase
AMP

FIGURE 22-37 **Regulatory mechanisms in the biosynthesis of adenine and guanine nucleotides in *E. coli*.** Regulation of these pathways differs in other organisms.

In the second control mechanism, exerted at a later stage, an excess of GMP in the cell inhibits formation of xanthylate from inosinate by IMP dehydrogenase, without affecting the formation of AMP. Conversely, an accumulation of adenylate inhibits formation of adenylosuccinate by adenylosuccinate synthetase, without affecting the biosynthesis of GMP. When both products are present in sufficient quantities, IMP builds up, and it inhibits an earlier step in the pathway; this is another example of the regulatory strategy called **sequential feedback inhibition**.

In the third mechanism, GTP is required in the conversion of IMP to AMP, whereas ATP is required for conversion of IMP to GMP (Fig. 22-36), a reciprocal arrangement that tends to balance the synthesis of the two ribonucleotides.

The fourth and final control mechanism is the inhibition of PRPP synthesis by the allosteric regulation of ribose phosphate pyrophosphokinase. This enzyme is inhibited by ADP and GDP, in addition to metabolites from other pathways for which PRPP is a starting point.

Pyrimidine Nucleotides Are Made from Aspartate, PRPP, and Carbamoyl Phosphate

The common pyrimidine ribonucleotides are cytidine 5′-monophosphate (CMP; cytidylate) and uridine 5′-monophosphate (UMP; uridylate), which contain the pyrimidines cytosine and uracil. De novo pyrimidine nucleotide biosynthesis (**Fig. 22-38**) proceeds in a somewhat different manner from purine nucleotide synthesis; the six-membered pyrimidine ring is made first and then attached to ribose 5-phosphate. Required in this process is carbamoyl phosphate, also an intermediate in the urea

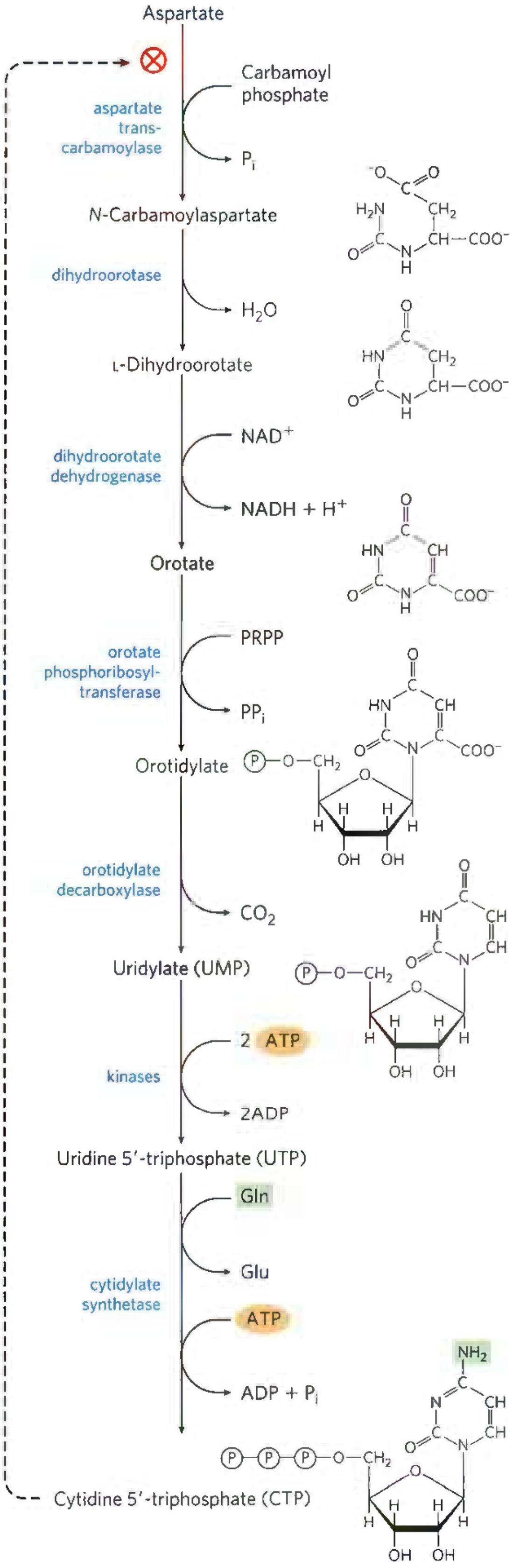

Aspartate
aspartate trans-carbamoylase
Carbamoyl phosphate
Pi
N-Carbamoylaspartate
dihydroorotase
H2O
L-Dihydroorotate
dihydroorotate dehydrogenase
NAD+
NADH + H+
Orotate
orotate phosphoribosyl-transferase
PRPP
PPi
Orotidylate
orotidylate decarboxylase
CO2
Uridylate (UMP)
kinases
2 ATP
2ADP
Uridine 5′-triphosphate (UTP)
cytidylate synthetase
Gln
Glu
ATP
ADP + Pi
Cytidine 5′-triphosphate (CTP)

FIGURE 22-38 De novo synthesis of pyrimidine nucleotides: biosynthesis of UTP and CTP via orotidylate. The pyrimidine is constructed from carbamoyl phosphate and aspartate. The ribose 5-phosphate is then added to the completed pyrimidine ring by orotate phosphoribosyltransferase. The first step in this pathway (not shown here; see Fig. 18-11a) is the synthesis of carbamoyl phosphate from CO_2, NH_4^+, and ATP. In eukaryotes, the first step is catalyzed by carbamoyl phosphate synthetase II.

cycle. However, in animals the carbamoyl phosphate required in urea synthesis is made in mitochondria by carbamoyl phosphate synthetase I, whereas the carbamoyl phosphate required in pyrimidine biosynthesis is made in the cytosol by a different form of the enzyme, **carbamoyl phosphate synthetase II**. In bacteria, a single enzyme supplies carbamoyl phosphate for the synthesis of arginine and pyrimidines. The bacterial enzyme has three separate active sites, spaced along a channel nearly 100 Å long (**Fig. 22-39**). Bacterial carbamoyl phosphate synthetase provides a vivid illustration of the channeling of unstable reaction intermediates between active sites so that products are formed efficiently.

Carbamoyl phosphate reacts with aspartate to yield *N*-carbamoylaspartate in the first committed step of pyrimidine biosynthesis (Fig. 22-38). This reaction is catalyzed by **aspartate transcarbamoylase**. In bacteria, this step is highly regulated, and bacterial aspartate transcarbamoylase is one of the most thoroughly studied allosteric enzymes (see below). By removal of water from *N*-carbamoylaspartate, a reaction catalyzed by **dihydroorotase**, the pyrimidine ring is closed to form L-dihydroorotate. This compound is oxidized to the pyrimidine derivative orotate, a reaction in which NAD^+ is the ultimate electron acceptor. In eukaryotes, the first

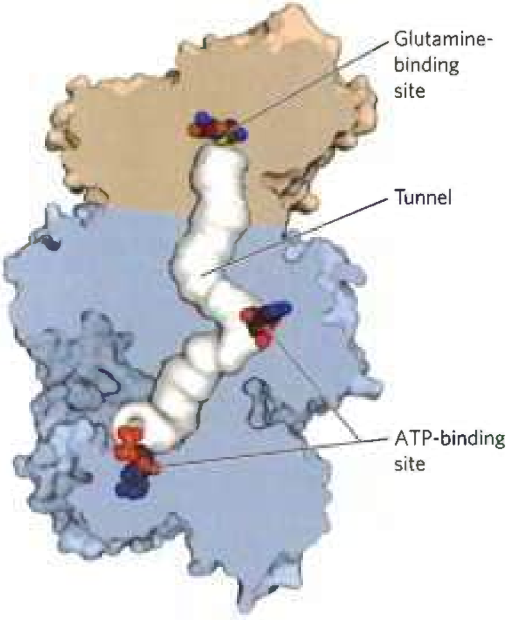

Glutamine-binding site
Tunnel
ATP-binding site

FIGURE 22-39 Channeling of intermediates in bacterial carbamoyl phosphate synthetase. The reaction catalyzed by this enzyme (and its mitochondrial counterpart) is illustrated in Figure 18-11a. In this cutaway, the small and large subunits are shown in tan and blue, respectively; the tunnel between active sites (almost 100 Å long) is shown as white. In this reaction, a glutamine molecule binds to the small subunit, donating its amido nitrogen as NH_4^+ in a glutamine amidotransferase–type reaction. The NH_4^+ enters the tunnel, which takes it to a second active site, where it combines with bicarbonate in a reaction requiring ATP. The carbamate then reenters the tunnel to reach the third active site, where it is phosphorylated by ATP to carbamoyl phosphate. To solve this structure, the enzyme was crystallized with ornithine bound to the glutamine-binding site and ADP bound to the ATP-binding sites. [Data from PDB ID 1M6V, J. B. Thoden et al., *J. Biol. Chem.* 277:39,722, 2002.]

three enzymes in this pathway—carbamoyl phosphate synthetase II, aspartate transcarbamoylase, and dihydroorotase—are part of a single trifunctional protein. The protein, known by the acronym CAD, contains three identical polypeptide chains (each of M_r 230,000), each with active sites for all three reactions. This suggests that metabolons may be the rule in this pathway.

Once orotate is formed, the ribose 5-phosphate side chain, provided once again by PRPP, is attached to yield orotidylate (Fig. 22-38). Orotidylate is then decarboxylated to uridylate, which is phosphorylated to UTP. P6 CTP is formed from UTP by the action of **cytidylate synthetase**, by way of an acyl phosphate intermediate (consuming one ATP). The nitrogen donor is normally glutamine, although the cytidylate synthetases in many species can use NH_4^+ directly.

Pyrimidine Nucleotide Biosynthesis Is Regulated by Feedback Inhibition

P4 Regulation of the rate of pyrimidine nucleotide synthesis in bacteria occurs in large part through aspartate transcarbamoylase (ATCase), which catalyzes the first reaction in the sequence and is inhibited by CTP, the end product of the sequence (Fig. 22-38). The bacterial ATCase molecule consists of six catalytic subunits and six regulatory subunits (see Fig. 6-36). The catalytic subunits bind the substrate molecules, and the allosteric subunits bind the allosteric inhibitor, CTP. The entire ATCase molecule, as well as its subunits, exists in two conformations, active and inactive. When CTP is not bound to the regulatory subunits, the enzyme is maximally active. As CTP accumulates and binds to the regulatory subunits, they undergo a change in conformation. This change is transmitted to the catalytic subunits, which then also shift to an inactive conformation. ATP prevents the changes induced by CTP. **Figure 22-40** shows the effects of the allosteric regulators on the activity of ATCase.

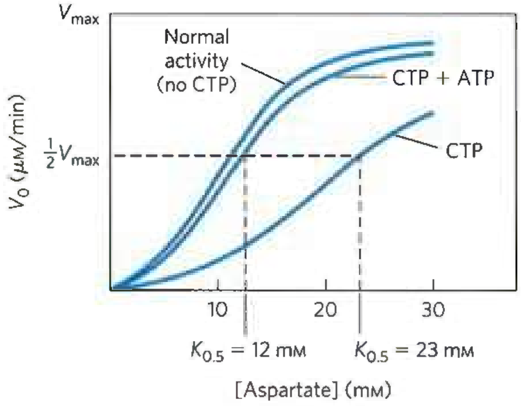


FIGURE 22-40 Allosteric regulation of aspartate transcarbamoylase by CTP and ATP. Addition of 0.8 mM CTP, the allosteric inhibitor of ATCase, increases the $K_{0.5}$ for aspartate (lower curve), thereby reducing the rate of conversion of aspartate to *N*-carbamoylaspartate. ATP at 0.6 mM fully reverses this inhibition by CTP (middle curve).

Nucleoside Monophosphates Are Converted to Nucleoside Triphosphates

Nucleotides to be used in biosynthesis are generally converted to nucleoside triphosphates. The conversion pathways are common to all cells. Phosphorylation of AMP to ADP is promoted by **adenylate kinase**, in the reaction

$$\text{ATP} + \text{AMP} \rightleftharpoons 2\ \text{ADP}$$

The ADP so formed is phosphorylated to ATP by the glycolytic enzymes or through oxidative phosphorylation.

ATP also brings about the formation of other nucleoside diphosphates by the action of a class of enzymes called **nucleoside monophosphate kinases**. These enzymes, which are generally specific for a particular base but nonspecific for the sugar (ribose or deoxyribose), catalyze the reaction

$$\text{ATP} + \text{NMP} \rightleftharpoons \text{ADP} + \text{NDP}$$

The efficient cellular systems for rephosphorylating ADP to ATP (ATP synthase; Chapter 19) tend to remove ADP and pull this reaction in the direction of products.

Nucleoside diphosphates are converted to triphosphates by the action of a ubiquitous enzyme, **nucleoside diphosphate kinase**, which catalyzes the reaction

$$\text{NTP}_\text{D} + \text{NDP}_\text{A} \rightleftharpoons \text{NDP}_\text{D} + \text{NTP}_\text{A}$$

This enzyme is notable in that it is not specific for the base (purines or pyrimidines) or the sugar (ribose or deoxyribose). This nonspecificity applies to both phosphate acceptor (A) and donor (D), although the donor (NTP_D) is almost invariably ATP because it is present in higher concentration than other nucleoside triphosphates under aerobic conditions.

Ribonucleotides Are the Precursors of Deoxyribonucleotides

Deoxyribonucleotides, the building blocks of DNA, are derived from the corresponding ribonucleotides by direct reduction at the 2′-carbon atom of the D-ribose to form the 2′-deoxy derivative. For example, adenosine diphosphate (ADP) is reduced to 2′-deoxyadenosine diphosphate (dADP), and GDP is reduced to dGDP. This reaction is somewhat unusual in that the reduction occurs at a nonactivated carbon; no closely analogous chemical reactions are known. The reaction is catalyzed by **ribonucleotide reductase**, best characterized in *E. coli*, in which its substrates are ribonucleoside diphosphates.

The reduction of the D-ribose portion of a ribonucleoside diphosphate to 2′-deoxy-D-ribose requires a pair of hydrogen atoms, which are ultimately donated by NADPH via an intermediate hydrogen-carrying protein, **thioredoxin**. This ubiquitous protein serves a similar redox function in photosynthesis (see Fig. 20-37) and other processes. Thioredoxin has pairs of —SH groups that carry hydrogen atoms from NADPH to the ribonucleoside diphosphate. P6 Its oxidized (disulfide) form is reduced by NADPH in a reaction catalyzed by **thioredoxin reductase** (**Fig. 22-41**), and reduced thioredoxin is then used by ribonucleotide reductase to reduce the nucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs). A second source of reducing equivalents for ribonucleotide reductase is glutathione (GSH). Glutathione serves as the reductant for a protein closely related to thioredoxin, **glutaredoxin**, which then transfers the reducing power to ribonucleotide reductase.

Ribonucleotide reductase is notable in that its reaction mechanism provides the best-characterized example of the involvement of free radicals in biochemical transformations, once thought to be rare in biological systems. The enzyme in *E. coli* and most eukaryotes is an $\alpha_2\beta_2$ dimer, with two catalytic subunits, α_2, and two radical-generation subunits, β_2 (**Fig. 22-42**). Each catalytic subunit contains two kinds of regulatory sites, as described below. The two active sites of the enzyme are formed at the interface between the catalytic (α_2) and radical-generation (β_2) subunits. At each active site, an α subunit contributes two sulfhydryl groups required for activity, and the β_2 subunits contribute a stable tyrosyl radical. The β_2 subunits also have a binuclear iron (Fe^{3+}) cofactor that helps generate and stabilize the Tyr^{122} radical. The tyrosyl radical is too far from the active site to interact directly with the site, but several aromatic residues form a long-range radical-transfer pathway to the active site (Fig. 22-42c). A likely mechanism for the ribonucleotide reductase reaction is illustrated in **Figure 22-43**. In *E. coli*, the sources of the required reducing equivalents for this reaction are thioredoxin and glutaredoxin, as noted above.

Three classes of ribonucleotide reductase have been reported. Their mechanisms (where known) generally conform to the scheme in Figure 22-43, but they differ in the identity of the group supplying the active-site radical and in the cofactors used to generate it. The *E. coli* enzyme (class I) requires oxygen to regenerate

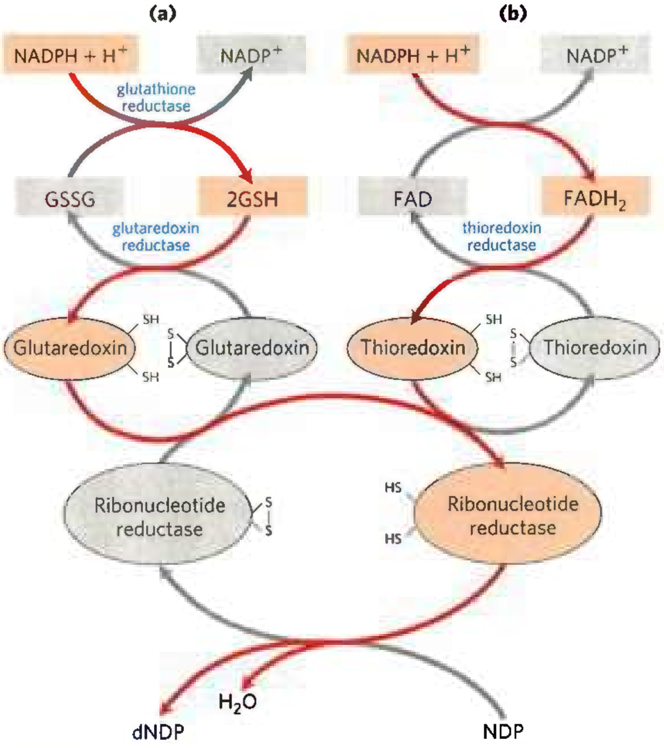

(a)
(b)
NADPH + H+
NADP+
glutathione reductase
GSSG
2GSH
glutaredoxin reductase
Glutaredoxin
SH
SH
S
S
Glutaredoxin
NADPH + H+
NADP+
FAD
FADH2
thioredoxin reductase
Thioredoxin
SH
SH
S
S
Thioredoxin
Ribonucleotide reductase
S
S
HS
HS
Ribonucleotide reductase
H2O
dNDP
NDP

FIGURE 22-41 Reduction of ribonucleotides to deoxyribonucleotides by ribonucleotide reductase. Electrons are transmitted (red arrows) to the enzyme from NADPH via (a) glutaredoxin or (b) thioredoxin. The sulfhydryl groups in glutaredoxin reductase are contributed by two molecules of bound glutathione (GSH; GSSG indicates oxidized glutathione). Note that thioredoxin reductase is a flavoenzyme, with FAD as a prosthetic group.

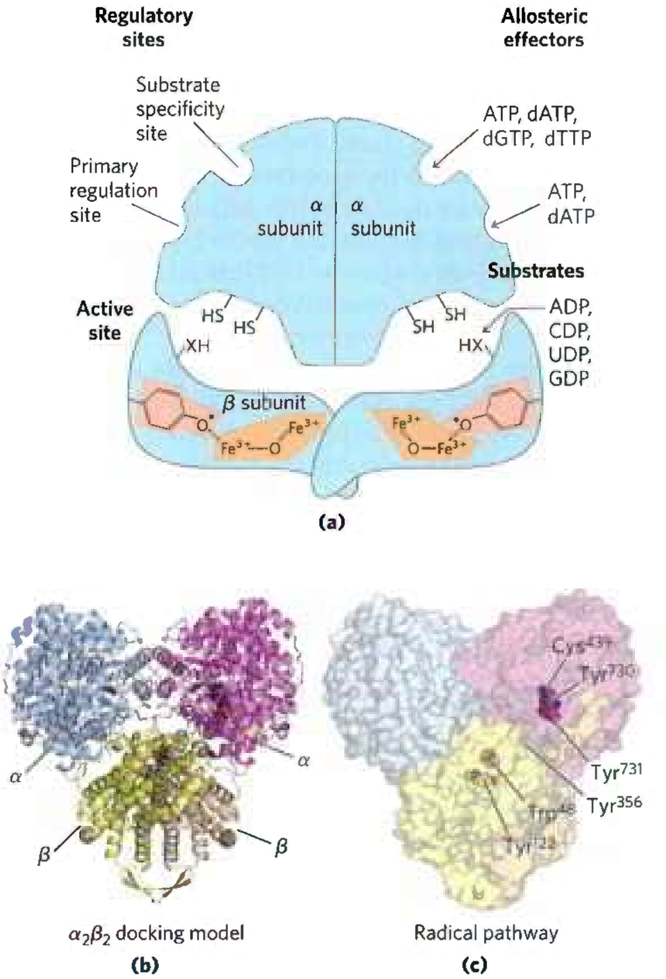
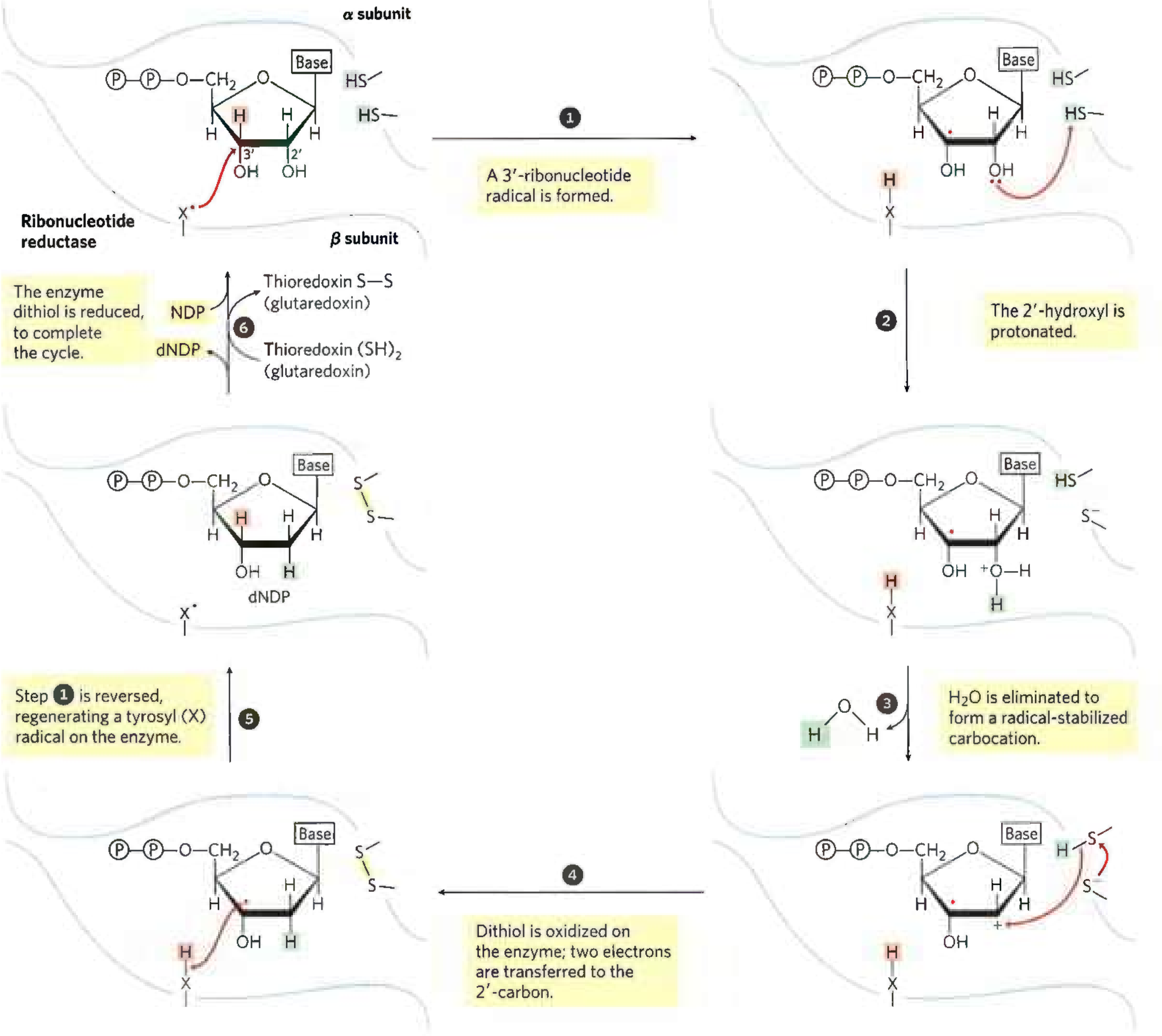

Regulatory sites
Allosteric effectors
Substrate specificity site
Primary regulation site
ATP, dATP, dGTP, dTTP
ATP, dATP
α subunit
α subunit
Substrates
ADP, CDP, UDP, GDP
Active site
HS
HS
XH
SH
SH
HX
β subunit
Fe3+
Fe3+
O
(a)
α
α
β
β
Cys439
Tyr730
Tyr731
Tyr356
Trp48
Tyr122
α2β2 docking model
(b)
Radical pathway
(c)

FIGURE 22-42 Ribonucleotide reductase. (a) A schematic diagram of the subunit structures. Each catalytic subunit (α; also called R1) contains the two regulatory sites described in Figure 22-44 and two Cys residues central to the reaction mechanism. The radical-generation subunits (β; also called R2) each contain a critical Tyr^{122} residue and binuclear iron center. (b) The likely structure of $\alpha_2\beta_2$. (c) The likely path of radical formation from the initial Tyr^{122} in a β subunit to the active-site Cys^{439}, which is used in the mechanism shown in Figure 22-43. Several aromatic amino acid residues participate in long-range transfer of the radical from the point of its formation at Tyr^{122} to the active site, where the nucleotide substrate is bound. [(a) Information from L. Thelander and P. Reichard, *Annu. Rev. Biochem.* 48:133, 1979. (b, c) Data from PDB ID 3UUS, N. Ando et al., *Proc. Natl. Acad. Sci. USA* 108:21,046, 2011.]



① A 3′-ribonucleotide radical is formed.

② The 2′-hydroxyl is protonated.

③ H_2O is eliminated to form a radical-stabilized carbocation.

④ Dithiol is oxidized on the enzyme; two electrons are transferred to the 2′-carbon.

⑤ Step ① is reversed, regenerating a tyrosyl (X) radical on the enzyme.

⑥ The enzyme dithiol is reduced, to complete the cycle.

MECHANISM FIGURE 22-43 Proposed mechanism for ribonucleotide reductase. In the enzyme of *E. coli* and most eukaryotes, the active thiol groups are on the α subunit. The active-site radical (—X•) is on the β subunit and in *E. coli* is probably a thiyl radical of Cys^{439} (see Fig. 22-42).

the tyrosyl radical if it is quenched, so this enzyme functions only in an aerobic environment. Class II enzymes, found in other microorganisms, have 5′-deoxyadenosylcobalamin (see Box 17-2) rather than a binuclear iron center. Class III enzymes have evolved to function in an anaerobic environment. *E. coli* contains a separate class III ribonucleotide reductase when grown anaerobically; this enzyme contains an iron-sulfur cluster (structurally distinct from the binuclear iron center of the class I enzyme) and requires NADPH and *S*-adenosylmethionine for activity. It uses nucleoside triphosphates rather than nucleoside diphosphates as substrates. The evolution of different classes of ribonucleotide reductase for production of DNA precursors in different environments reflects the importance of this reaction in nucleotide metabolism.

P4 Regulation of *E. coli* ribonucleotide reductase is unusual in that not only its *activity* but also its *substrate specificity* is regulated by the binding of effector molecules. Each α subunit has two types of regulatory sites (Fig. 22-42). One type affects overall enzyme activity and binds either ATP, which activates the enzyme, or dATP, which inactivates it. The second type alters substrate specificity in response to the effector molecule—ATP, dATP, dTTP, or dGTP—that is bound there (**Fig. 22-44**). When ATP or dATP is bound, reduction of UDP and CDP is

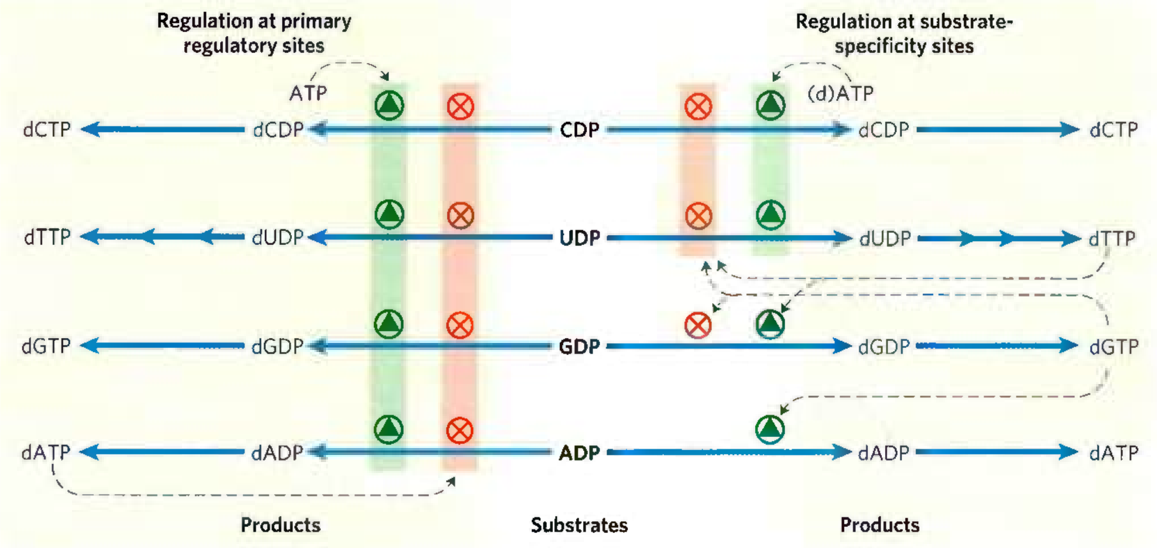


FIGURE 22-44 Regulation of ribonucleotide reductase by deoxynucleoside triphosphates. The overall activity of the enzyme is affected by binding at the primary regulatory site (left). The substrate specificity of the enzyme is affected by the nature of the effector molecule bound at the second type of regulatory site, the substrate-specificity site (right). The diagram indicates inhibition or stimulation of enzyme activity with the four different substrates. The pathway from dUDP to dTMP is described below (see Figs 22-46, 22-47).

favored. When dTTP or dGTP is bound, reduction of GDP or ADP, respectively, is stimulated. **P1** The scheme is designed to provide a balanced pool of precursors for DNA synthesis. ATP is also a general activator for biosynthesis and ribonucleotide reduction. The presence of dATP in small amounts increases the reduction of pyrimidine nucleotides. An oversupply of the pyrimidine dNTPs is signaled by high levels of dTTP. Abundant dTTP shifts the specificity to favor reduction of GDP. High levels of dGTP, in turn, shift the specificity to ADP reduction, and high levels of dATP shut the enzyme down. These effectors are thought to induce several distinct enzyme conformations with altered specificities.

These regulatory effects are accompanied by, and presumably mediated by, large structural rearrangements in the enzyme. When the active form of the *E. coli* enzyme ($\alpha_2\beta_2$) is inhibited by the addition of the allosteric inhibitor dATP, a ringlike $\alpha_4\beta_4$ structure forms, with alternating α_2 and β_2 subunits (**Fig. 22-45**). In this altered structure, the radical-forming path from β to α is disrupted and the residues in the path are exposed to solvent, effectively preventing radical transfer and thus inhibiting the reaction. The formation of ringlike $\alpha_4\beta_4$ structures is reversed when dATP levels are reduced. The yeast ribonucleotide reductase also undergoes oligomerization in the presence of dATP, forming a hexameric ring structure, $\alpha_6\beta_6$.

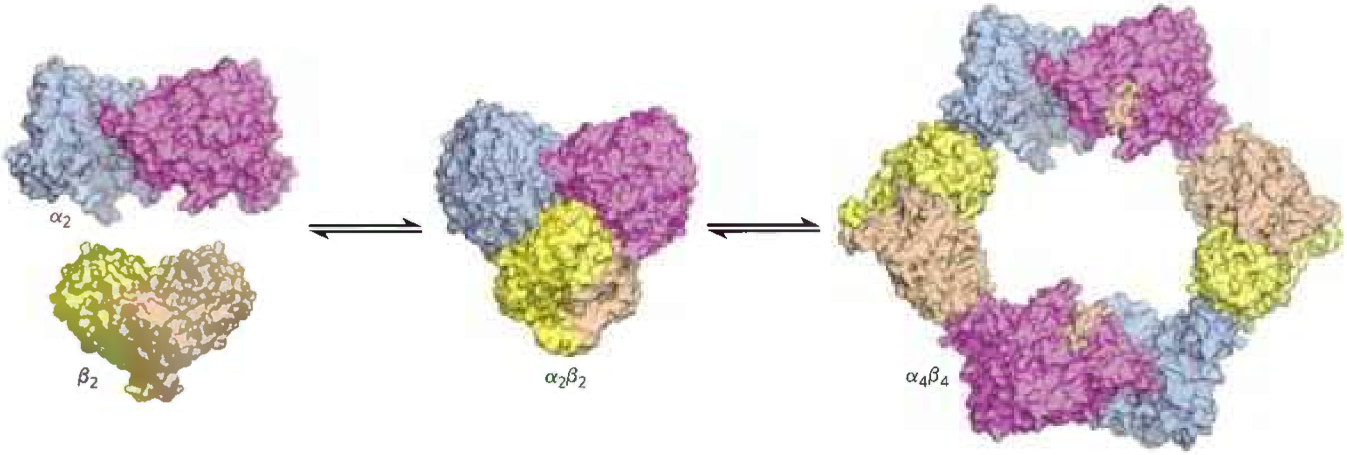


FIGURE 22-45 Oligomerization of ribonucleotide reductase induced by the allosteric inhibitor dATP. At high concentrations of dATP (50 μM), ring-shaped $\alpha_4\beta_4$ structures form. In this conformation, the residues in the radical-forming path are exposed to the solvent, blocking the radical reaction and inhibiting the enzyme. The oligomerization is reversed at lower dATP concentrations. [Data from PDB ID 3UUS, N. Ando et al., *Proc. Natl. Acad. Sci. USA* 108:21,046, 2011.]

Thymidylate Is Derived from dCDP and dUMP

DNA contains thymine rather than uracil, and the de novo pathway to thymine involves only deoxyribonucleotides. The immediate precursor of thymidylate (dTMP) is dUMP. In bacteria, the pathway to dUMP begins with formation of dUTP, either by deamination of dCTP or by phosphorylation of dUDP (**Fig. 22-46**). The dUTP is converted to dUMP by a dUTPase. The latter reaction must be efficient to keep dUTP pools low and prevent incorporation of uridylate into DNA.

Conversion of dUMP to dTMP is catalyzed by **thymidylate synthase**. A one-carbon unit at the hydroxymethyl (—CH_2OH) oxidation level (see Fig. 18-17) is transferred from N^5,N^{10}-methylenetetrahydrofolate to dUMP, then reduced to a methyl group (**Fig. 22-47**). The reduction occurs at the expense of oxidation of tetrahydrofolate to dihydrofolate, which is unusual in tetrahydrofolate-requiring reactions. (The mechanism of this reaction is shown in Fig. 22-52.) The dihydrofolate is reduced to tetrahydrofolate by **dihydrofolate reductase**—a regeneration that is essential for the many processes that require tetrahydrofolate. In plants and at least one protist, thymidylate synthase and dihydrofolate reductase reside on a single, bifunctional protein.

About 10% of the human population (and up to 50% of people in impoverished communities) suffers from folic acid deficiency. When the deficiency is severe, the symptoms can include heart disease, cancer, and some types of brain dysfunction. Folic acid deficiency during pregnancy can also produce neural tube defects in infants. At least some of these symptoms arise from a reduction in thymidylate synthesis, leading to an abnormal incorporation of uracil into DNA. Uracil is recognized by DNA repair pathways (described in Chapter 25) and is cleaved from the DNA. The presence of high levels of uracil in DNA leads to strand breaks that can greatly affect the function and regulation of nuclear DNA, ultimately causing the observed effects on the heart and brain, as well as increased mutagenesis that leads to cancer. ■

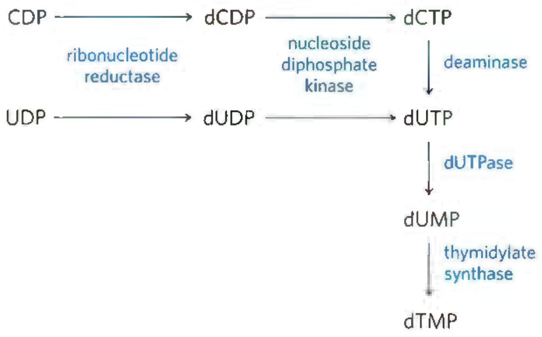


FIGURE 22-46 Biosynthesis of thymidylate (dTMP). The pathways are shown beginning with the reaction catalyzed by ribonucleotide reductase.

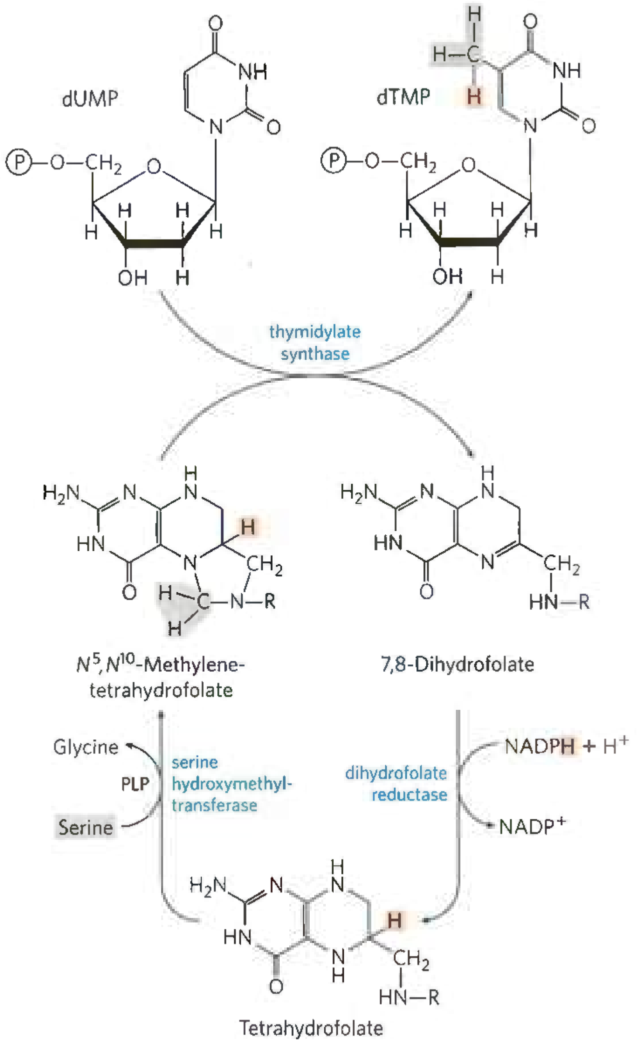


FIGURE 22-47 Conversion of dUMP to dTMP by thymidylate synthase and dihydrofolate reductase. Serine hydroxymethyltransferase is required for regeneration of the N^5,N^{10}-methylene form of tetrahydrofolate. In the synthesis of dTMP, all three hydrogens of the added methyl group are derived from N^5,N^{10}-methylenetetrahydrofolate (light red and gray).

Degradation of Purines and Pyrimidines Produces Uric Acid and Urea, Respectively

Purine nucleotides are degraded by a pathway in which they lose their phosphate through the action of **5′-nucleotidase** (**Fig. 22-48**). Adenylate yields adenosine, which is deaminated to inosine by **adenosine deaminase**, and inosine is hydrolyzed to hypoxanthine (its purine base) and D-ribose. Hypoxanthine is oxidized successively to xanthine and then uric acid by **xanthine oxidase**, a flavoenzyme with an atom of molybdenum and four iron-sulfur centers in its prosthetic group. Molecular oxygen is the electron acceptor in this complex reaction.

GMP catabolism also yields uric acid as an end product. GMP is first hydrolyzed to guanosine, which is then cleaved to free guanine. Guanine undergoes hydrolytic removal of its amino group to yield xanthine, which is converted to uric acid by xanthine oxidase.

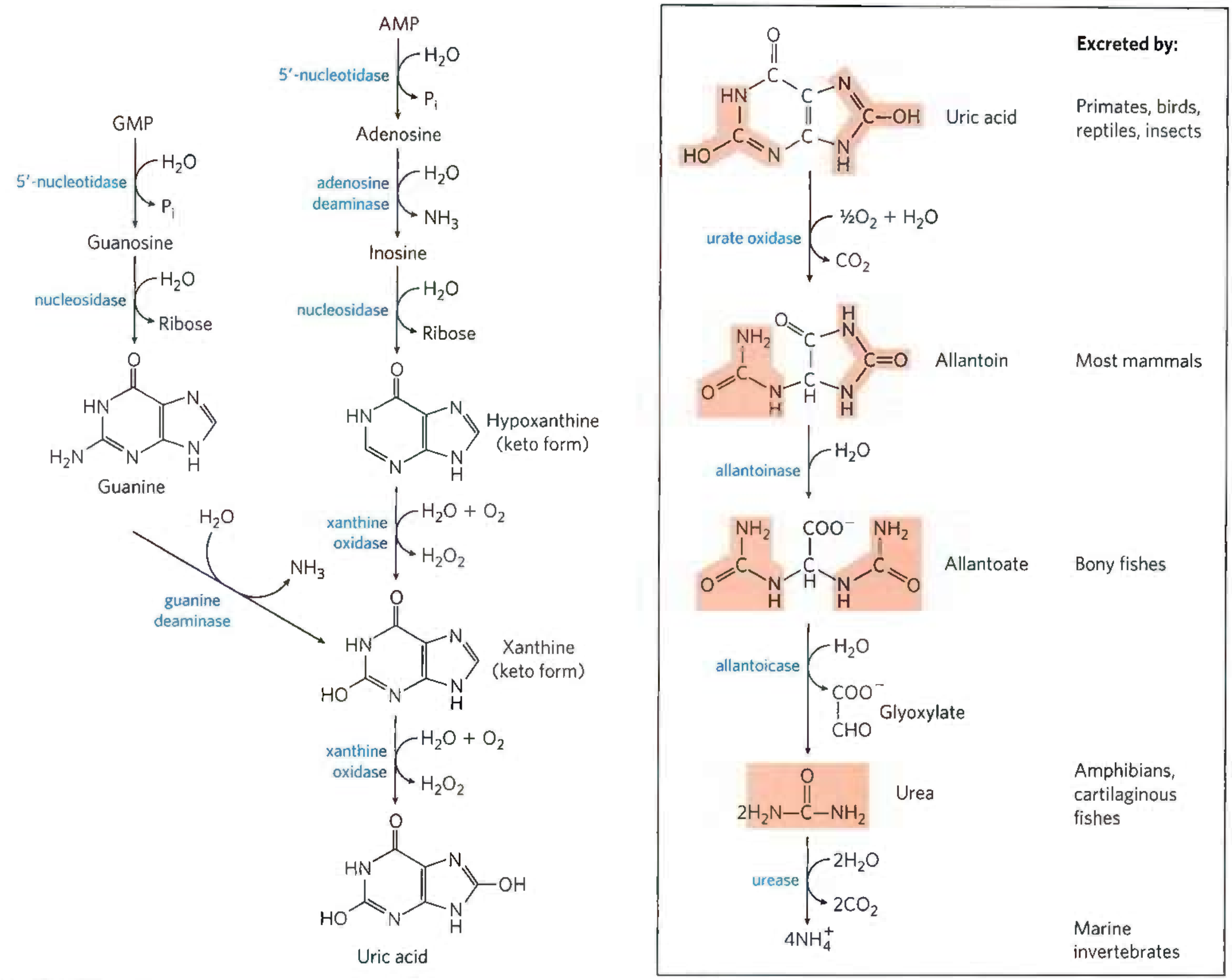

AMP
5′-nucleotidase
H_2O
P_i
Adenosine
adenosine deaminase
H_2O
NH_3
Inosine
nucleosidase
H_2O
Ribose
Hypoxanthine (keto form)
GMP
5′-nucleotidase
H_2O
P_i
Guanosine
nucleosidase
H_2O
Ribose
Guanine
H_2O
guanine deaminase
NH_3
xanthine oxidase
$H_2O + O_2$
H_2O_2
Xanthine (keto form)
xanthine oxidase
$H_2O + O_2$
H_2O_2
Uric acid
Excreted by:
Uric acid
Primates, birds, reptiles, insects
urate oxidase
$\frac{1}{2}O_2 + H_2O$
CO_2
Allantoin
Most mammals
allantoinase
H_2O
Allantoate
Bony fishes
allantoicase
H_2O
COO^-
CHO
Glyoxylate
Urea
Amphibians, cartilaginous fishes
urease
$2H_2O$
$2CO_2$
$4NH_4^+$
Marine invertebrates

FIGURE 22-48 Catabolism of purine nucleotides. Note that primates excrete much more nitrogen as urea via the urea cycle (Chapter 18) than as uric acid from purine degradation. Similarly, fish excrete much more nitrogen as NH_4^+ than as urea produced by the pathway shown here.

Uric acid is the excreted end product of purine catabolism in primates, birds, and some other animals. A healthy adult human excretes uric acid at a rate of about 0.6 g/24 h; the excreted product arises in part from ingested purines and in part from turnover of the purine nucleotides of nucleic acids. In most mammals and many other vertebrates, uric acid is degraded to **allantoin** by the action of **urate oxidase**. In other organisms the pathway is further extended, as shown in Figure 22-48.

The pathways for degradation of pyrimidines generally lead to NH_4^+ production and thus to urea synthesis. The carbons of thymine are degraded to succinyl-CoA; those of cytosine and uracil are degraded to acetyl-CoA (**Fig. 22-49**).

Genetic aberrations in human purine metabolism have been found, some with serious consequences. For example, **adenosine deaminase (ADA) deficiency** leads to severe immunodeficiency disease in which T lymphocytes and B lymphocytes do not develop properly. Lack of ADA leads to a 100-fold increase in the cellular concentration of dATP, a strong inhibitor of ribonucleotide reductase (Fig. 22-44). High levels of dATP produce a general deficiency of other dNTPs in T lymphocytes. The basis for B-lymphocyte toxicity is less clear. Individuals with ADA deficiency lack an effective immune system and do not survive unless treated. Current therapies include bone marrow transplants from a matched donor to replace the hematopoietic stem cells that mature into B and T lymphocytes. However, transplant recipients often suffer a variety of cognitive and physiological problems. Enzyme replacement therapy, requiring once- or twice-weekly intramuscular injection of active ADA, is effective, but the therapeutic benefit often declines after 8 to 10 years; complications may then arise, including malignancies. For many people, a permanent cure requires replacing the defective gene with a functional one in bone marrow cells. ADA deficiency was one of the first targets of human gene therapy trials (in 1990). Mixed results in early trials have given way to significant successes, and gene therapy is rapidly becoming a viable path for long-term restoration of immune

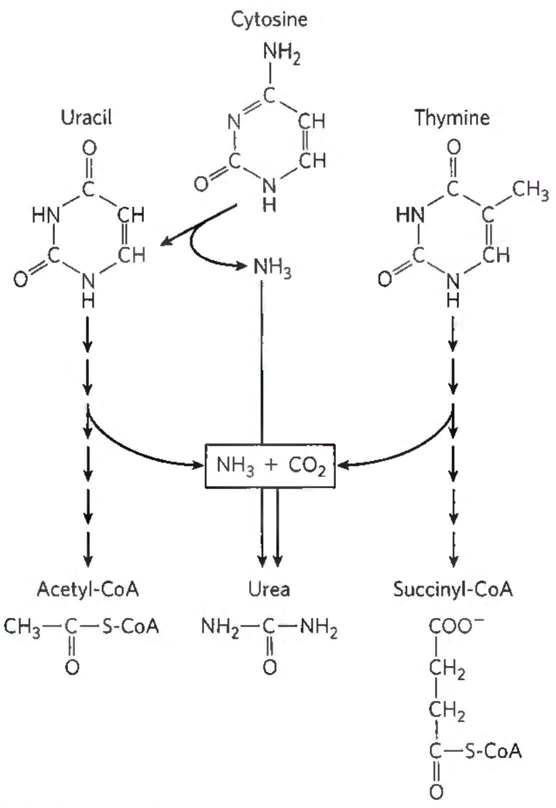


FIGURE 22-49 Catabolism of pyrimidines. These simplified pathways show end products but no intermediates.

function for these patients. Newer approaches based on CRISPR-mediated gene editing (see Fig. 9-21) may eventually be even more effective. ■

Purine and Pyrimidine Bases Are Recycled by Salvage Pathways

Free purine and pyrimidine bases are constantly released in cells during the metabolic degradation of nucleotides. Free purines are in large part salvaged and reused to make nucleotides, in a pathway much simpler than the de novo synthesis of purine nucleotides described earlier. One of the primary salvage pathways consists of a single reaction catalyzed by **adenosine phosphoribosyltransferase**, in which free adenine reacts with PRPP to yield the corresponding adenine nucleotide:

$$\text{Adenine} + \text{PRPP} \longrightarrow \text{AMP} + \text{PP}_i$$

Free guanine and hypoxanthine (the deamination product of adenine; Fig. 22-48) are salvaged in the same way by **hypoxanthine-guanine phosphoribosyltransferase**. A similar salvage pathway exists for pyrimidine bases in microorganisms, and possibly in mammals.

A genetic lack of hypoxanthine-guanine phosphoribosyltransferase activity, seen almost exclusively in young boys, results in a set of symptoms called **Lesch-Nyhan syndrome**. Children with this genetic disorder, which becomes manifest by the age of 2 years, are sometimes poorly coordinated and have intellectual deficits. In addition, they are extremely hostile and show compulsive self-destructive tendencies: they mutilate themselves by biting off their fingers, toes, and lips.

The devastating effects of Lesch-Nyhan syndrome illustrate the importance of the salvage pathways. Hypoxanthine and guanine arise constantly from the breakdown of nucleic acids. In the absence of hypoxanthine-guanine phosphoribosyltransferase, PRPP levels rise and purines are overproduced by the de novo pathway, resulting in high levels of uric acid production and goutlike damage to tissue (see below). The brain is especially dependent on the salvage pathways, and this may account for the central nervous system damage in children with Lesch-Nyhan syndrome. This syndrome is another potential target for gene therapy. ■

Excess Uric Acid Causes Gout

Long thought (erroneously) to be due to "high living," gout is a disease of the joints caused by an elevated concentration of uric acid in the blood and tissues. The joints become inflamed, painful, and arthritic, owing to the abnormal deposition of sodium urate crystals. The kidneys are also affected, as excess uric acid is deposited in the kidney tubules. Gout occurs predominantly in males. Its precise cause is not known, but it often involves an underexcretion of urate. A genetic deficiency of one or another enzyme of purine metabolism may also be a factor in some cases.

Gout is effectively treated by a combination of nutritional and drug therapies. Patients exclude foods especially rich in nucleotides and nucleic acids, such as liver or glandular products, from the diet. Major alleviation of the symptoms is provided by the drug **allopurinol** (**Fig. 22-50**), which inhibits xanthine oxidase, the enzyme

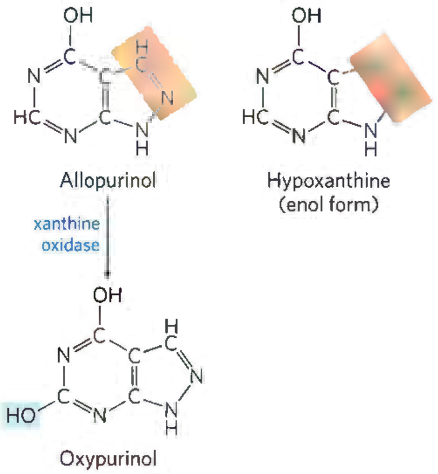
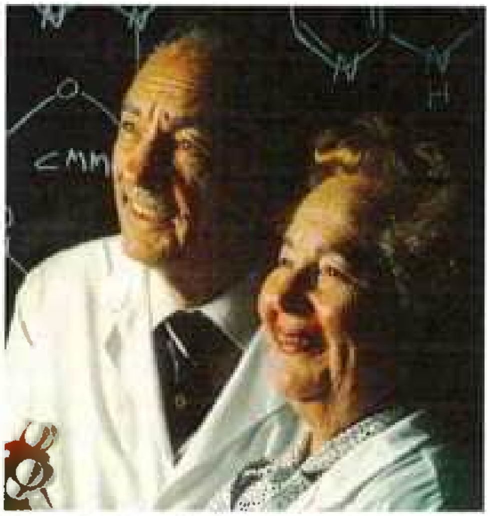


FIGURE 22-50 Allopurinol, an inhibitor of xanthine oxidase. Hypoxanthine is the normal substrate of xanthine oxidase. A slight alteration in the structure of hypoxanthine (shaded light red) yields the medically effective enzyme inhibitor allopurinol. At the active site, allopurinol is converted to oxypurinol, a strong competitive inhibitor that remains tightly bound to the reduced form of the enzyme.

that catalyzes the conversion of purines to uric acid. Allopurinol is a substrate of xanthine oxidase, which converts allopurinol to oxypurinol (alloxanthine). Oxypurinol inactivates the reduced form of the enzyme by remaining tightly bound in its active site. When xanthine oxidase is inhibited, the excreted products of purine metabolism are xanthine and hypoxanthine, which are more water-soluble than uric acid and less likely to form crystalline deposits. Allopurinol was developed by Gertrude Elion and George Hitchings, who also developed acyclovir, used in treating people with genital and oral herpes infections, and other purine analogs used in cancer chemotherapy. ■



George Hitchings, 1905–1998, and Gertrude Elion, 1918–1999. [Will and Deni McIntyre/Science Source.]

Many Chemotherapeutic Agents Target Enzymes in Nucleotide Biosynthetic Pathways

The growth of cancer cells is not controlled in the same way as cell growth in most normal tissues. Cancer cells have greater requirements for nucleotides as precursors of DNA and RNA, and consequently are generally more sensitive than normal cells to inhibitors of nucleotide biosynthesis. A number of important chemotherapeutic agents—for cancer and other diseases—act by inhibiting one or more enzymes in these pathways. Several well-studied examples can illustrate productive approaches to treatment and help us understand how these enzymes work.

Important targets for pharmaceutical agents include thymidylate synthase and dihydrofolate reductase, enzymes that provide the only cellular pathway for thymine synthesis (Fig. 22-51). One inhibitor that acts on thymidylate synthase, **fluorouracil**, is an important chemotherapeutic agent. Fluorouracil itself is not the enzyme inhibitor. In the cell, salvage pathways convert it to the deoxynucleoside monophosphate FdUMP, which binds to and inactivates the enzyme. Inhibition by FdUMP (Fig. 22-52) is a classic example of mechanism-based enzyme inactivation. Another prominent chemotherapeutic agent, **methotrexate**, is an inhibitor of dihydrofolate reductase. This folate analog acts as a competitive inhibitor; the enzyme binds methotrexate with about 100 times higher affinity than dihydrofolate. **Aminopterin** is a related compound that acts similarly.

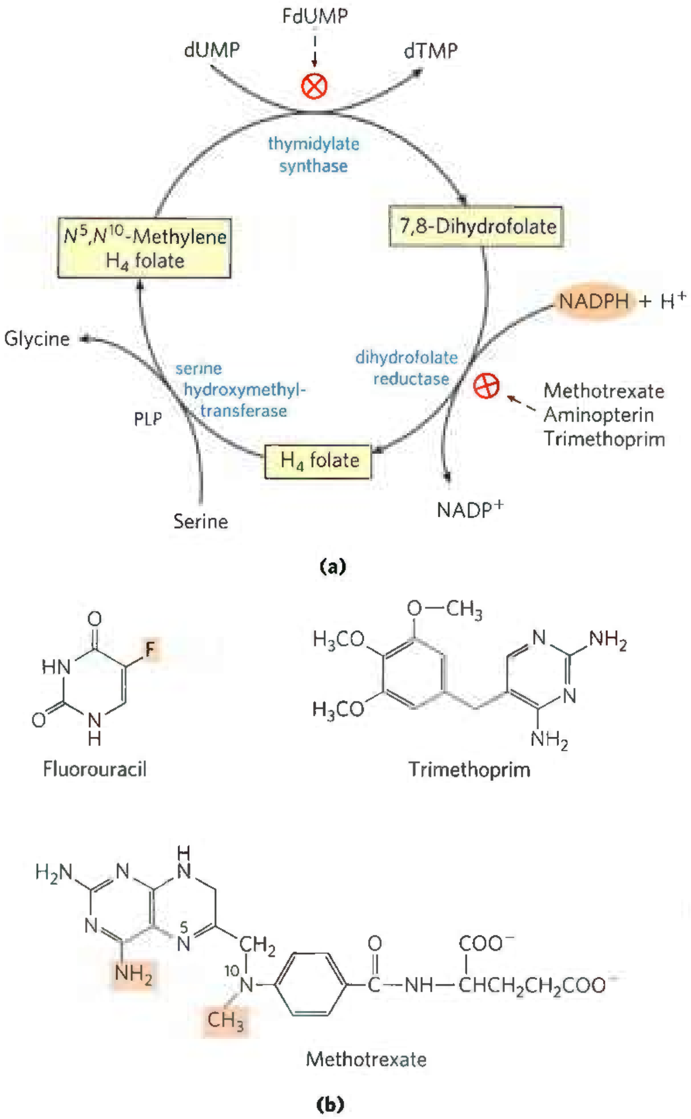
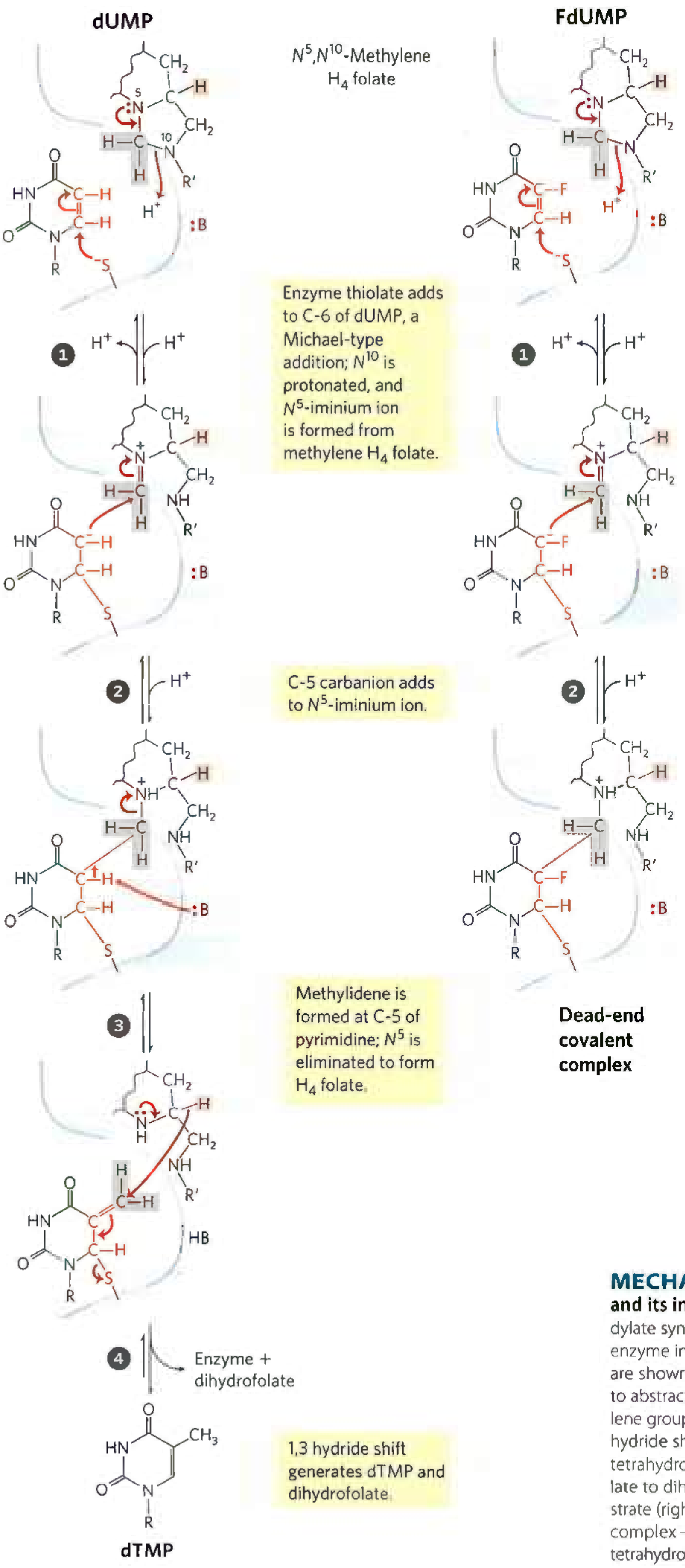


FIGURE 22-51 Thymidylate synthesis and folate metabolism as targets of chemotherapy. (a) During thymidylate synthesis, N^5,N^{10}-methylenetetrahydrofolate is converted to 7,8-dihydrofolate; the N^5,N^{10}-methylenetetrahydrofolate is regenerated in two steps (see Fig. 22-47). This cycle is a major target of several chemotherapeutic agents. (b) Fluorouracil and methotrexate are important chemotherapeutic agents. In cells, fluorouracil is converted to FdUMP, which inhibits thymidylate synthase. Methotrexate, a structural analog of tetrahydrofolate, inhibits dihydrofolate reductase; the shaded amino and methyl groups replace a carbonyl oxygen and a proton, respectively, in folate. Another important folate analog, aminopterin, is identical to methotrexate except that it lacks the shaded methyl group. Trimethoprim, a tight-binding inhibitor of bacterial dihydrofolate reductase, was developed as an antibiotic.


dUMP
FdUMP
N^5,N^{10}-Methylene H_4 folate
Dead-end covalent complex
dTMP
Enzyme + dihydrofolate
:B
HB

① Enzyme thiolate adds to C-6 of dUMP, a Michael-type addition; N^{10} is protonated, and N^5-iminium ion is formed from methylene H_4 folate.

② C-5 carbanion adds to N^5-iminium ion.

③ Methylidene is formed at C-5 of pyrimidine; N^5 is eliminated to form H_4 folate.

④ 1,3 hydride shift generates dTMP and dihydrofolate.

MECHANISM FIGURE 22-52 Conversion of dUMP to dTMP and its inhibition by FdUMP. The normal reaction mechanism of thymidylate synthase (left). The nucleophilic sulfhydryl group contributed by the enzyme in step ① and the ring atoms of dUMP taking part in the reaction are shown in red; :B denotes an amino acid side chain that acts as a base to abstract a proton after step ③. The hydrogens derived from the methylene group of N^5,N^{10}-methylenetetrahydrofolate are shaded in gray. The 1,3 hydride shift (step ④) moves a hydride ion (shaded light red) from C-6 of tetrahydrofolate to the methyl group of thymidine, oxidizing tetrahydrofolate to dihydrofolate. This hydride shift is blocked when FdUMP is the substrate (right). Steps ① and ② proceed normally, but they result in a stable complex—consisting of FdUMP linked covalently to the enzyme and to tetrahydrofolate—that inactivates the enzyme.

The medical potential of inhibitors of nucleotide biosynthesis is not limited to cancer treatment. All fast-growing cells (including bacteria and protists) are potential targets. **Trimethoprim**, an antibiotic developed by Hitchings and Elion, binds to bacterial dihydrofolate reductase nearly 100,000 times better than to the mammalian enzyme. It is used to treat certain urinary and middle-ear bacterial infections. Parasitic protists, such as the trypanosomes that cause African sleeping sickness (African trypanosomiasis), lack pathways for de novo nucleotide biosynthesis and are particularly sensitive to agents that interfere with their ability to use salvage pathways to scavenge nucleotides from the surrounding environment. Allopurinol (Fig. 22-50) and several similar purine analogs have shown promise for the treatment of African trypanosomiasis and related afflictions. See Box 6-1 for another approach to combating African trypanosomiasis, made possible by advances in our understanding of metabolism and enzyme mechanisms. ■

SUMMARY 22.4 *Biosynthesis and Degradation of Nucleotides*

- The purine ring system is built up step by step, beginning with 5-phosphoribosylamine. The amino acids glutamine, glycine, and aspartate furnish all the nitrogen atoms of purines. Two ring-closure steps form the purine nucleus.
- Purine biosynthesis is regulated by an elaborate system of feedback inhibition.
- Pyrimidines are synthesized from carbamoyl phosphate and aspartate, and ribose 5-phosphate is then attached to yield the pyrimidine ribonucleotides.
- Pyrimidine biosynthesis is regulated by feedback inhibition of aspartate transcarbamoylase.
- Nucleoside monophosphates are converted to their triphosphates by enzymatic phosphorylation reactions.
- Ribonucleotides are converted to deoxyribonucleotides by ribonucleotide reductase, an enzyme with novel mechanistic and regulatory characteristics.
- The thymine nucleotides are derived from dCDP and dUMP.
- Uric acid and urea are the end products of purine and pyrimidine degradation.
- Free purines can be salvaged and rebuilt into nucleotides. Genetic deficiencies in certain salvage enzymes cause serious disorders such as Lesch-Nyhan syndrome.
- Accumulation of uric acid crystals in the joints, possibly caused by another genetic deficiency, results in gout.
- Enzymes of the nucleotide biosynthetic pathways are targets for an array of chemotherapeutic agents used to treat cancer and other diseases.

KEY TERMS

Terms in bold are defined in the glossary.

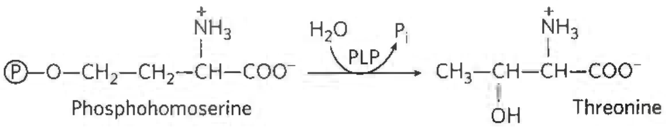
nitrogen fixation	795
nitrification	795
denitrification	795
anammox	796
symbionts	797
nitrogenase complex	797
FeMo cofactor	800
ferredoxin	800
leghemoglobin	801
glutamate	802
glutamine	802
glutamine synthetase	802
glutamate synthase	802
glutamine amidotransferases	804
5-phosphoribosyl-1-pyrophosphate (PRPP)	806
porphyrin	817
porphyria	817
bilirubin	817
phosphocreatine	819
creatine	819
glutathione (GSH)	819
auxin	821
dopamine	821
norepinephrine	821
epinephrine	821
γ-aminobutyrate (GABA)	821
serotonin	822
histamine	822
spermine	822
spermidine	822
ornithine decarboxylase	822
de novo pathway	823
salvage pathway	823
inosinate (IMP)	825
carbamoyl phosphate synthetase II	828
aspartate transcarbamoylase	828
nucleoside monophosphate kinase	829
nucleoside diphosphate kinase	829
ribonucleotide reductase	829
thioredoxin	829
thymidylate synthase	833
dihydrofolate reductase	833
adenosine deaminase (ADA) deficiency	834
Lesch-Nyhan syndrome	835
allopurinol	835
fluorouracil	836
methotrexate	836
aminopterin	836
trimethoprim	838

PROBLEMS

1. ATP Consumption by Root Nodules in Legumes Bacteria residing in the root nodules of the pea plant consume more than 20% of the ATP produced by the plant. Suggest why these bacteria consume so much ATP.

2. Nitrate Fertilizers and Oceanic Dead Zones Farmers apply industrially fixed nitrogen, in the form of ammonia or nitrate, to agricultural fields worldwide to increase crop yields. Agricultural runoff feeds into rivers and creates large hypoxic dead zones at the point where rivers meet oceans. How does an increase in soluble fixed nitrogen create dead zones?

3. PLP Reaction Mechanisms Pyridoxal phosphate (PLP) can help catalyze transformations one or two carbons removed from the α carbon of an amino acid. The enzyme threonine synthase promotes the PLP-dependent conversion of phosphohomoserine to threonine. Suggest a mechanism for this reaction.



$$\text{Ⓟ}{-}O{-}CH_2{-}CH_2{-}\underset{}{\overset{\overset{+}{N}H_3}{\underset{}{CH}}}{-}COO^- \xrightarrow[\text{PLP}]{H_2O \quad P_i} CH_3{-}\underset{OH}{CH}{-}\overset{\overset{+}{N}H_3}{CH}{-}COO^-$$

4. Transformation of Aspartate to Asparagine There are two routes for transforming aspartate to asparagine at the expense of ATP. Many bacteria have an asparagine synthetase that uses ammonium ion as the nitrogen donor. Mammals have an asparagine synthetase that uses glutamine as the nitrogen donor. Given that the latter requires an extra ATP (for the synthesis of glutamine), why do mammals use this route?

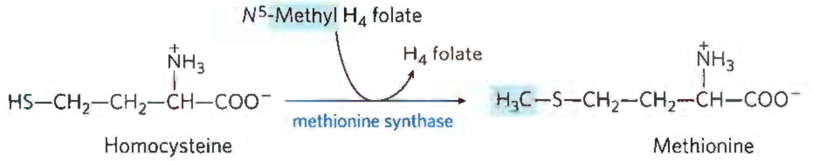
5. Equation for the Synthesis of Aspartate from Glucose Write the net equation for the synthesis of aspartate (a nonessential amino acid) from glucose, carbon dioxide, and ammonia.

6. Asparagine Synthetase Inhibitors in Leukemia Therapy Mammalian asparagine synthetase is a glutamine-dependent amidotransferase. Efforts to identify an effective inhibitor of human asparagine synthetase for use in chemotherapy for patients with leukemia have focused not on the amino-terminal glutaminase domain but on the carboxyl-terminal synthetase active site. Explain why the glutaminase domain is not a promising target for a useful drug.

7. Phenylalanine Hydroxylase Deficiency and Diet Tyrosine is normally a nonessential amino acid, but individuals with a genetic defect in phenylalanine hydroxylase require tyrosine in their diet for normal growth. Explain.

8. Arginine Biosynthesis The first step of arginine biosynthesis from glutamate acetylates glutamate on the α-amino group. A subsequent step late in the same pathway removes the added acetyl group. What chemical problem is solved by adding and then removing an acetyl group, with none of the acetyl atoms appearing in the arginine product of the pathway?

9. Cofactors for One-Carbon Transfer Reactions Most one-carbon transfers are promoted by one of three cofactors: biotin, tetrahydrofolate, or *S*-adenosylmethionine. *S*-Adenosylmethionine generally serves as a methyl group donor; the transfer potential of the methyl group in N^5-methyltetrahydrofolate is insufficient for most biosynthetic reactions. However, one example of the use of N^5-methyltetrahydrofolate in methyl group transfer is in methionine formation by the methionine synthase reaction; methionine is the immediate precursor of *S*-adenosylmethionine (see Fig. 18-18).


N^5-Methyl H_4 folate
H_4 folate
$HS-CH_2-CH_2-\overset{\overset{+}{N}H_3}{CH}-COO^-$
methionine synthase
$H_3C-S-CH_2-CH_2-\overset{\overset{+}{N}H_3}{CH}-COO^-$
Homocysteine
Methionine

Explain how the methyl group of *S*-adenosylmethionine can be derived from N^5-methyltetrahydrofolate, even though the transfer potential of the methyl group in N^5-methyltetrahydrofolate is one-thousandth of that of *S*-adenosylmethionine.

10. Concerted Regulation in Amino Acid Biosynthesis Various products of glutamine metabolism independently modulate the glutamine synthetase of *E. coli* (see Fig. 22-8). In this concerted inhibition, the extent of enzyme inhibition is greater than the sum of the separate inhibitions caused by each product. For *E. coli* grown in a medium rich in histidine, what is the advantage of concerted inhibition?

11. Relationship between Folic Acid Deficiency and Anemia Folic acid deficiency, believed to be the most common vitamin deficiency, causes a type of anemia in which hemoglobin synthesis is impaired and erythrocytes do not mature properly. What is the metabolic relationship between hemoglobin synthesis and folic acid deficiency?

12. Synthesis of Polyamines The metabolic amino acid ornithine is a direct precursor of the polyamine putrescine, shown here.

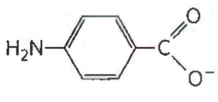
$$H_3\overset{+}{N}-CH_2-CH_2-CH_2-CH_2-\overset{+}{N}H_3$$

Subsequent reactions convert putrescine to spermine and spermidine. What type of reaction is required to convert ornithine to putrescine, and what enzymatic cofactor is needed?

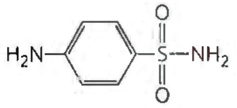
13. Nucleotide Biosynthesis in Amino Acid Auxotrophic Bacteria Wild-type *E. coli* cells can synthesize all 20 common amino acids, but some mutants, called amino acid auxotrophs, are unable to synthesize a specific amino acid and require its addition to the culture medium for optimal growth. Besides their role in protein synthesis, some amino acids are also precursors for other nitrogenous cell products. Consider the three amino acid auxotrophs that are unable to synthesize glycine, glutamine, and aspartate, respectively. For each mutant, what nitrogenous products other than proteins would the cell fail to synthesize?

14. Inhibitors of Nucleotide Biosynthesis Suggest a mechanism for the inhibition of alanine racemase by L-fluoroalanine.

15. Mode of Action of Sulfa Drugs Some bacteria require *p*-aminobenzoate in the culture medium for normal growth, and their growth is severely inhibited by the addition of sulfanilamide, one of the earliest sulfa drugs. Moreover, in the presence of this drug, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; see Fig. 22-35) accumulates in the culture medium. Addition of excess *p*-aminobenzoate reverses these effects.



p-Aminobenzoate



Sulfanilamide

(a) What is the role of *p*-aminobenzoate in these bacteria? (Hint: See Fig. 18-16.)
(b) Why does AICAR accumulate in the presence of sulfanilamide?
(c) Why does addition of excess *p*-aminobenzoate reverse the inhibition and accumulation?

16. Purine Biosynthesis Which atoms of the purine ring derive from the amide nitrogen of glutamine? **(a)** N-1 **(b)** N-3 **(c)** N-7 **(d)** N-9

17. Pathway of Carbon in Pyrimidine Biosynthesis Predict the locations of ^{14}C in orotate isolated from cells grown on a small amount of uniformly labeled [^{14}C]succinate. Justify your prediction.

18. Nucleotides as Poor Sources of Energy Under starvation conditions, organisms can use proteins and amino acids as sources of energy. Deamination of amino acids produces carbon skeletons that can enter the glycolytic pathway and the citric acid cycle to produce energy in the form of ATP. Nucleotides are not similarly degraded for use as energy-yielding fuels. What observations about cellular physiology support this statement? What aspect of the structure of nucleotides makes them a relatively poor source of energy?

19. Treatment of Gout Physicians use allopurinol (see Fig. 22-50), an inhibitor of xanthine oxidase, to treat chronic gout. Explain the biochemical basis for this treatment. Patients treated with allopurinol sometimes develop xanthine stones in the kidneys, although the incidence of kidney damage is much lower than in untreated gout. Explain this observation in light of these solubilities in urine: uric acid, 0.15 g/L; xanthine, 0.05 g/L; and hypoxanthine, 1.4 g/L.

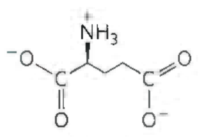
20. Antibiotics That Inhibit Dihydrofolate Reductase Trimethoprim, a commonly used antibiotic, inhibits the bacterial form of dihydrofolate reductase much more than it inhibits the mammalian enzyme. What metabolic processes described in this chapter are affected by depleting tetrahydrofolate?

DATA ANALYSIS PROBLEM

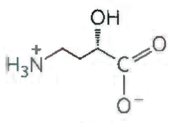
21. Use of Modern Molecular Techniques to Determine the Synthetic Pathway of a Novel Amino Acid Most of the biosynthetic pathways described in this chapter were determined before the development of recombinant DNA technology and genomics, so the techniques were quite different from those that researchers would use today. Here we explore an example of the use of modern molecular techniques to investigate the pathway of synthesis of a novel amino acid, (2*S*)-4-amino-2-hydroxybutyrate (AHBA). The techniques mentioned here are described in various places in the text; this problem is designed to show how they can be integrated in a comprehensive study.

AHBA is a γ-amino acid that is a component of some aminoglycoside antibiotics, including the antibiotic butirosin. Antibiotics modified by the addition of an AHBA residue are often more resistant to inactivation by bacterial antibiotic-resistance enzymes. As a result, understanding how AHBA is synthesized and added to antibiotics is useful in the design of pharmaceuticals.

In an article published in 2005, Li and coworkers describe how they determined the synthetic pathway of AHBA from glutamate.



Glutamate



AHBA

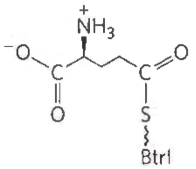
(a) Briefly describe the chemical transformations needed to convert glutamate to AHBA. At this point, don't be concerned about the *order* of the reactions.

Li and colleagues began by cloning the butirosin biosynthetic gene cluster from the bacterium *Bacillus circulans*, which makes large quantities of butirosin. They identified five genes that are essential for the pathway: *btrI*, *btrJ*, *btrK*, *btrO*, and *btrV*. They cloned these genes into *E. coli* plasmids that allow overexpression of the genes, producing proteins with "histidine tags" fused to their amino termini to facilitate purification (see p. 313).

The predicted amino acid sequence of the BtrI protein showed strong homology to known acyl carrier proteins (see Fig. 21-5). Using mass spectrometry, Li and colleagues found a molecular mass of 11,812 for the purified BtrI protein (including the His tag). When the purified BtrI was incubated with coenzyme A and an enzyme known to attach CoA to other acyl carrier proteins, the majority molecular species had an M_r of 12,153.

(b) How would you use these data to argue that BtrI can function as an acyl carrier protein with a CoA prosthetic group?

Using standard terminology, Li and coauthors called the form of the protein lacking CoA apo-BtrI and the form with CoA (linked as in Fig. 21-5) holo-BtrI. When holo-BtrI was incubated with glutamine, ATP, and purified BtrJ protein, the holo-BtrI species of M_r 12,153 was replaced with a species of M_r 12,281, corresponding to the thioester of glutamate and holo-BtrI. Based on these data, the authors proposed the following structure for the M_r 12,281 species, γ-glutamyl-*S*-BtrI:



(c) What other structure(s) is (are) consistent with the data above?

(d) Li and coauthors argued that the structure shown here (γ-glutamyl-*S*-BtrI) is likely to be correct because the α-carboxyl group must be removed at some point in the synthetic process. Explain the chemical basis of this argument. (Hint: See Fig. 18-6, reaction C.)

The BtrK protein showed significant homology to PLP-dependent amino acid decarboxylases, and BtrK isolated from *E. coli* was found to contain tightly bound PLP. When γ-glutamyl-*S*-BtrI was incubated with purified BtrK, a molecular species of M_r 12,240 was produced.

(e) What is the most likely structure of this species?

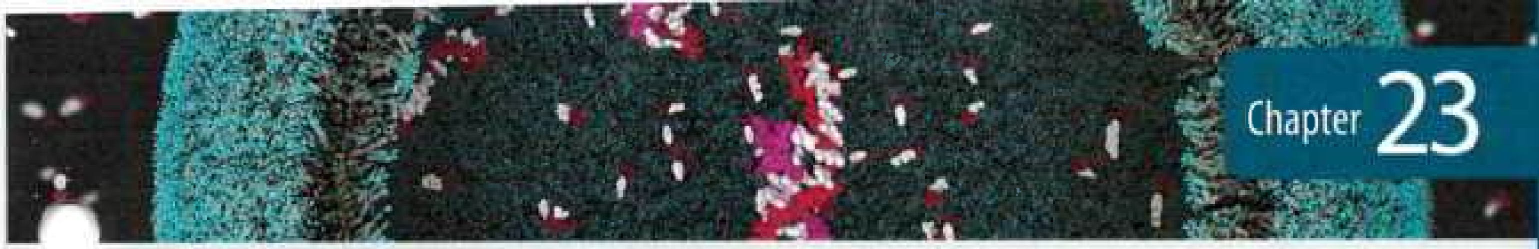
(f) When the investigators incubated glutamate and ATP with purified BtrI, BtrJ, and BtrK, they found a molecular species of M_r 12,370. What is the most likely structure of this species? Hint: Remember that BtrJ can use ATP to γ-glutamylate nucleophilic groups.

Li and colleagues found that BtrO is homologous to monooxygenase enzymes (see Box 21-1) that hydroxylate alkanes, using FMN as a cofactor, and BtrV is homologous to an NAD(P)H oxidoreductase. Two other genes in the cluster, *btrG* and *btrH*, probably encode enzymes that remove the γ-glutamyl group and attach AHBA to the target antibiotic molecule.

(g) Based on these data, propose a plausible pathway for the synthesis of AHBA and its addition to the target antibiotic. Include the enzymes that catalyze each step and any other substrates or cofactors needed (ATP, NAD, etc.).

Reference

Li, Y., N.M. Llewellyn, R. Giri, F. Huang, and J.B. Spencer. 2005. Biosynthesis of the unique amino acid side chain of butirosin: possible protective-group chemistry in an acyl carrier protein–mediated pathway. *Chem. Biol.* 12:665–675.



Chapter 23

HORMONAL REGULATION AND INTEGRATION OF MAMMALIAN METABOLISM

23.1 Hormone Structure and Action *842*
23.2 Tissue-Specific Metabolism *848*
23.3 Hormonal Regulation of Fuel Metabolism *859*
23.4 Obesity and the Regulation of Body Mass *867*
23.5 Diabetes Mellitus *875*

In Chapters 13 through 22 our focus has been on metabolism at the level of the individual cell, emphasizing central pathways common to almost all cells—bacterial, archaeal, and eukaryotic. We have seen how metabolic processes within cells are regulated at the level of individual enzyme reactions by substrate availability, by allosteric mechanisms, and by reversible covalent modification of enzymes.

To fully appreciate the significance of individual metabolic pathways and their regulation, we must view these pathways in the context of the whole organism. An essential characteristic of multicellular organisms is cell differentiation and division of labor. Specialized functions of the tissues and organs require specialized fuels and patterns of metabolism. Hormonal and neuronal signals integrate and coordinate the metabolic activities of different tissues and optimize the allocation of fuels and precursors to each organ. Although our focus is on mammalian systems, mammals are hardly unique in possessing hormonal signaling systems. Insects and nematode worms have highly developed systems for hormonal regulation, with fundamental mechanisms similar to those in mammals. Plants, too, use hormonal signals to coordinate the activities of their differentiated, specialized tissues.

In this chapter we will look at the specialized metabolism of several major organs and tissues and the integration of metabolism in mammals. We begin with an overview of the broad range of hormones and hormonal mechanisms, then turn to the tissue-specific functions regulated by these mechanisms. We discuss the distribution of nutrients to various organs, emphasizing the central role of the liver, and the metabolic cooperation among these organs. To illustrate the integrative role of hormones, we describe the interplay of insulin, glucagon, and epinephrine in coordinating fuel metabolism in muscle, liver, and adipose tissue. We also introduce other hormones, produced in adipose tissue, muscle, gut, and brain, that play key roles in coordinating metabolism and behavior. We discuss the long-term hormonal regulation of body mass and the role of obesity in the development of metabolic syndrome and type 2 diabetes. Finally, we discuss interventions used to manage diabetes.

In this chapter, we illustrate the following principles:

P1 **The tissues in a mammal are connected by a neurosecretory system that coordinates their activities.** Mammals use chemically diverse hormones in a highly specific and multidirectional signaling system, connecting the tissues with each other and with the central nervous system.

P2 **Among the tissues and organs of an animal, there is a striking division of labor.** The specialized role of each organ is reflected in its metabolic activities and capabilities. The circulatory system connects all of the tissues by carrying hormonal signals and metabolites between and among them.

P3 **Because the brain requires a continuous supply of glucose, maintaining an adequate concentration of glucose in the blood is a high priority in the activities of the other tissues.** The liver integrates the use of fuels (glucose, fatty acids, and amino acids) by each tissue to keep the blood glucose level within the optimal range. Hormones carried in the blood (insulin, glucagon, epinephrine, cortisol) mediate this regulation.

P4 **Maintaining an optimal body mass is an important priority in the adult mammal.** Body mass is a function of dietary intake, physical activity, and the choice of metabolic fuel, all of which are subject to hormonal regulation. Hormonal signals between the brain, the adipose tissue, and the gastrointestinal tract help to set activity and feeding behavior.

P5 **The metabolic activities of cells and organisms are complex and intertwined; perturbation at one point in the system has far-reaching consequences for health.** When the normal energy-yielding metabolism of glucose and fatty acids is impeded by defective insulin signaling, the result is the disease diabetes.

23.1 Hormone Structure and Action

Hormones are small molecules or proteins that are produced in one tissue, released into the bloodstream, and carried to other tissues, where they act through specific receptors to bring about changes in cellular activities. Hormones serve to coordinate the metabolic activities of several tissues or organs. Virtually every process in a complex organism is regulated by one or more hormones: maintenance of blood pressure, blood volume, and electrolyte balance; embryogenesis; sexual differentiation, development, and reproduction; hunger, eating behavior, digestion, and fuel allocation—to name but a few.

The coordination of metabolism in mammals is achieved by the **neuroendocrine system**. Individual cells in one tissue sense a change in the organism's circumstances and respond by secreting a chemical messenger that passes to another cell in the same or different tissue, where the messenger binds to a specific receptor molecule and triggers a change in this target cell. These chemical messengers may relay information over very short or very long distances. In neuronal signaling (**Fig. 23-1a**), the chemical messenger is a neurotransmitter (acetylcholine, for example) that may travel only a fraction of a micrometer across a synaptic cleft to the next neuron in a network. In endocrine signaling (Fig. 23-1b), the messenger is a hormone that is carried in the bloodstream to neighboring cells or to distant organs and tissues; it may travel a meter or more before encountering its target cell. Except for this anatomic difference, the neuronal and endocrine signaling mechanisms are remarkably similar, and the same molecule can sometimes act as both neurotransmitter and hormone. Epinephrine and norepinephrine, for example, serve as neurotransmitters at certain synapses of the brain and at neuromuscular junctions of smooth muscle and as hormones that regulate fuel metabolism in liver and muscle. The following discussion of cellular signaling emphasizes hormone action, drawing on discussions of fuel metabolism in earlier chapters, but most of the fundamental mechanisms described here also occur in neurotransmitter action.

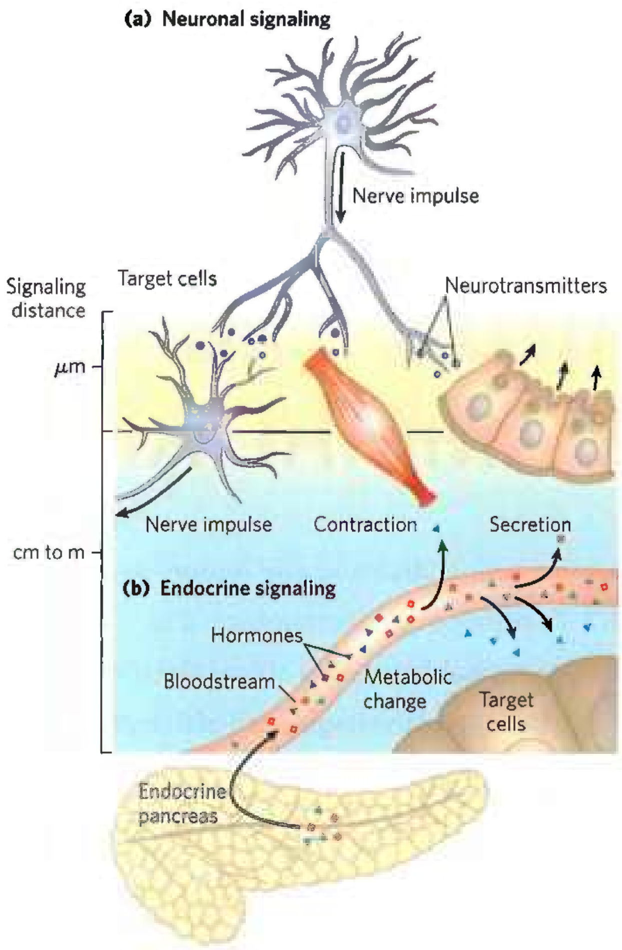


FIGURE 23-1 Signaling by the neuroendocrine system. (a) In neuronal signaling, electrical signals (nerve impulses) originate in the cell body of a neuron and travel very rapidly over long distances to the axon tip, where neurotransmitters are released and diffuse to the target cell. The target cell (another neuron, a myocyte, or a secretory cell) is only a fraction of a micrometer or a few micrometers away from the site of neurotransmitter release. (b) In endocrine signaling, hormones (such as insulin produced in pancreatic β cells) are secreted into the bloodstream, which carries them throughout the body to target tissues that may be a meter or more away from the secreting cell. Both neurotransmitters and hormones interact with specific receptors on or in their target cells, triggering responses.

Hormones Act through Specific High-Affinity Cellular Receptors

Hormones exert their effects through specific receptors in target cells. The high affinity of the hormone-receptor interaction allows cells to respond to very low concentrations of hormone. Recall from Chapter 12 (Fig. 12-2) that there are four general types of intracellular consequences of ligand-receptor interaction: (1) a second messenger, such as cAMP, cGMP, or inositol trisphosphate, generated inside the cell, acts as an allosteric regulator of one or more enzymes; (2) a receptor tyrosine kinase is activated by the extracellular hormone; (3) a change in membrane potential results from the opening or closing of a hormone-gated ion channel; and (4) a steroid or steroidlike molecule causes a change in the level of expression (transcription of DNA into mRNA) of one or more genes, mediated by a nuclear hormone receptor protein. It can be helpful to characterize cell surface hormone receptors as **metabotropic**, those that activate or inhibit an enzyme downstream from the receptor, or **ionotropic**, those that open or close an ion channel in the plasma membrane, resulting in a change in membrane potential (ΔV_m) or in the concentration of an ion such as Ca^{2+} (**Fig. 23-2**).

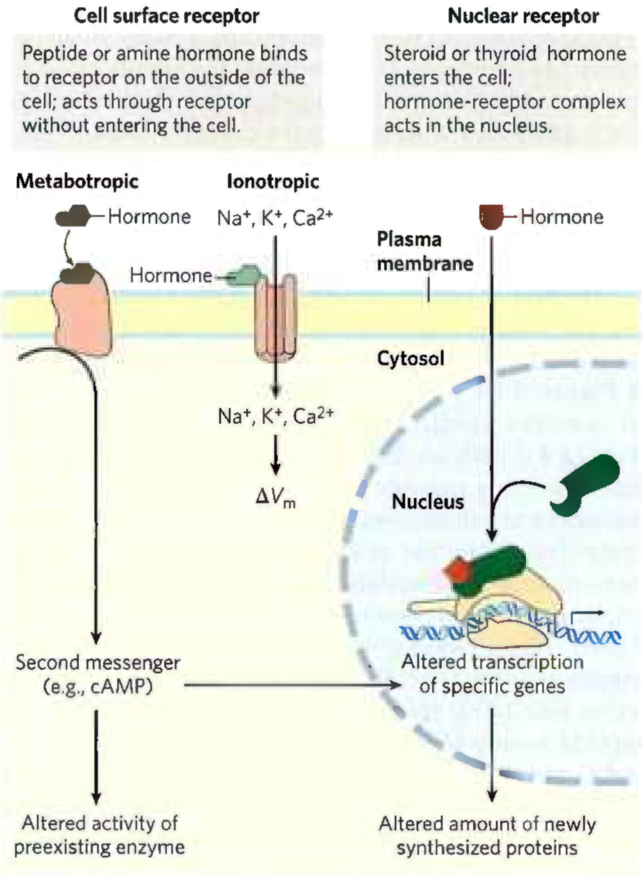

Cell surface receptor
Peptide or amine hormone binds to receptor on the outside of the cell; acts through receptor without entering the cell.
Nuclear receptor
Steroid or thyroid hormone enters the cell; hormone-receptor complex acts in the nucleus.
Metabotropic
Ionotropic
Hormone
Na^+, K^+, Ca^{2+}
Hormone
Hormone
Plasma membrane
Cytosol
Na^+, K^+, Ca^{2+}
ΔV_m
Nucleus
Second messenger (e.g., cAMP)
Altered transcription of specific genes
Altered activity of preexisting enzyme
Altered amount of newly synthesized proteins

FIGURE 23-2 Two general mechanisms of hormone action. The peptide and amine hormones are faster-acting than steroid and thyroid hormones. Peptide hormones act from outside the cell, binding a plasma membrane hormone receptor. Steroid and thyroid hormones pass through the plasma membrane and enter the nucleus, where they regulate the expression of specific genes.

A single hormone molecule, in forming a hormone-receptor complex, activates a catalyst that produces many molecules of second messenger, so the receptor serves as both signal transducer and signal amplifier. The signal may be further amplified by a signaling cascade, such as we saw in the regulation of glycogen synthesis and breakdown by epinephrine (see Fig. 12-7). Signal amplification allows one epinephrine molecule to result in the production of many thousands or millions of molecules of glucose 1-phosphate from glycogen.

Water-insoluble hormones, including the steroid, retinoid, and thyroid hormones, readily pass through the plasma membrane of their target cells to reach their receptor proteins in the nucleus (Fig. 23-2). The hormone-receptor complex itself carries the message: it interacts with DNA to alter the expression of specific genes, changing the enzyme content of the cell and thereby changing cellular metabolism (see Fig. 12-34).

Hormones that act through plasma membrane receptors generally trigger very rapid physiological or biochemical responses. Just seconds after the adrenal medulla secretes epinephrine into the bloodstream, skeletal muscle responds by accelerating the breakdown of glycogen. By contrast, the thyroid hormones and the sex (steroid) hormones promote maximal responses in their target tissues only after hours or even days. These differences in response time correspond to different modes of action. In general, the fast-acting hormones lead to a change in the activity of one or more preexisting enzymes in the cell, by allosteric mechanisms or covalent modification. The slower-acting hormones generally alter gene expression, resulting in the synthesis of more (upregulation) or less (downregulation) of the regulated protein(s).

Hormones Are Chemically Diverse

P1 Mammals have several classes of hormones, distinguishable by their diverse chemical structures and their modes of action (**Table 23-1**). Peptide, catecholamine, eicosanoid, and endocannabinoid hormones act from outside the target cell via cell surface receptors. Steroid, vitamin D, retinoid, and thyroid hormones enter the cell and act through nuclear receptors. Nitric oxide (a gas) also enters the cell, but activates a cytosolic enzyme, guanylyl cyclase.

Hormones can also be classified by the way they get from their point of release to their target tissue. **Endocrine** (from the Greek *endon*, "within," and *krinein*, "to release") hormones are released into the blood and carried to target cells throughout the body (insulin and glucagon are examples). **Paracrine** hormones are released into the extracellular space and diffuse to neighboring target cells (the eicosanoid

TABLE 23-1 Classes of Hormones

Type	Example	Synthetic path	Mode of action
Protein	Insulin (Fig. 23-4)	Proteolytic processing of prohormone	Plasma membrane RTK
Protein	Glucagon		Plasma membrane GPCRs; second messengers
Peptide	Vasopressin (p. 880)		
Catecholamine	Epinephrine (Fig. 22-31)	From tyrosine	
Eicosanoid	Prostaglandin E_2 (Fig. 10-17)	From arachidonate	
Endocannabinoid	Anandamide (Fig. 23-40)		
Steroid	Testosterone (Fig. 10-18)	From cholesterol	Nuclear receptors; transcriptional regulation
Corticosteroid	Cortisol (Fig. 10-18)		
Vitamin D	Calcitriol (Fig. 10-19)		
Retinoid	Retinoic acid (Fig. 10-20)	From vitamin A	
Thyroid	Triiodothyronine (T_3)	From Tyr in thyroglobulin	
Nitric oxide	$NO^•$ (Fig. 22-33)	From arginine	Cytosolic receptor; second messenger

hormones are of this type). **Autocrine** hormones affect the same cell that releases them, binding to receptors on the cell surface.

The **peptide hormones** vary in size from 3 (thyrotropin-releasing hormone; **Fig. 23-3**) to more than 200 (human chorionic gonadotropin) amino acid residues. They include the pancreatic hormones insulin, glucagon, and somatostatin; the parathyroid hormone calcitonin; and all the hormones of the hypothalamus and pituitary.

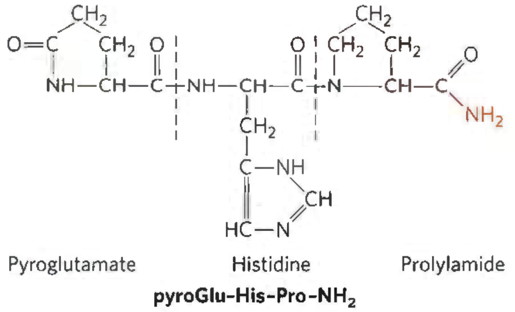


FIGURE 23-3 The structure of thyrotropin-releasing hormone (TRH). Purified (through heroic efforts) from extracts of hypothalamus, TRH proved to be a derivative of the tripeptide Glu–His–Pro. The side-chain carboxyl group of the amino-terminal Glu forms an amide (red bond) with the residue's α-amino group, creating pyroglutamate, and the carboxyl group of the carboxyl-terminal Pro is converted to an amide (red $—NH_2$). Such modifications are common among the small peptide hormones. In a typical protein of M_r ~50,000, the charges on the amino- and carboxyl-terminal groups contribute relatively little to the overall charge on the molecule, but in a tripeptide these two charges dominate the properties of the molecule. Formation of the amide derivatives removes these charges.

Peptide hormones (some of which are actually small proteins) are synthesized as proproteins (prohormones) that are activated upon release by proteolytic cleavage, much as we saw with zymogen activation of pancreatic enzymes (Fig. 6-42) and the blood-clotting cascade (Fig. 6-44). In some cases, a preproprotein is synthesized and processed to create more than one active peptide product.

Insulin is a small protein (M_r 5,800) with two polypeptide chains, A and B, joined by two disulfide bonds. (The amino acid sequence of bovine insulin is shown in Figure 3-24.) It is synthesized in the pancreas as an inactive single-chain precursor, preproinsulin (**Fig. 23-4**), with an amino-terminal "signal sequence" that directs its passage into secretory vesicles. (Signal sequences are discussed in Chapter 27; see Fig. 27-38.) Proteolytic removal of the signal sequence and formation of three disulfide bonds produces proinsulin, which is stored in secretory granules in pancreatic β cells. When blood glucose is elevated sufficiently to trigger insulin secretion, proinsulin is converted to active insulin by specific proteases, which cleave two peptide bonds to form the mature insulin molecule and C peptide, which are released into the blood by exocytosis. The capillaries that serve peptide-producing endocrine glands are fenestrated (punctuated with tiny holes or "windows"), so the hormone molecules readily enter the bloodstream for transport to target cells elsewhere. Insulin, acting through its receptor tyrosine kinase (Fig. 12-21) has profound effects on both anabolic and catabolic processes in many tissues, which we explore in detail below.

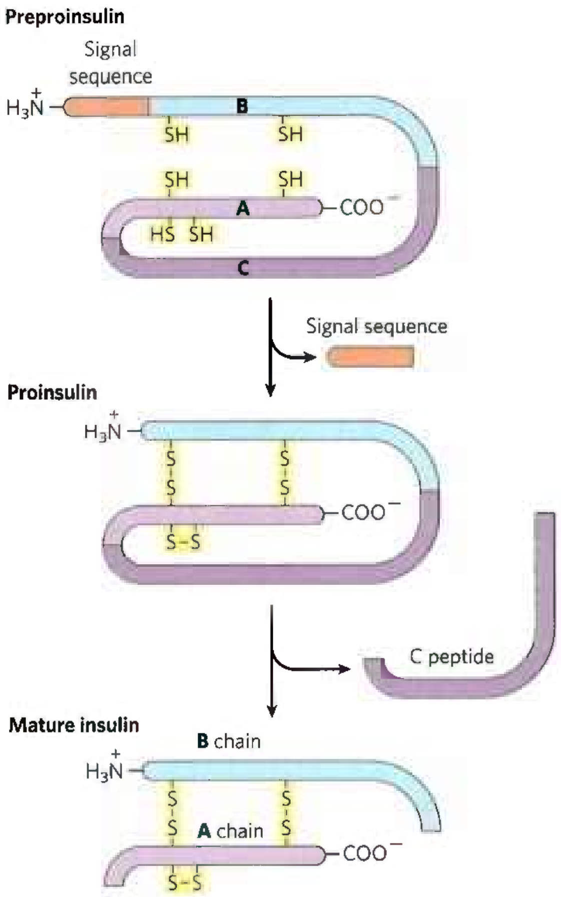


FIGURE 23-4 Insulin. Mature insulin is formed from its larger precursor preproinsulin by proteolytic processing. Removal of a 23 amino acid segment (the signal sequence) at the amino terminus of preproinsulin and formation of three disulfide bonds produce proinsulin. Further proteolytic cuts remove the C peptide from proinsulin to produce mature insulin, composed of A and B chains.

Pro-opiomelanocortin (POMC) is a spectacular example of a proprotein that undergoes specific cleavage to produce several active hormones. The POMC gene encodes a large polypeptide that contains within it at least nine biologically active peptides (**Fig. 23-5**). The proprotein is processed differently in different tissues, depending on which proteases the cells express. The active products influence an astonishing number of physiological systems.

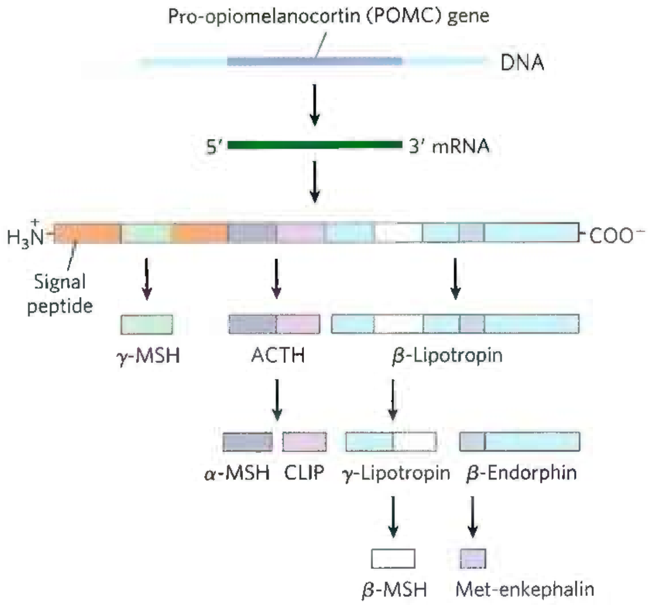


FIGURE 23-5 Proteolytic processing of the pro-opiomelanocortin (POMC) precursor. The initial gene product of the POMC gene is a long polypeptide that undergoes cleavage by a series of specific proteases to produce ACTH (corticotropin), β- and γ-lipotropin, α-, β-, and γ-MSH (melanocyte-stimulating hormone, or melanocortin), CLIP (corticotropin-like intermediary peptide), β-endorphin, and Met-enkephalin. The points of cleavage are pairs of basic residues, Arg–Lys, Lys–Arg, or Lys–Lys.

Some Hormones Are Released by a "Top-Down" Hierarchy of Neuronal and Hormonal Signals

The changing levels of specific hormones regulate specific cellular processes, but what regulates the regulators — what sets the level of each hormone? The brief answer is that P1 the central nervous system receives input from many internal and external sensors — signals about danger, hunger, dietary intake, blood composition, for example — and orchestrates the production of appropriate hormonal signals by the endocrine tissues. For a more complete answer, consider the path of cortisol release by the adrenal gland, triggered by a stress detected by the central nervous system. **Figure 23-6** illustrates the "chain of command" in this top-down hormonal signaling hierarchy. The **hypothalamus**, a pea-size region of the brain (**Fig. 23-7**), is the coordination center of the endocrine system; it receives and integrates messages from the central nervous system. In response, the hypothalamus produces releasing factors, including corticotropin-releasing hormone (CRH), that pass directly to the nearby pituitary gland through blood vessels and neurons that connect the two glands. The pituitary gland secretes adrenocorticotropic hormone (also called corticotropin or ACTH), which travels through the blood to the adrenal cortex and triggers the release of cortisol. Cortisol, the ultimate hormone in this cascade, acts through its receptor in many types of target cells to alter their metabolism. In hepatocytes, one effect of cortisol is to increase the rate of gluconeogenesis.

Hormonal cascades such as those responsible for the release of cortisol result in large amplifications of the initial signal and allow exquisite fine-tuning of the output of the ultimate hormone (Fig. 23-6). At each level in the cascade, a small signal elicits a larger response. For example, the initial electrical signal to the hypothalamus results in the release of a few

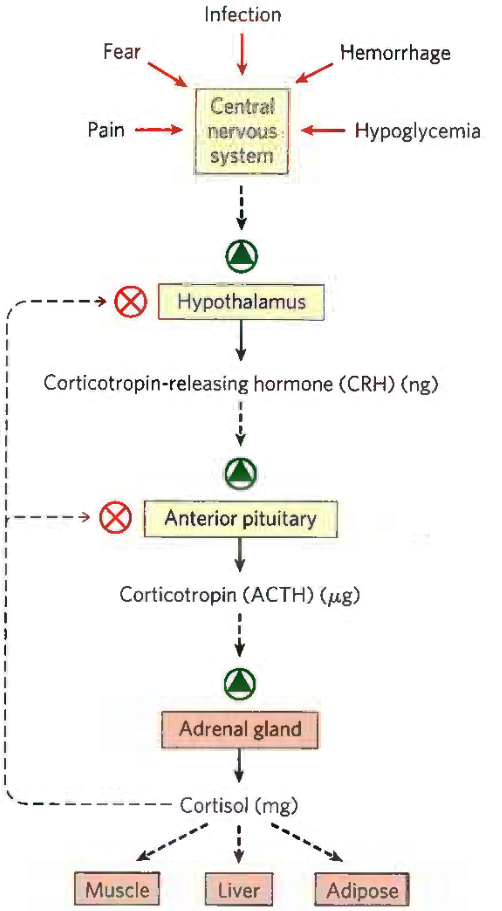

Infection
Fear
Hemorrhage
Pain
Central nervous system
Hypoglycemia
Hypothalamus
Corticotropin-releasing hormone (CRH) (ng)
Anterior pituitary
Corticotropin (ACTH) (μg)
Adrenal gland
Cortisol (mg)
Muscle
Liver
Adipose

FIGURE 23-6 Cascade of top-down hormone release following central nervous system input to the hypothalamus. Solid black arrows indicate hormone production and release; broken black arrows indicate the action of hormones on target tissues. In each endocrine tissue along the pathway, a stimulus from the level above is received, amplified, and transduced into release of the next hormone in the cascade. The cascade is sensitive to regulation at several levels through feedback inhibition (thin, dashed arrows) by the ultimate hormone (in this case, cortisol). The product therefore regulates its own production, as in feedback inhibition of biosynthetic pathways within a single cell.

nanograms of corticotropin-releasing hormone, which elicits the release of a few *micrograms* of corticotropin. Corticotropin acts on the adrenal cortex to cause the release of *milligrams* of cortisol, for an overall amplification of at least a millionfold.

At each level of a hormonal cascade, feedback inhibition of earlier steps in the cascade is possible; an unnecessarily elevated level of the ultimate hormone or of an intermediate hormone inhibits the release of earlier hormones in the cascade. These feedback mechanisms accomplish the same end as those that limit the output of a biosynthetic pathway (compare Fig. 23-6 with Fig. 22-37): a product is synthesized (or released) only until the necessary concentration is reached. In the blood-clotting cascade (Fig. 6-44) we saw a similar pattern: a signal stimulates a cascade of protein activations; feedback mechanisms limit their action and the duration of the response.

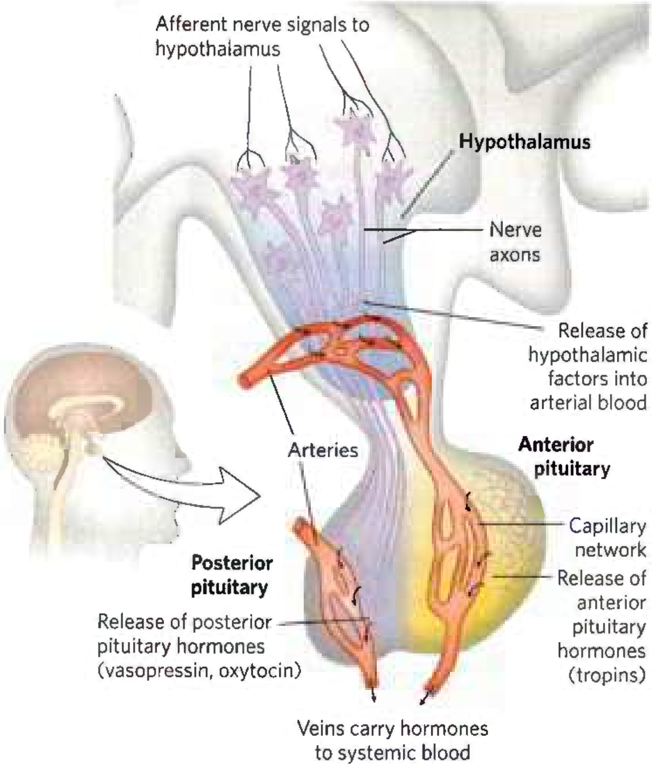

Afferent nerve signals to hypothalamus
Hypothalamus
Nerve axons
Release of hypothalamic factors into arterial blood
Arteries
Anterior pituitary
Capillary network
Release of anterior pituitary hormones (tropins)
Posterior pituitary
Release of posterior pituitary hormones (vasopressin, oxytocin)
Veins carry hormones to systemic blood

FIGURE 23-7 Neuroendocrine origins of hormone signals. Location of the hypothalamus and pituitary gland and details of the hypothalamus-pituitary system. Signals from connecting neurons stimulate the hypothalamus to secrete releasing factors into a blood vessel that carries the hormones directly to a capillary network in the anterior pituitary. In response to each releasing factor, the anterior pituitary releases the appropriate hormone into the general circulation. Posterior pituitary hormones are synthesized in neurons arising in the hypothalamus; the hormones are transported along axons to nerve endings in the posterior pituitary and stored there until released into the blood in response to a neuronal signal.

"Bottom-Up" Hormonal Systems Send Signals Back to the Brain and to Other Tissues

In addition to the top-down hierarchy of hormonal signaling shown in Figure 23-6, P1 some hormones are produced in the digestive tract, muscle, and adipose tissue and communicate the current metabolic state to the hypothalamus (**Fig. 23-8**). These signals are integrated in the hypothalamus, and an appropriate neuronal or hormonal response is elicited.

Adipokines, for example, are peptide hormones, produced *in adipose tissue*, that signal the adequacy of fat reserves. The adipokine **leptin**, released when adipose tissue is well-filled with triacylglycerols, acts in the brain to inhibit feeding behavior, whereas **adiponectin** signals depletion of fat reserves and stimulates feeding. Other tissues produce and release other hormones. **Ghrelin** is produced in the gastrointestinal tract when the stomach is empty and acts in the hypothalamus to stimulate feeding behavior; when the stomach fills, ghrelin release ceases. **Incretins** are peptide hormones produced in the gut after ingestion of a meal; they

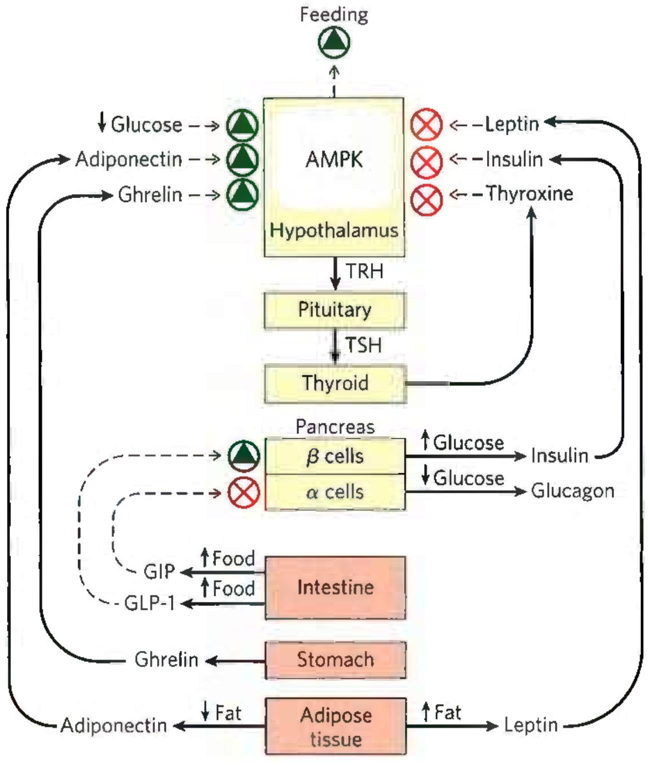


FIGURE 23-8 Regulation of feeding behavior by two-way information flow between tissues and the hypothalamus. When food intake and energy production are adequate, peptide hormones released by the stomach, intestine, and adipose tissue feed back on the hypothalamus to signal satiety and reduce feeding behavior. Other tissue-specific peptide hormones signal inadequate supplies of stored triacylglycerols or low blood glucose levels. All of these signals impinge, directly or indirectly, on AMP-activated protein kinase (AMPK) in the hypothalamus, which integrates these signals and influences feeding behavior and energy-yielding metabolism in the tissues. Nerves carry electrical signals from the brain to the other tissues to complete the information circuit and achieve homeostasis (not shown). TRH, thyrotropin-releasing hormone; GLP-1, glucagon-like peptide-1; GIP, gastric inhibitory polypeptide.

increase secretion of insulin and decrease secretion of glucagon from the pancreas. **Neuropeptide Y (NPY)** is a hormone produced in the hypothalamus and in the adrenal glands. Its release promotes feeding and reduces energy expenditure for nonessential activities. **Peptide YY (PYY_{3-36})**, produced in the intestine, signals satiety in the brain. **Irisin** is a peptide hormone produced in muscle as a result of exercise; it acts to convert white adipose tissue into beige adipose tissue, which dissipates energy as heat (see Section 23.2).

We return to these hormones (summarized in Table 23-2) when we discuss the regulation of body mass in humans (Section 23.4).

SUMMARY 23.1 *Hormone Structure and Action*

- Hormones connect all of the organs in the body, carrying information and signals between the central nervous system and all of the tissues.
- Hormones are chemically diverse with a wide range of biological roles. Peptide hormones are synthesized as proteins on ribosomes, then cleaved proteolytically to form the active peptide(s).
- Hormones are regulated by a top-down hierarchy of interactions between the brain and endocrine glands: nerve impulses stimulate the hypothalamus to send specific hormones to the pituitary gland, thus stimulating (or inhibiting) the release of a second rank of hormones. The anterior pituitary hormones in turn stimulate other endocrine glands (adrenals, for example) to secrete their characteristic hormones, which in turn stimulate specific target tissues.
- Some hormones act in bottom-up signaling: adipose tissue, muscle, and the gastrointestinal tract release peptide hormones that act on other tissues or in the central nervous system.

TABLE 23-2 Some Peptide Hormones That Act on Feeding Behavior and Fuel Selection in Mammals

Hormone	Production site(s)	Target tissue(s)	Action(s)
Insulin	Pancreatic β cells	Muscle, adipose, liver	Stimulates glucose uptake and synthesis of glycogen and fat
Glucagon	Pancreatic α cells	Liver, adipose	Stimulates gluconeogenesis and glucose release to blood
Leptin	Adipose tissue	Hypothalamus	Reduces hunger
Adiponectin	Adipose tissue	Muscle, liver, others	Stimulates catabolism and feeding behavior
Ghrelin	Stomach, intestine	Brain	Signals hunger
Incretins: GLP-1, GIP	Intestine	Pancreas	Stimulate insulin release
NPY	Hypothalamus, adrenals	Brain, autonomic nervous system	Stimulates feeding behavior
PYY_{3-36}	Intestine	Brain	Signals satiety
Irisin	Muscle (after exercise)	Adipose	Turns white adipose tissue to beige

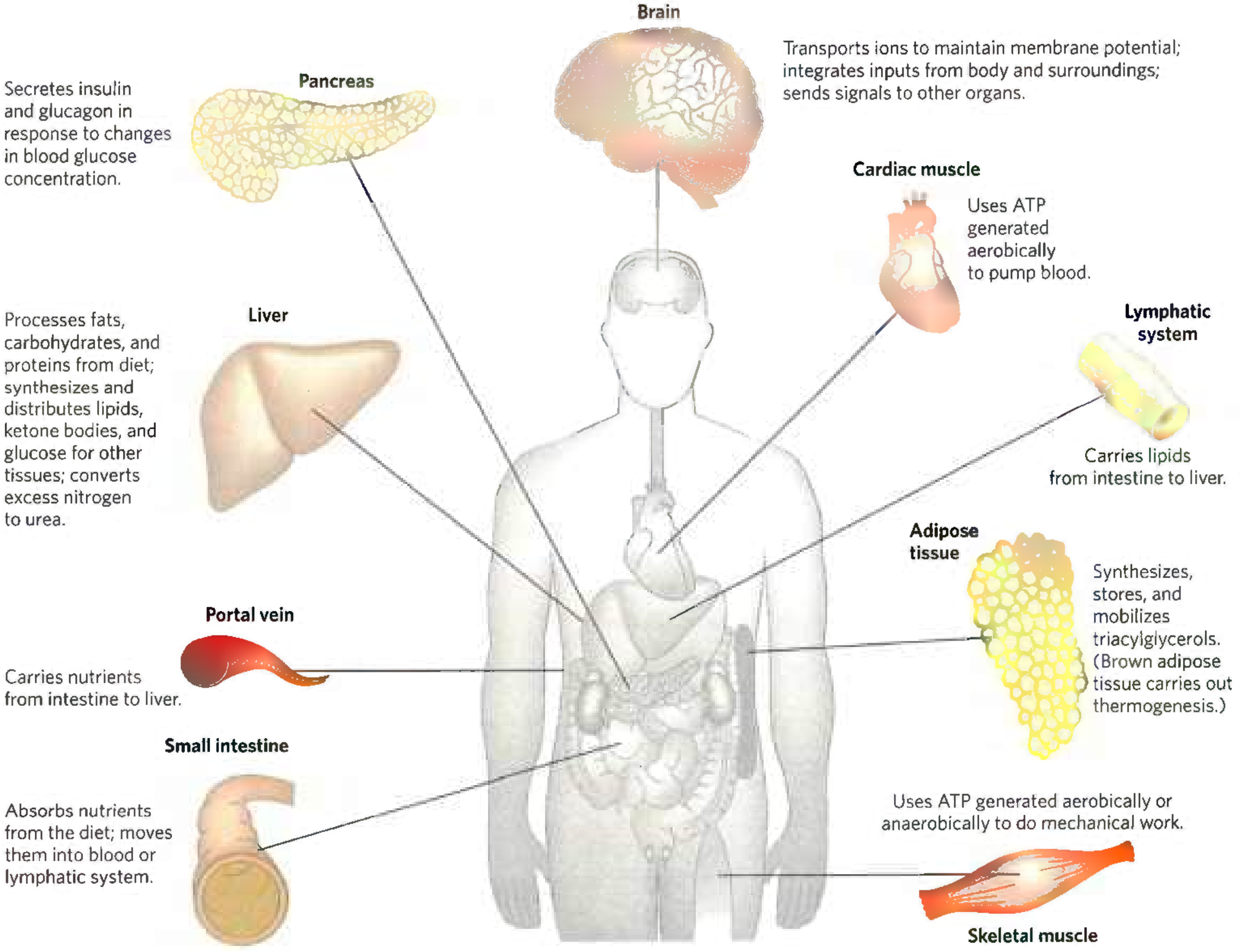
23.2 Tissue-Specific Metabolism

Each tissue of the human body has a specialized function, reflected in its anatomy and metabolic activity (**Fig. 23-9**). Skeletal muscle allows directed motion; adipose tissue stores and distributes energy in the form of fats, which serve as fuel throughout the body and as thermal insulation; in the brain, cells pump ions across their plasma membranes to produce electrical signals. The liver plays a central processing and distribution role in metabolism and furnishes all other organs and tissues with an appropriate mix of nutrients via the bloodstream. The functional centrality of the liver is indicated by the common reference to all other tissues and organs as "extrahepatic." We therefore begin our discussion of the division of metabolic labor by considering the transformations of carbohydrates, amino acids, and fats in the mammalian liver. This is followed by brief descriptions of the primary metabolic functions of adipose tissue, muscle, brain, and the tissue that interconnects all others: the blood.

The Liver Processes and Distributes Nutrients

During digestion in mammals, the three main classes of nutrients (carbohydrates, fats, and proteins) undergo enzymatic hydrolysis into their simple constituents (monosaccharides, fatty acids, and amino acids). This breakdown is necessary because the epithelial cells lining the intestinal lumen absorb only relatively small molecules. Many of the fatty acids and monoacylglycerols released by digestion of fats in the intestine are reassembled within these epithelial cells into triacylglycerols (TAGs).

After being absorbed, most sugars and amino acids and some reconstituted TAGs pass from intestinal epithelial cells into blood capillaries and travel in the bloodstream to the liver; the remaining TAGs enter adipose tissue via the lymphatic system. The portal vein (Fig. 23-9) is a direct route from the digestive organs to the liver, and the liver therefore has first access to ingested nutrients. The liver has two main cell types.



Transports ions to maintain membrane potential; integrates inputs from body and surroundings; sends signals to other organs.

Secretes insulin and glucagon in response to changes in blood glucose concentration.

Uses ATP generated aerobically to pump blood.

Processes fats, carbohydrates, and proteins from diet; synthesizes and distributes lipids, ketone bodies, and glucose for other tissues; converts excess nitrogen to urea.

Carries lipids from intestine to liver.

Synthesizes, stores, and mobilizes triacylglycerols. (Brown adipose tissue carries out thermogenesis.)

Carries nutrients from intestine to liver.

Absorbs nutrients from the diet; moves them into blood or lymphatic system.

Uses ATP generated aerobically or anaerobically to do mechanical work.

FIGURE 23-9 Specialized metabolic functions of mammalian tissues.

Kupffer cells are phagocytes, important in immune function. **Hepatocytes**, of primary interest here, transform dietary nutrients into the fuels and precursors required by other tissues and export them via the blood. The kinds and amounts of nutrients supplied to the liver are determined by the composition of the diet, the interval between meals, and several other factors. The demand of extrahepatic tissues for fuels and precursors varies from one organ to another, and it also varies with the level of activity and overall nutritional state of the individual.

To meet these changing circumstances, the liver has remarkable metabolic flexibility. For example, when the diet is rich in protein, hepatocytes synthesize more of the enzymes needed for amino acid catabolism and gluconeogenesis. Within hours after a shift to a high-carbohydrate diet, the levels of these enzymes begin to drop and the hepatocytes increase their synthesis of enzymes essential to carbohydrate metabolism and fat synthesis. Liver enzymes turn over (that is, are synthesized and degraded) at 5 to 10 times the rate of enzyme turnover in other tissues, such as muscle. Extrahepatic tissues also can adjust their metabolism to prevailing conditions, but none of these tissues is as adaptable as the liver, and none is so central to the organism's overall metabolism.

We turn now to a survey of the possible fates of sugars, amino acids, and lipids that enter the liver from the bloodstream.

Carbohydrates The glucose transporter of hepatocytes (GLUT2) allows rapid, passive diffusion of glucose, so that the concentration of glucose in a hepatocyte is essentially the same as that in the blood. Glucose entering hepatocytes is phosphorylated by glucokinase (hexokinase IV) to yield glucose 6-phosphate. Glucokinase has a much higher K_m for glucose (10 mM) than do the hexokinase isozymes in other cells (p. 539) and, unlike these other isozymes, it is not inhibited by its product, glucose 6-phosphate. The presence of glucokinase allows hepatocytes to continue phosphorylating glucose when the glucose concentration rises well above levels that would overwhelm other hexokinases. The high K_m of glucokinase also ensures that the phosphorylation of glucose in hepatocytes is minimal when the glucose concentration is low, preventing the liver from consuming glucose as fuel via glycolysis. This spares glucose for other tissues. Fructose, galactose, and mannose, all absorbed from the small intestine, are also converted to glucose 6-phosphate by enzymatic pathways examined in Chapter 14 (see Fig. 14-9). Glucose 6-phosphate is at the crossroads of carbohydrate metabolism in the liver. It may take any of several major metabolic routes (**Fig. 23-10**), depending on the current metabolic needs of the organism. By the action of various allosterically regulated enzymes, and through hormonal regulation of enzyme synthesis and activity, the liver directs the flow of glucose into one or more of these pathways.

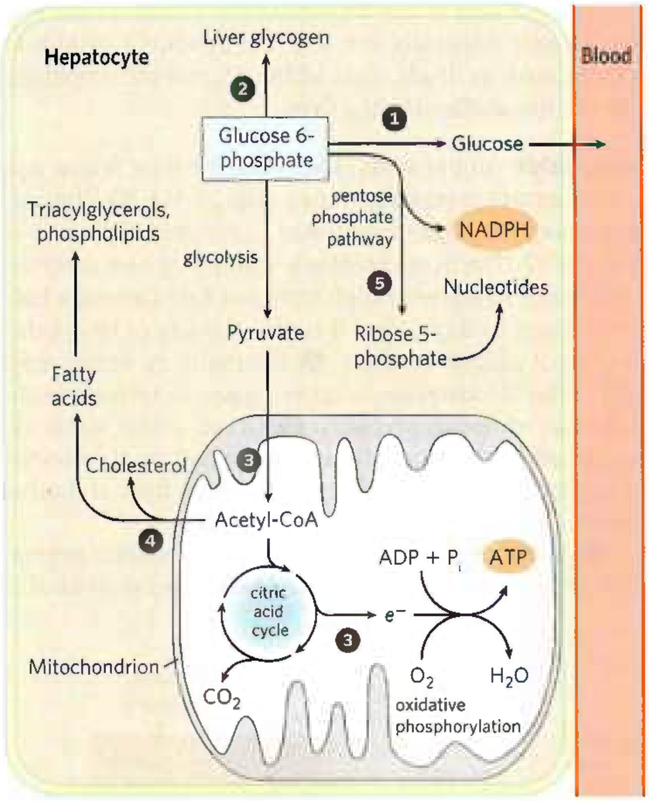


FIGURE 23-10 Metabolic pathways for glucose 6-phosphate in the liver. Here and in Figures 23-11 and 23-12, anabolic pathways are generally shown leading upward, catabolic pathways leading downward, and distribution to other organs leading horizontally. The numbered processes in each figure are described in the text.

① Glucose 6-phosphate is dephosphorylated by glucose 6-phosphatase to yield free glucose (see Fig. 15-6), which is exported to replenish blood glucose. Export is the predominant pathway when glucose 6-phosphate is in limited supply, because the blood glucose concentration must be kept sufficiently high (4 to 5 mM) to provide adequate energy for the brain and other tissues. ② Glucose 6-phosphate not immediately needed to form blood glucose is converted to liver glycogen, or has one of several other fates. Following glycolysis and the pyruvate dehydrogenase reaction, ③ the acetyl-CoA so formed can be oxidized for ATP production by the citric acid cycle, with ensuing electron transfer and oxidative phosphorylation yielding ATP. (Normally, however, fatty acids are the preferred fuel for ATP production in hepatocytes.) ④ Acetyl-CoA can also serve as the precursor of fatty acids, which are incorporated into TAGs and phospholipids, and of cholesterol. Much of the lipid synthesized in the liver is transported to other tissues by blood lipoproteins. ⑤ Alternatively, glucose 6-phosphate can enter the pentose phosphate pathway, yielding both reducing power (NADPH), needed for the biosynthesis of fatty acids and cholesterol, and ribose 5-phosphate, a precursor for nucleotide biosynthesis. NADPH is also an essential cofactor in the detoxification and elimination of many drugs and other **xenobiotics** (compounds that don't occur naturally but are the products of human activity, such as drugs, food additives, and preservatives) that are metabolized in the liver.

Amino Acids Amino acids that enter the liver follow several important metabolic routes (**Fig. 23-11**). ① They are precursors for protein synthesis, a process discussed in Chapter 27. The liver constantly renews its own proteins, which have a relatively high turnover rate (average half-life of hours to days), and it is also the site of biosynthesis of most plasma proteins. ② Alternatively, amino acids pass in the bloodstream to other organs to be used in the synthesis of tissue proteins. ③ Other amino acids are precursors in the biosynthesis of nucleotides, hormones, and other nitrogenous compounds in the liver and other tissues.

(4a) Amino acids not needed as biosynthetic precursors are transaminated or deaminated and degraded to yield pyruvate and citric acid cycle intermediates, with various fates; (4b) the ammonia released is converted to the excretory product urea. ⑤ Pyruvate can be converted to glucose and glycogen via gluconeogenesis, or ⑥ can be converted to acetyl-CoA, which has several possible fates: ⑦ oxidation via the citric acid cycle and ⑧ oxidative phosphorylation to produce ATP, or ⑨ conversion to lipids for storage. ⑩ Citric acid cycle intermediates can be siphoned off into glucose synthesis by gluconeogenesis.

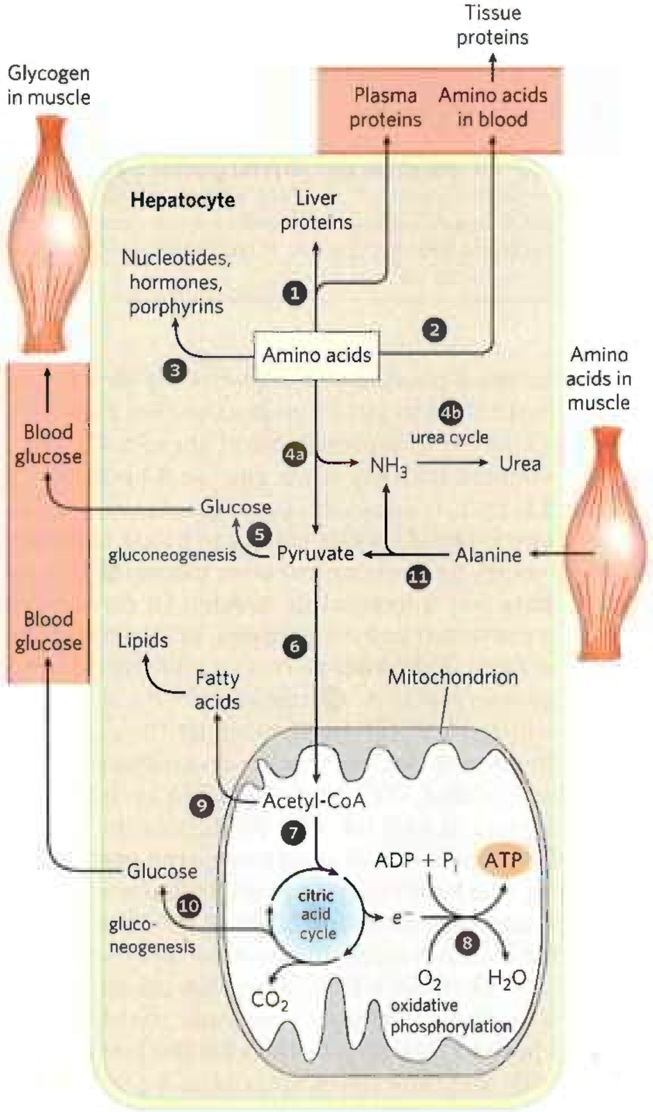


FIGURE 23-11 **Metabolism of amino acids in the liver.**

P2 The liver also metabolizes amino acids that arrive intermittently from other tissues. The blood is adequately supplied with glucose just after the digestion and absorption of dietary carbohydrate or, between meals, by the conversion of liver glycogen to blood glucose. During the interval between meals, especially if prolonged, some muscle protein is degraded to amino acids. These amino acids donate their amino groups (by transamination) to pyruvate, the product of glycolysis, to yield alanine, which ⑪ is transported to the liver and deaminated. Hepatocytes convert the resulting pyruvate to blood glucose via gluconeogenesis ⑤, and the ammonia to urea for excretion (4b). One benefit of this glucose-alanine cycle is the smoothing out of fluctuations in blood glucose between meals. The amino acid deficit incurred in muscles is made up after the next meal by incoming dietary amino acids.

Lipids The fatty acid components of lipids entering hepatocytes also have several different fates (**Fig. 23-12**). ① Some are converted to liver lipids. ② Under most circumstances, fatty acids are the primary oxidative fuel in the liver. Free fatty acids may be activated and oxidized to yield acetyl-CoA and NADH. ③ The acetyl-CoA is further oxidized via the citric acid cycle, and ④ oxidations in the cycle drive the synthesis of ATP by oxidative phosphorylation. ⑤ Excess acetyl-CoA, not required by the liver, is converted to acetoacetate and β-hydroxybutyrate; these ketone bodies circulate in the blood to other tissues to be used as fuel for the citric acid cycle. Ketone bodies, unlike fatty acids, can cross the blood-brain barrier, providing the brain with a source of acetyl-CoA for energy-yielding oxidation. Ketone bodies can supply a significant fraction of the energy in some extrahepatic tissues — up to one-third in the heart and as much as 60% to 70% in the brain during prolonged fasting. ⑥ Some of the acetyl-CoA derived from fatty acids (and from glucose) is used for the biosynthesis of cholesterol, which is required for membrane synthesis. Cholesterol is also the precursor of all steroid hormones and of the bile salts, which are essential for the digestion and absorption of lipids.

The other two metabolic fates of lipids require specialized mechanisms for the transport of insoluble lipids in blood. ⑦ Fatty acids are converted to the phospholipids and TAGs of plasma lipoproteins, which carry lipids to adipose tissue for storage. ⑧ Some free fatty acids

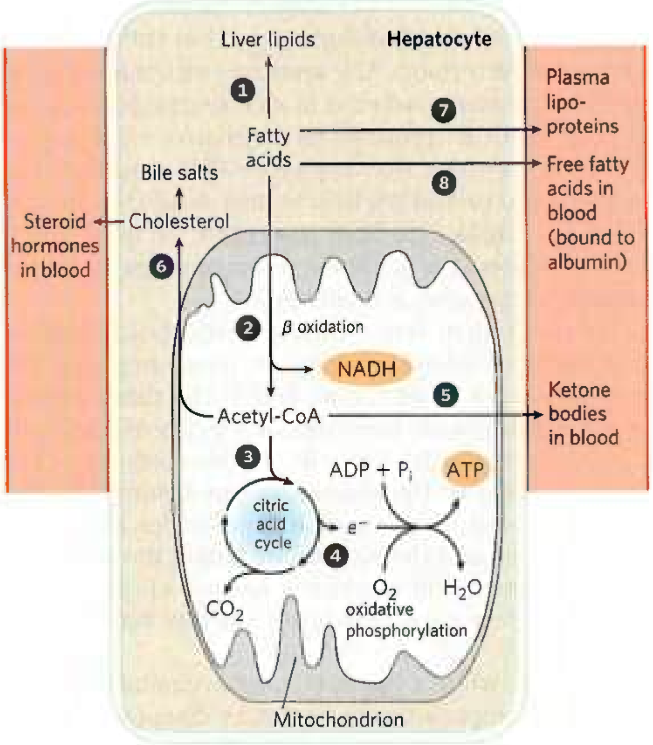
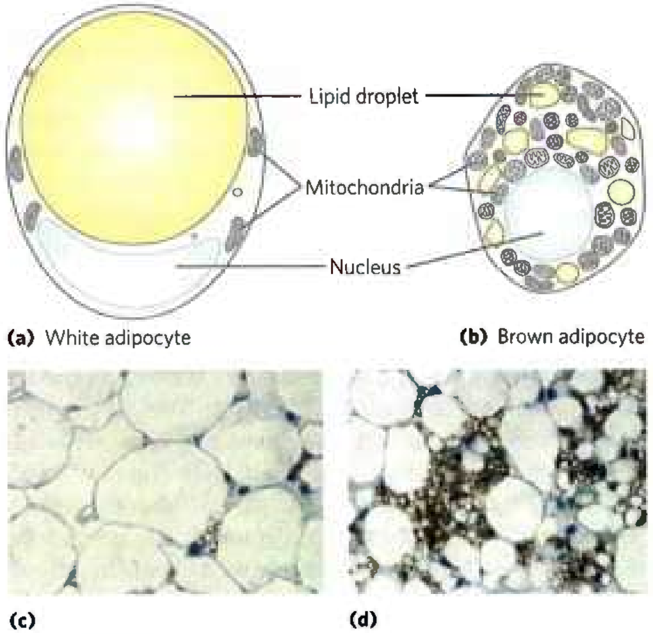


FIGURE 23-12 **Metabolism of fatty acids in the liver.**



(a) White adipocyte **(b)** Brown adipocyte

FIGURE 23-13 **White and brown adipose tissue.** Schematic views of typical mouse adipocytes from (a) white adipose tissue (WAT) and (b) brown adipose tissue (BAT). White adipocytes are larger and contain a single huge lipid droplet, which squeezes the mitochondria and nucleus against the plasma membrane. In brown adipocytes, mitochondria are much more prominent, the nucleus is near the center of the cell, and multiple small fat droplets are present. Below are light micrographs of (c) adipocytes in white adipose tissue, stained to show nuclei, and (d) a region of mixed white and brown adipocytes, stained with an antibody specific to UCP1, the uncoupling protein responsible for thermogenesis. [(c, d) Courtesy of Dr. Patrick Seale.]

(FFAs) are bound to serum albumin and carried to the heart and skeletal muscles, which take up and oxidize FFAs as a major fuel. Serum albumin is the most abundant plasma protein; one molecule can carry up to seven FFA molecules (see Fig. 17-3).

The liver thus serves as the body's distribution center, exporting nutrients in the correct proportions to other organs, smoothing out fluctuations in metabolism caused by intermittent food intake, and processing excess amino groups into urea and other products to be disposed of by the kidneys. Certain nutrients are stored in the liver, including iron ions and vitamin A. The liver also detoxifies xenobiotics. Detoxification often includes the cytochrome P-450–dependent hydroxylation of relatively insoluble organic compounds, making them sufficiently soluble for further breakdown and excretion (see Box 21-1).

Adipose Tissues Store and Supply Fatty Acids

There are two primary types of adipose tissue, white and brown (**Fig. 23-13**), with different roles, and we focus first on the more abundant of the two. **White adipose tissue (WAT)** is amorphous and widely distributed in the body: under the skin, around deep blood vessels, and in the abdominal cavity. The **adipocytes** of white adipose tissue are large (diameter 30 to 70 μm), spherical cells, completely filled with a single large lipid droplet that constitutes about 65% of the cell mass and squeezes the mitochondria and nucleus into a thin layer against the plasma membrane (Fig. 23-13a, c). The lipid droplet contains TAGs and sterol esters and is coated with a monolayer of phospholipids, oriented with their polar head groups facing the cytosol. Specific proteins are associated with the surface of the droplets, including perilipin and the enzymes for synthesis and breakdown of TAGs (see Fig. 17-2). White adipose tissue typically makes up about 15% of the mass of a healthy young adult human. Adipocytes are metabolically active, responding quickly to hormonal stimuli in a metabolic interplay with the liver, skeletal muscles, and heart.

Adipocytes have an active glycolytic metabolism, oxidize pyruvate and fatty acids via the citric acid cycle, and carry out oxidative phosphorylation. During periods of high carbohydrate intake, adipose tissue can convert glucose (via pyruvate and acetyl-CoA) to fatty acids, convert the fatty acids to TAGs, and store the TAGs as large lipid droplets—although in humans, much of the fatty acid synthesis occurs in hepatocytes. Adipocytes store TAGs arriving from the liver (carried in the blood as very-low-density lipoproteins) and from the intestinal tract (carried in chylomicrons), particularly after meals rich in fat.

When the demand for fuel rises (between meals, for example), lipases in adipocytes hydrolyze stored TAGs to release FFAs, which can travel in the bloodstream to skeletal muscle, the heart, and, during starvation, the liver. The release of fatty acids from adipocytes is greatly accelerated by epinephrine, which stimulates the cAMP-dependent phosphorylation of perilipin and thus gives lipases that are specific for tri-, di-, and monoacylglycerols access to TAGs in lipid droplets (see Fig. 17-2). Hormone-sensitive lipase is also stimulated by phosphorylation. Insulin counterbalances this effect of epinephrine, decreasing the activity of the lipase.

The breakdown and synthesis of TAGs in adipose tissue constitute a substrate cycle; up to 70% of the fatty acids released by the three lipases are reesterified in adipocytes, re-forming TAGs. Each cycle consumes ATP (used to activate the fatty acids as acyl-CoA esters), so the net effect of the substrate cycling is the breakdown of ATP and the accompanying release of heat. In adipose tissue, glycerol liberated by adipocyte lipases cannot be reused in the synthesis of TAGs, because adipocytes lack glycerol kinase. Instead, the glycerol phosphate required for TAG synthesis is made from pyruvate by glyceroneogenesis, requiring the action of the cytosolic PEP carboxykinase (see Fig. 21-22).

In addition to its central function as a fuel depot, adipose tissue plays an important role as an endocrine organ, producing and releasing hormones that signal the state of energy reserves and coordinate metabolism of fats and carbohydrates throughout the body. We return to this function in Section 23.4 when we discuss the hormonal regulation of body mass.

Brown and Beige Adipose Tissues Are Thermogenic

In small vertebrates and hibernating animals, a significant proportion of the adipose tissue is **brown adipose tissue (BAT)**, distinguished from white adipose tissue by its smaller (diameter 20 to 40 μm), differently shaped (polygonal, not round) adipocytes (Fig. 23-13b, d). Like white adipocytes, brown adipocytes store TAGs, but in several smaller lipid droplets per cell rather than as a single central droplet. Brown adipocytes have more mitochondria and a richer supply of capillaries and innervation than white adipocytes; it is the cytochromes of mitochondria and the hemoglobin in capillaries that give brown adipose tissue its characteristic color. A unique feature of brown adipocytes is their production of **uncoupling protein 1 (UCP1)**, also called thermogenin (see Fig. 19-36). This protein is responsible for one of the principal functions of brown adipose tissue: **thermogenesis**.

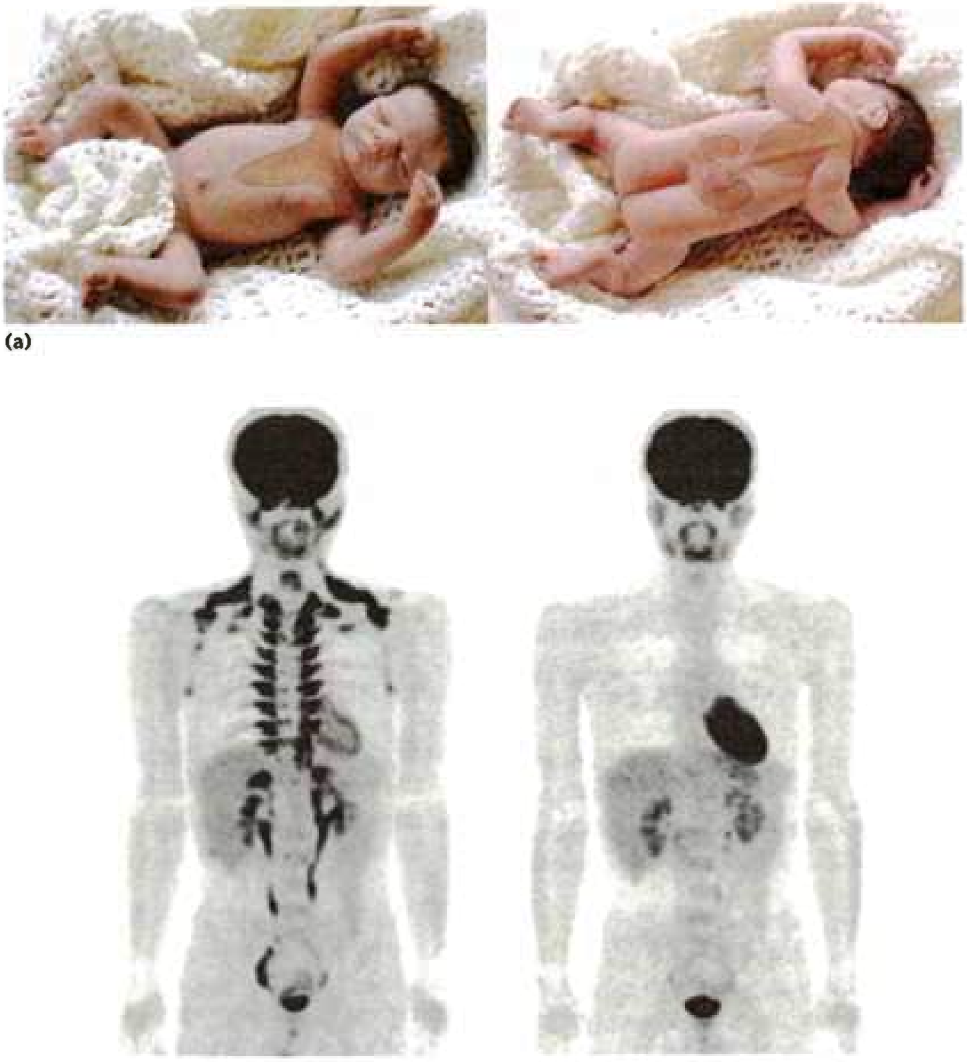
In brown adipocytes, fatty acids stored in lipid droplets are released, enter mitochondria, and undergo complete conversion to CO_2 by β oxidation and the citric acid cycle. The reduced $FADH_2$ and NADH so generated pass their electrons through the respiratory chain to molecular oxygen. In white adipocytes, protons pumped out of the mitochondria during electron transfer reenter the matrix through ATP synthase, with the energy of electron transfer conserved in ATP synthesis. In brown adipocytes, UCP1 provides an alternative route for the reentry of protons that bypasses ATP synthase. The energy of the proton gradient is thus dissipated as heat, which can maintain the body (especially the nervous system and viscera) at its optimal temperature when the ambient temperature is relatively low.

In the human fetus, differentiation of fibroblast preadipocytes into brown adipose tissue begins at the twentieth week of gestation, and at the time of birth, brown adipose tissue represents 1% to 5% of total body mass. The brown fat deposits are located where the heat generated by thermogenesis can ensure that vital tissues — blood vessels to the head, major abdominal blood vessels, and the viscera, including the pancreas, adrenal glands, and kidneys — are not chilled as the newborn enters a world of lower ambient temperature (**Fig. 23-14a**).

At birth, white adipose tissue development begins and brown adipose tissue begins to disappear. Young adult humans have much-diminished deposits of brown adipose tissue, ranging from 3% of all adipose tissue in males to 7% in females, making up less than 0.1% of body mass. However, adults have, distributed among their white adipose cells, significant numbers of adipocytes that can be converted by cold exposure or by β-adrenergic stimulation into cells very similar to brown adipocytes. These **beige adipocytes** have multiple lipid droplets, are richer in mitochondria than white adipocytes, and produce UCP1, so they function effectively as heat generators. Brown and beige adipocytes produce heat by oxidation of their own fatty acids, but they also take up and oxidize both fatty acids and glucose from the blood at rates out of proportion to their mass. In fact, the detection of brown adipose tissue by PET scanning depends on the adipocytes' relatively high rate of *glucose* uptake and metabolism (Fig. 23-14b). In adaptation to warm or cold surroundings, and in the normal differentiation of white, brown, and beige adipose tissue, the nuclear transcription factor PPARγ (described later in the chapter) plays a central role. And as we noted above, the peptide hormone irisin, produced in muscle by exercise, triggers the development of beige adipose tissue that continues to burn fuel long after the exercise ends.

Muscles Use ATP for Mechanical Work

Metabolism in skeletal muscle cells — **myocytes** — is specialized to generate ATP as the immediate source of energy for contraction. Moreover, skeletal muscle is adapted to do its mechanical work intermittently, on demand. Sometimes skeletal muscles must work at their maximum capacity for a short time, as in a 100 m sprint;



(b) After cold exposure   Room-temperature control

FIGURE 23-14 **Brown adipose tissue in infants and adults.** (a) At birth, human infants have brown fat distributed as shown here, to protect the spine, major blood vessels, and internal organs. (b) Positron emission tomography (PET) scanning can show metabolic activity in a living person, in real time. PET scans allow visualization of isotopically labeled glucose in precisely localized regions of the body. A positron-emitting glucose analog, 2-[^{18}F]-fluoro-2-deoxy-D-glucose (FDG), is injected into the bloodstream; a short time later, a PET scan shows how much of the glucose has been taken up by each part of the body—a measure of metabolic activity. On the left is a PET scan of a healthy 25-year-old man who fasted for 12 hours, then stayed for 1 hour in a cold (19 °C) room, with his legs on ice to thoroughly chill him. At the end of the hour, he was injected with [^{18}F]-FDG, then remained under cold conditions for another hour. Whole-body PET scans were then done at 24 °C. For the control scan, the same man underwent the same PET scan protocol two weeks later, but this time following 2 hours at 27 °C instead of chilling (right). Intense labeling of the brain and heart shows high rates of glucose uptake; labeling of the kidneys and bladder indicates clearance of FDG. In the scan after chilling (left), [^{18}F]-FDG labels brown adipose tissue in the region above the collarbone and along the vertebrae. [(a) Adam Steinberg. (b) Reprinted with permission of American Diabetes Association, from M. Saito et al., *Diabetes* 58:1526, 2009, Fig. 1; permission conveyed through Copyright Clearance Center, Inc.]

at other times more prolonged work is required, as in running a marathon or in prolonged physical labor.

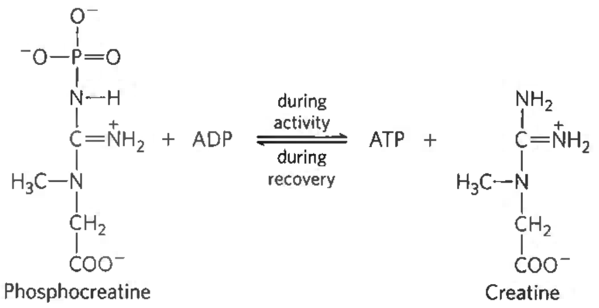
There are two general classes of muscle tissue, which differ in physiological role and fuel utilization. **Slow-twitch muscle**, also called red muscle, provides relatively low tension but is highly resistant to fatigue. It produces ATP by the relatively slow but steady process of oxidative phosphorylation. Red muscle is very rich in mitochondria and is served by dense networks of blood vessels, which bring the oxygen essential to ATP production. **Fast-twitch muscle**, or white muscle, has fewer mitochondria than red muscle and is less well supplied with blood vessels, but it can develop greater tension and do so faster. White muscle is quicker to fatigue because, when active, it uses ATP faster than it can replace it. There is a genetic component to the proportion of red and white muscle in any individual, but with training, an athlete can improve the endurance of fast-twitch muscle.

Skeletal muscle can use free fatty acids or glucose as fuel, depending on the degree of muscular activity

(**Fig. 23-15**). In resting muscle, the primary fuels are FFAs from adipose tissue. These are oxidized and degraded to yield acetyl-CoA, which enters the citric acid cycle, ultimately yielding the energy for ATP synthesis by oxidative phosphorylation. Muscle in light activity uses blood glucose in addition to fatty acids. The glucose is phosphorylated, then degraded by glycolysis to pyruvate, which is converted to acetyl-CoA and oxidized via the citric acid cycle and oxidative phosphorylation.

In maximally active fast-twitch muscles, the demand for ATP is so great that the blood flow cannot provide O_2 and fuels fast enough to supply sufficient ATP by aerobic respiration alone. Under these conditions, stored muscle glycogen is broken down to lactate by fermentation (p. 525). Each glucose unit degraded yields three ATP, because phosphorolysis of glycogen produces glucose 6-phosphate (via glucose 1-phosphate), sparing the ATP normally consumed in the hexokinase reaction. Lactic acid fermentation thus responds more quickly than oxidative phosphorylation to an increased need for ATP, supplementing basal ATP production by aerobic oxidation of other fuels via the citric acid cycle and respiratory chain. The secretion of epinephrine, which stimulates both the release of glucose from liver glycogen and the breakdown of glycogen in muscle tissue, greatly enhances the use of blood glucose and muscle glycogen as fuels for muscular activity. (Epinephrine mediates the so-called fight-or-flight response, discussed more fully below.)

The relatively small amount of glycogen (about 1% of the total weight of skeletal muscle) limits the glycolytic energy available during all-out exertion. Moreover, the accumulation of lactate and consequent decrease in pH in maximally active muscles reduces their efficiency. Skeletal muscle, however, contains another source of ATP, phosphocreatine (10 to 30 mM), which can rapidly regenerate ATP from ADP by the creatine kinase reaction:



During periods of active contraction and glycolysis, this reaction proceeds predominantly in the direction of ATP synthesis; during recovery from exertion, the same enzyme resynthesizes phosphocreatine from creatine and ATP. Because of the relatively high levels of ATP and phosphocreatine in muscle, these compounds can be detected in intact muscle, in real time, by NMR spectroscopy (**Fig. 23-16**). Creatine serves to shuttle ATP equivalents from the mitochondrion to sites of ATP consumption and can be the limiting factor in the development of new muscle tissue (**Box 23-1**).

After a period of intense muscular activity, the individual continues breathing heavily for some time, using much of the extra O_2 for oxidative phosphorylation in the liver. The ATP produced is used for gluconeogenesis (in the liver) from lactate that has been carried in the blood from the muscles. The glucose thus formed returns to the muscles to replenish their glycogen, completing the Cori cycle (**Fig. 23-17**; see also Box 15-1).

Actively contracting skeletal muscle generates heat as a byproduct of imperfect coupling of the chemical

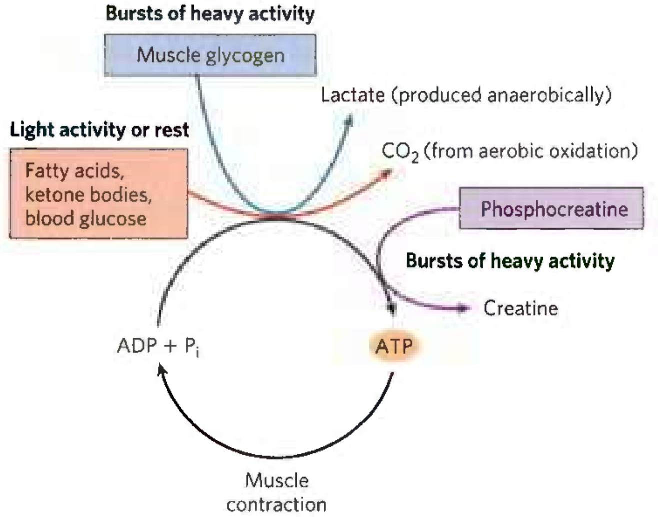


FIGURE 23-15 Energy sources for muscle contraction. Different fuels are used for ATP synthesis during bursts of heavy activity and during light activity or rest. Phosphocreatine can rapidly supply ATP.

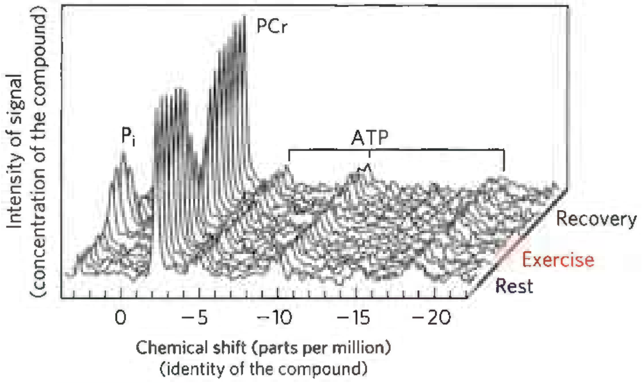


FIGURE 23-16 Phosphocreatine buffers ATP concentration during exercise. A "stack plot" of magnetic resonance spectra (of ^{31}P) shows inorganic phosphate (P_i), phosphocreatine (PCr), and ATP (each of its three phosphates giving a signal). The series of plots represents the passage of time, from a period of rest to one of exercise, and then of recovery. Notice that the ATP signal barely changes during exercise, kept high by continued respiration and by the reservoir of phosphocreatine, which diminishes during exercise. During recovery, when ATP production by catabolism is greater than ATP use by the (now resting) muscle, the phosphocreatine reservoir is refilled. [Data from M. L. Blei, K. E. Conley, and M. J. Kushmerick, *J. Physiol.* 465:203, 1993, Fig. 4.]

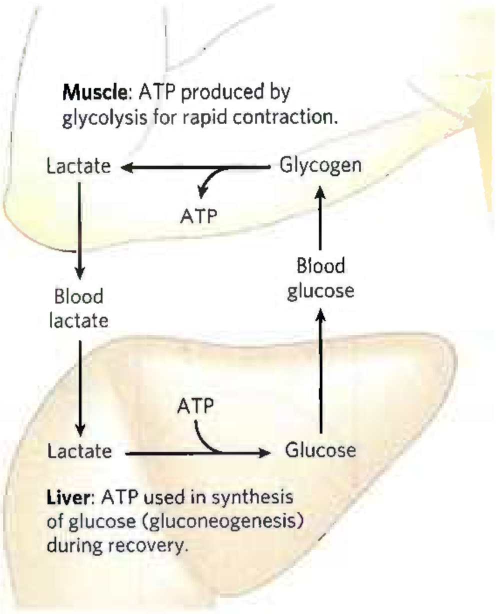

Muscle: ATP produced by glycolysis for rapid contraction.
Lactate
Glycogen
ATP
Blood glucose
Blood lactate
ATP
Lactate
Glucose
Liver: ATP used in synthesis of glucose (gluconeogenesis) during recovery.

FIGURE 23-17 Metabolic cooperation between skeletal muscle and the liver: the Cori cycle. Extremely active muscles use glycogen as their energy source, generating lactate by glycolysis. During recovery, some of the lactate is transported to the liver and converted to glucose by gluconeogenesis. The glucose is released to the blood and returned to the muscles to replenish their glycogen stores. The overall pathway, glucose ⟶ lactate ⟶ glucose, constitutes the Cori cycle.

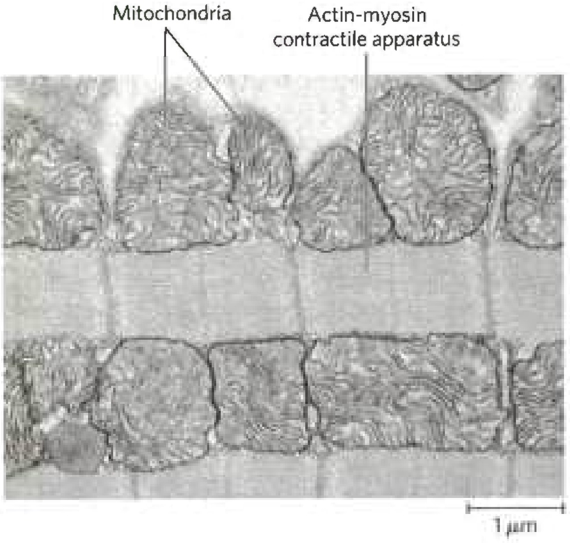

Mitochondria
Actin-myosin contractile apparatus
1 μm

FIGURE 23-18 Electron micrograph of heart muscle. In the profuse mitochondria of heart tissue, pyruvate (from glucose), fatty acids, and ketone bodies are oxidized to drive ATP synthesis. This steady aerobic metabolism allows the human heart to pump blood at a rate of nearly 6 L/min, or about 350 L/h—which amounts to 200 × 10^6 L of blood over 70 years. [D. W. Fawcett/Science Source.]

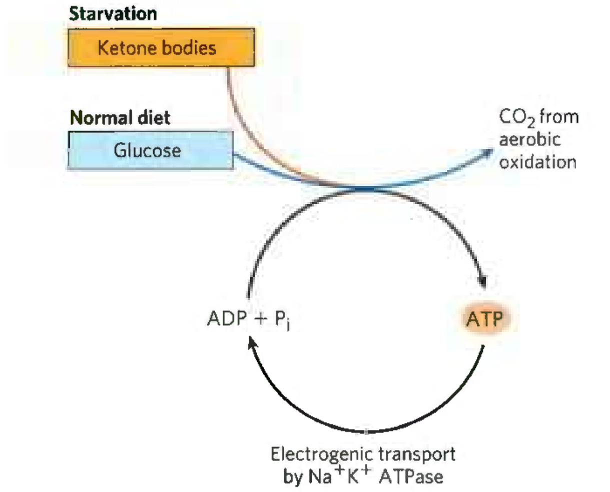

Starvation
Ketone bodies
Normal diet
Glucose
CO_2 from aerobic oxidation
ADP + P_i
ATP
Electrogenic transport by Na^+K^+ ATPase

FIGURE 23-19 The fuels that supply ATP in the brain. The energy source used by the brain varies with nutritional state. The ketone body used during starvation is β-hydroxybutyrate. Electrogenic transport by the Na^+K^+ ATPase maintains the transmembrane potential essential to information transfer among neurons.

energy of ATP with the mechanical work of contraction. This heat production can be put to good use when ambient temperature is low: skeletal muscle carries out **shivering thermogenesis**, rapidly repeated muscle contraction that produces heat but little motion, helping to maintain the body at its preferred temperature of 37 °C.

Heart muscle differs from skeletal muscle in that it is continuously active in a regular rhythm of contraction and relaxation, and has a completely aerobic metabolism at all times. Mitochondria are much more abundant in heart muscle than in skeletal muscle, making up almost half the volume of the cells (**Fig. 23-18**). The heart uses mainly FFAs as a source of energy, but also some glucose and ketone bodies taken up from the blood; these fuels are oxidized aerobically to generate ATP. Like skeletal muscle, heart muscle does not store lipids or glycogen in large amounts. It does have small amounts of reserve energy in the form of phosphocreatine, enough for a few seconds of contraction. Because the heart is normally aerobic and obtains its energy from oxidative phosphorylation, the failure of O_2 to reach part of the heart muscle when the blood vessels are blocked by lipid deposits (atherosclerosis) or blood clots (coronary thrombosis) can cause that region of the heart muscle to die. This is what happens in myocardial infarction (heart attack). ■

The Brain Uses Energy for Transmission of Electrical Impulses

The metabolism of the brain is remarkable in several respects. The neurons of the adult mammalian brain normally use only glucose as fuel (**Fig. 23-19**). (Astrocytes, the other major cell type in the brain, can oxidize fatty acids.) The brain, which constitutes about 2% of total body mass, has a very active respiratory metabolism (Fig. 23-15); more than 90% of the ATP produced in the neurons comes from oxidative phosphorylation. The brain uses O_2 at a fairly constant rate, accounting for almost 20% of the total O_2 consumed by the body at rest. Because the brain contains very little glycogen, it is constantly dependent on incoming glucose in the blood.

BOX 23-1

Creatine and Creatine Kinase: Invaluable Diagnostic Aids and the Muscle Builder's Friends

Animal tissues that have a high and fluctuating need for ATP, primarily skeletal muscle, cardiac muscle, and brain, contain several isozymes of creatine kinase. A cytosolic isozyme (cCK) is present in regions of high ATP use (myofibrils and sarcoplasmic reticulum, for example). By converting ADP produced during periods of high ATP use back to ATP, cCK prevents the accumulation of ADP to concentrations that could inhibit ATP-using enzymes by mass action. Another isozyme of creatine kinase is located in regions where the inner and outer membranes of mitochondria come into contact. This mitochondrial isozyme (mCK) probably serves to shuttle ATP equivalents produced in mitochondria to cytosolic sites of ATP use (Fig. 1). The species that diffuses from the mitochondrion to ATP-consuming activities in the cytosol is therefore creatine phosphate, not ATP. The mCK isozyme co-localizes with the adenine nucleotide transporter (in the inner mitochondrial membrane) and porin (in the outer mitochondrial membrane), which suggests that these three components may function together to transport ATP equivalents formed in mitochondria into the cytosol.

In knockout mice lacking the mitochondrial isozyme, myocytes compensate by producing more mitochondria, closely associated with myofibrils and sarcoplasmic reticulum, allowing quick diffusion of mitochondrial ATP to the sites of ATP use. Nevertheless, these mice have a reduced capacity for running, indicating a defect in some aspect of energy-supplying metabolism.

Creatine and phosphocreatine spontaneously break down to form creatinine (Fig. 2). To maintain high creatine levels, these losses have to be compensated for, either by dietary creatine, obtained primarily from meat (muscle) and dairy products, or by de novo synthesis from glycine, arginine, and methionine (see Fig. 22-28), which occurs primarily in liver and kidney. De novo synthesis of creatine is a major consumer of these amino acids, particularly in vegans, for whom this is the only source of creatine; plants do not contain creatine. Muscle tissue has a specific system to take up creatine (exported by liver or kidney) from the blood, against a substantial concentration gradient. Efficient uptake of dietary creatine requires continuous exercise; without exercise, creatine supplementation is of little value.

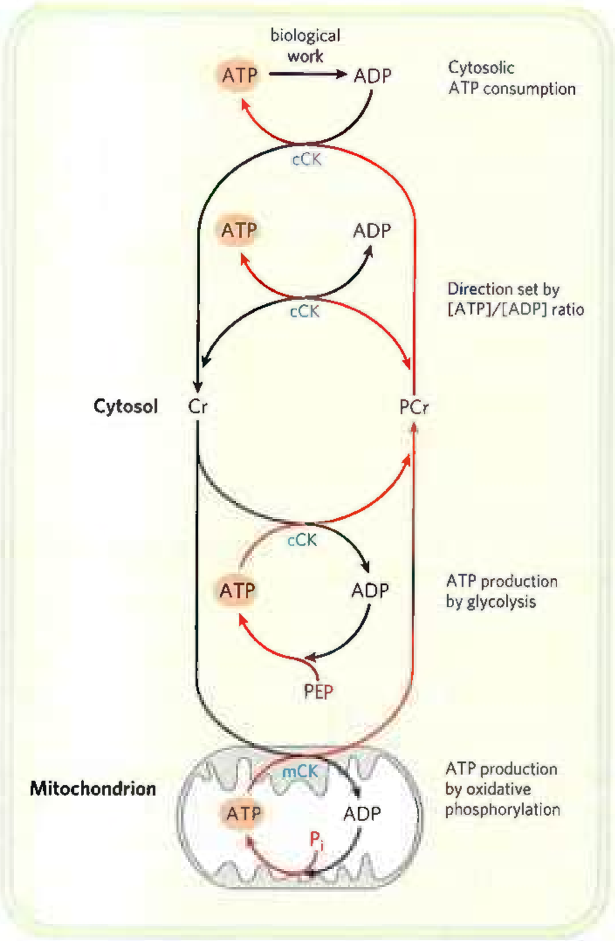

biological work
ATP
ADP
Cytosolic ATP consumption
cCK
ATP
ADP
Direction set by [ATP]/[ADP] ratio
cCK
Cytosol
Cr
PCr
cCK
ATP
ADP
ATP production by glycolysis
PEP
Mitochondrion
mCK
ATP
ADP
P_i
ATP production by oxidative phosphorylation

FIGURE 1 Mitochondrial creatine kinase (mCK) transfers a phosphoryl group from ATP to creatine (Cr) to form phosphocreatine (PCr), which diffuses to sites of ATP use; at these sites, cytosolic creatine kinase (cCK) passes the phosphoryl group into ATP. Cytosolic CK can also use ATP produced by glycolysis to synthesize PCr. During periods of little ATP demand, the pools of ATP and PCr are replenished in preparation for the next period of intense demand for ATP. In resting muscle, the concentration of PCr is three to five times that of ATP, buffering the cell against rapid depletion of ATP during short bursts of ATP demand. [Information from U. Schlattner et al., *Biochim. Biophys. Acta* 1762:164, 2006, Fig. 1.]

Should blood glucose fall significantly below a critical level for even a short time, severe and sometimes irreversible changes in brain function may result.

P3 Although the neurons of the brain cannot directly use free fatty acids or lipids from the blood as fuels, they can, when necessary, get up to 60% of their energy requirement from the oxidation of β-hydroxybutyrate (a ketone body), formed in the liver from fatty acids. The capacity of the brain to oxidize β-hydroxybutyrate via acetyl-CoA becomes important during prolonged fasting or starvation, after liver glycogen has been depleted, because it allows the brain to use body fat as an energy source. This spares muscle proteins—until they become the brain's ultimate source of glucose, via gluconeogenesis in the liver, during severe starvation.

In neurons, energy is required to create and maintain an electrical potential across the plasma membrane. The membrane contains an electrogenic ATP-driven antiporter, the Na^+K^+ ATPase, which simultaneously pumps two K^+ ions into and three Na^+ ions out of the neuron (see Fig. 11-39). The resulting transmembrane potential changes transiently as an electrical signal, an

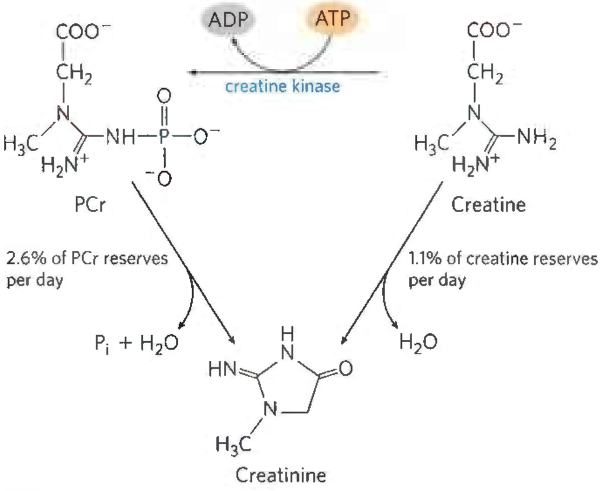

ADP
ATP
creatine kinase
PCr
Creatine
2.6% of PCr reserves per day
1.1% of creatine reserves per day
$P_i + H_2O$
H_2O
Creatinine

FIGURE 2 Spontaneous (nonenzymatic) formation of creatinine from phosphocreatine or creatine consumes a few percent of the body's total creatine per day, which must be replaced by biosynthesis or from the diet.

Heart muscle contains a unique isozyme of creatine kinase (MB, for myocardial band), which is not normally found in the blood but appears there when released from heart muscle damaged by a heart attack. The blood level of MB begins to rise within 2 hours of the heart attack, typically peaks 12 to 36 hours after the heart attack, and returns to normal levels in 3 to 5 days. Measurement of the MB isozyme in blood therefore confirms a diagnosis of heart attack and indicates approximately when it occurred.

Children with inborn errors in the enzymes of creatine synthesis or uptake suffer severe intellectual disability and seizures. They have much-reduced levels of brain creatine as measured by NMR (see Fig. 23-16). Creatine supplementation raises their brain creatine and creatine phosphate concentrations and brings about partial improvement of the symptoms.

FIGURE 3 Many body builders take supplemental creatine to supply phosphocreatine in new muscle tissue. [Steve Williams Photo/Getty Images.]

In the healthy kidney, creatinine from creatine breakdown is efficiently cleared from the blood into the urine. When renal function is defective, creatinine levels in the blood rise above the normal range of 0.8 to 1.4 mg/dL. Elevated blood creatinine is associated with renal failure in diabetes and other conditions in which renal function is temporarily or permanently compromised. Renal clearance of creatinine varies slightly with age, ethnicity, and gender, so correcting the calculation for those factors yields a more sensitive measure of the extent of renal function, the **glomerular filtration rate (GFR)**. ■

Body builders who are adding muscle mass have a greater need for creatine and commonly take creatine supplements of up to 20 g per day for a few days, followed by lower maintenance doses. The combination of exercise and creatine supplementation increases muscle mass (Fig. 3) and improves performance in high-intensity, short-duration work.

action potential, sweeps from one end of a neuron to the other (see Fig. 12-33). Action potentials are the chief mechanism of information transfer in the nervous system, so depletion of ATP in neurons would have disastrous effects on all activities coordinated by neuronal signaling.

Blood Carries Oxygen, Metabolites, and Hormones

P2 Blood mediates the metabolic interactions among all tissues. It transports nutrients from the small intestine to the liver and from the liver and adipose tissue to other organs; it also transports waste products from extrahepatic tissues to the liver for processing and to the kidneys for excretion. Oxygen moves in the bloodstream from the lungs to the tissues, and CO_2 generated by tissue respiration returns via the bloodstream to the lungs for exhalation. Blood also carries hormonal signals from one tissue to another. In its role as signal carrier, the circulatory system resembles the nervous system: both regulate and integrate the activities of different organs.

The average adult human has 5 to 6 L of blood. Almost half of this volume is occupied by three types of blood cells (**Fig. 23-20**): **erythrocytes** (red cells), filled with hemoglobin and specialized for carrying O_2 and CO_2; much smaller numbers of **leukocytes** (white cells) of several types (including **lymphocytes**, also found in lymphatic tissue), which are central to the immune system to defend against infections; and **platelets** (cell fragments), which help to mediate blood clotting. The liquid portion is the **blood plasma**, which is 90% water and 10% solutes. Dissolved or suspended in the plasma are many proteins, lipoproteins, nutrients, metabolites, waste products, inorganic ions, and hormones. More than 70% of the plasma solids are **plasma proteins**, primarily immunoglobulins (circulating antibodies), serum albumin, apolipoproteins (for lipid transport), transferrin (for iron transport), and blood-clotting proteins such as fibrinogen and prothrombin.

The ions and low molecular weight solutes in blood plasma are not fixed components; they are in constant flux between blood and various tissues. Dietary uptake of the inorganic ions that are the predominant electrolytes of blood and cytosol (Na^+, K^+, and Ca^{2+}) is, in general, counterbalanced by their excretion in the urine. For many blood components, something near a dynamic steady state is achieved: the concentration of a component changes little, although a continuous flux occurs between the digestive tract, blood, and urine. The plasma levels of Na^+, K^+, and Ca^{2+} remain close to 140, 5, and 2.5 mM, respectively, with little change in response to dietary intake. Any significant departure from these values can result in serious illness or death. The kidneys play an especially important role in maintaining ion balance by selectively filtering waste products and excess ions out of the blood while preventing the loss of essential nutrients and ions.

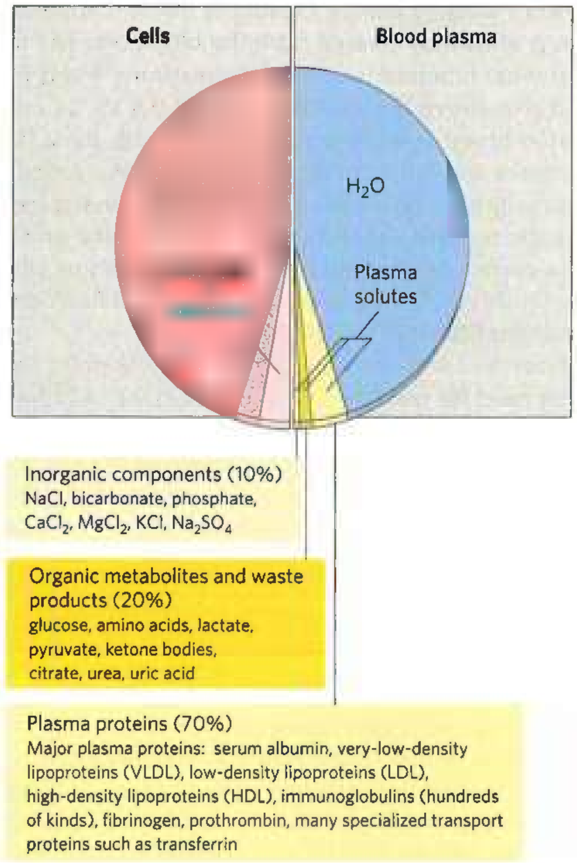


FIGURE 23-20 The composition of blood (by weight). Whole blood can be separated into blood plasma and cells by centrifugation. About 10% of blood plasma is solutes, about 10% of these consisting of inorganic salts, 20% small organic molecules, and 70% plasma proteins. The major dissolved components are listed here. Blood contains many other substances, often in trace amounts. These include other metabolites, enzymes, hormones, vitamins, trace elements, and bile pigments. Measurements of the concentrations of components in blood plasma are important in the diagnosis and treatment of many diseases.

The human erythrocyte loses its nucleus and mitochondria during differentiation. It therefore relies on glycolysis alone for its supply of ATP. The lactate produced by glycolysis returns to the liver, where gluconeogenesis converts it to glucose, to be stored as glycogen or recirculated to peripheral tissues. The erythrocyte has constant access to glucose in the bloodstream.

The concentration of glucose in plasma is subject to tight regulation. We have noted the constant requirement of the brain for glucose and the role of the liver in maintaining blood glucose in the normal range, 60 to 90 mg/100 mL of whole blood (~4.5 mM). (Because erythrocytes make up a significant fraction of blood volume, their removal by centrifugation leaves a supernatant fluid, the plasma, containing the "blood glucose" in a smaller volume. To convert blood glucose to plasma glucose concentration, multiply the blood glucose level by 1.14.) When blood glucose in a human drops to 70 mg/100 mL (the hypoglycemic condition), the person experiences discomfort and mental confusion (**Fig. 23-21**); further

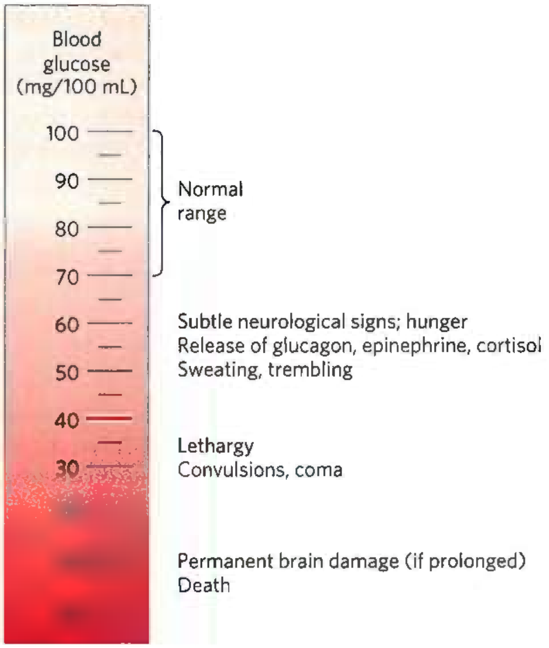

Blood glucose (mg/100 mL)
100
90
80
70
60
50
40
30
Normal range
Subtle neurological signs; hunger
Release of glucagon, epinephrine, cortisol
Sweating, trembling
Lethargy
Convulsions, coma
Permanent brain damage (if prolonged)
Death

FIGURE 23-21 Physiological effects of low blood glucose in humans. Blood glucose levels of 40 mg/100 mL and below constitute severe hypoglycemia.

reductions lead to coma, convulsions, and, in extreme hypoglycemia, death. Maintaining the normal concentration of glucose in blood is therefore a high priority, and a variety of regulatory mechanisms have evolved to achieve that end. Among the most important regulators of blood glucose are the hormones insulin, glucagon, and epinephrine, as discussed in the next section. ■

SUMMARY 23.2 ***Tissue-Specific Metabolism***

- In mammals there is a division of metabolic labor among specialized tissues and organs. The liver is the central processing and distribution organ for nutrients. Glucose 6-phosphate may be used to synthesize glycogen or fatty acids, or it may be sent into the citric acid cycle to produce ATP, or into the pentose phosphate pathway to yield NADPH and pentoses. Amino acids are used to synthesize liver and plasma proteins, or their carbon skeletons are converted to glucose and glycogen by gluconeogenesis; the ammonia formed by deamination is converted to urea. Fatty acids in the liver may undergo β oxidation, or be converted to triglycerides, phospholipids or cholesterol, or be converted into ketone bodies.
- White adipose tissue stores large reserves of triacylglycerol molecules and releases them into the blood in response to epinephrine or glucagon. Brown and beige adipose tissue are specialized for thermogenesis, the result of fatty acid oxidation in uncoupled mitochondria.
- Skeletal muscle is specialized to produce and use ATP for mechanical work, oxidizing fatty acids and glucose during low to moderate muscular activity. During strenuous muscular activity, glycogen is the ultimate fuel, supplying ATP through glycolysis and fermentation to lactate. Phosphocreatine directly replenishes ATP during active contraction. Heart muscle obtains nearly all its ATP from oxidative phosphorylation, with fatty acids as the primary fuel.
- The neurons of the brain use only glucose and β-hydroxybutyrate as fuels, the latter being important during fasting or starvation. The brain uses most of its ATP for the active transport of Na^+ and K^+ to maintain the electrical potential across the neuronal membrane.
- The blood transfers nutrients, oxygen, waste products, and hormonal signals among tissues and organs. It is made up of cells (erythrocytes, leukocytes, and platelets) and electrolyte-rich water (plasma) containing many dissolved proteins.

23.3 Hormonal Regulation of Fuel Metabolism

P3 The minute-by-minute adjustments that keep the blood glucose level near 4.5 mM involve the combined actions of insulin, glucagon, epinephrine, and cortisol on metabolic processes in many body tissues, but especially in liver, muscle, and adipose tissue. Insulin signals to these tissues that blood glucose is higher than necessary; as a result, cells take up excess glucose from the blood and convert it to glycogen and triacylglycerols for storage. Glucagon signals that blood glucose is too low, and tissues respond by producing glucose through glycogen breakdown and (in the liver) gluconeogenesis, and by oxidizing fats to reduce the need for glucose. Epinephrine is released into the blood to prepare the muscles, lungs, and heart for a burst of activity. Cortisol mediates the body's response to longer-term stresses.

In this section, we discuss these hormonal regulations in the context of three normal metabolic states—well fed, fasted, and starving.

Insulin Counters High Blood Glucose in the Well-Fed State

After a high-carbohydrate meal, blood glucose rises several-fold. In response, insulin is released and acts through its plasma membrane receptors in muscle and liver (see Figs. 12-22, 12-23) to stimulate glucose uptake, phosphorylation, and oxidation via glycolysis and the citric acid cycle (**Table 23-3**). In response to insulin, GLUT4 glucose transporters sequestered in intracellular vesicles move to the plasma membrane, dramatically increasing the uptake of glucose from the blood (see Box 11-1). In the liver, insulin activates glycogen synthase and inactivates glycogen phosphorylase, so that much of the glucose 6-phosphate derived from blood glucose is channeled into glycogen.

TABLE 23-3 Effects of Insulin on Blood Glucose: Uptake of Glucose by Cells and Storage as Triacylglycerols and Glycogen

Metabolic effect	Target enzyme
↑ Glucose uptake (muscle, adipose tissue)	↑ Glucose transporter (GLUT4)
↑ Glucose uptake (liver)	↑ Glucokinase (increased expression)
↑ Glycogen synthesis (liver, muscle)	↑ Glycogen synthase
↓ Glycogen breakdown (liver, muscle)	↓ Glycogen phosphorylase
↑ Glycolysis, acetyl-CoA production (liver, muscle)	↑ PFK-1 (by PFK-2) ↑ Pyruvate dehydrogenase complex
↑ Fatty acid synthesis (liver)	↑ Acetyl-CoA carboxylase
↑ Triacylglycerol synthesis (adipose tissue)	↑ Lipoprotein lipase

Insulin also stimulates the storage of excess fuel as fat in adipose tissue (**Fig. 23-22**). Excess acetyl-CoA not needed for energy production via the citric acid cycle is used for fatty acid synthesis. These fatty acids are converted to TAGs in the liver and exported to adipose tissue as components of plasma lipoproteins (VLDL; see Fig. 21-40). In adipose tissue, TAGs are released from VLDL as fatty acids, which are taken up by adipocytes and reconverted to TAGs for storage in response to insulin stimulation.

In summary, an important effect of insulin is to bring about the conversion of excess blood glucose after a meal to two storage forms: glycogen (in the liver and muscle) and TAGs (in adipose tissue).

Pancreatic β Cells Secrete Insulin in Response to Changes in Blood Glucose

The peptide hormones insulin, glucagon, and somatostatin are produced by clusters of specialized pancreatic cells, the islets of Langerhans (**Fig. 23-23**). Each cell type of the islets produces a single hormone: α cells produce glucagon; β cells, insulin; and δ cells, somatostatin. When glucose enters the bloodstream from the intestine after a carbohydrate-rich meal, the resulting increase in blood glucose causes the pancreas to secrete insulin (and to decrease the secretion of glucagon).

As shown in **Figure 23-24**, when blood glucose rises, ① GLUT2 transporters carry glucose into the β cells, where it is immediately converted to glucose 6-phosphate by glucokinase and enters glycolysis. With the higher rate of glucose catabolism, ② [ATP] increases, causing **ATP-gated K^+ channels** in the plasma membrane to close. ③ Reduced efflux of K^+ depolarizes the membrane. (Recall from Section 12.6 that exit of K^+ through an open K^+ channel hyperpolarizes the membrane; thus, closing the K^+ channel effectively depolarizes the membrane.) Membrane depolarization opens voltage-gated Ca^{2+} channels, and ④ the resulting

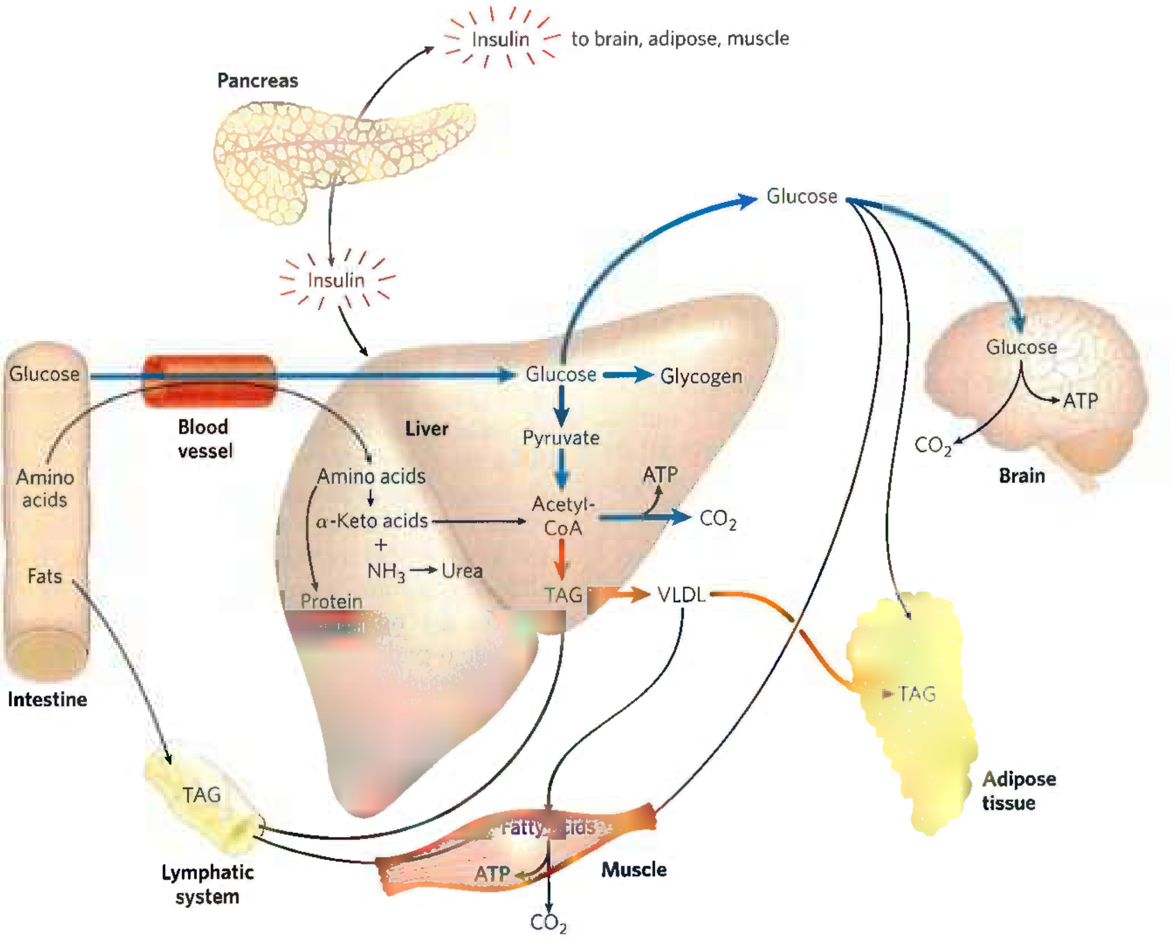


FIGURE 23-22 The well-fed state: the lipogenic liver. Immediately after a calorie-rich meal, glucose, fatty acids, and amino acids enter the liver. Blue arrows follow the path of glucose; orange arrows follow the path of lipids. Insulin released in response to the high blood glucose concentration stimulates glucose uptake by the tissues. Some glucose is exported to the brain for its energy needs, and some goes to adipose and muscle tissue. In the liver, excess glucose is oxidized to acetyl-CoA, which is used to make triacylglycerols for export to adipose and muscle tissue. The NADPH necessary for lipid synthesis is obtained by oxidation of glucose in the pentose phosphate pathway. Excess amino acids are converted to pyruvate and acetyl-CoA, which are also used for lipid synthesis. Dietary fats move from the intestine as chylomicrons, via the lymphatic system, to the liver, muscle, and adipose tissues.

increase in cytosolic [Ca^{2+}] triggers ⑤ the release of insulin by exocytosis. The brain integrates inputs on energy supply and demand, and signals from the parasympathetic and sympathetic nervous systems also affect (stimulate and inhibit, respectively) insulin release. A simple feedback loop limits hormone release: insulin lowers blood glucose by stimulating glucose uptake by the tissues; the reduced blood glucose is detected by the β cell as a diminished flux through the glucokinase reaction; this slows or stops the release of insulin. This feedback regulation holds blood glucose concentration nearly constant despite large fluctuations in dietary intake.

The activity of ATP-gated K^+ channels is central to the regulation of insulin secretion by β cells. The channels are octamers of four identical Kir6.2 subunits and four identical SUR1 subunits (**Fig. 23-25a**) and are constructed along the same lines as the K^+ channels of bacteria and those of other eukaryotic cells (see Fig. 11-45). The four Kir6.2 subunits form a cone around the K^+ channel and function as the selectivity filter and ATP-gating mechanism (Fig. 23-25b). When [ATP] rises, indicating increased blood glucose, the K^+ channels close, thus depolarizing the plasma membrane and triggering insulin release as shown in Figure 23-24. The **sulfonylurea**

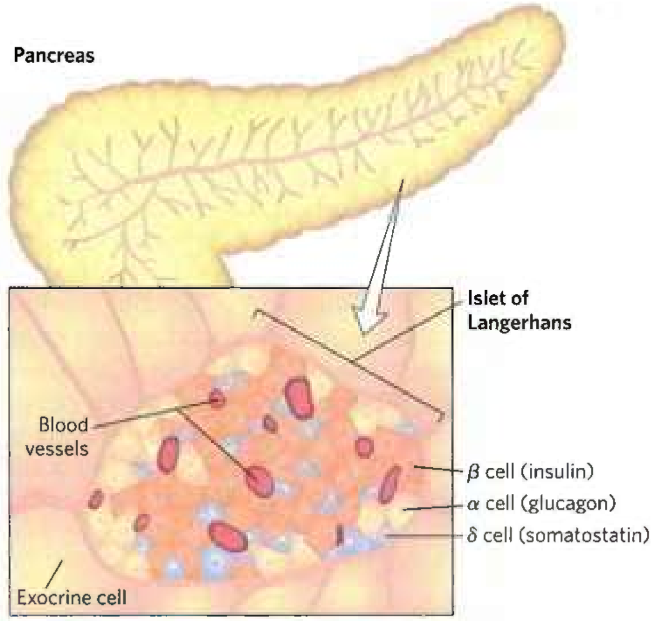

Pancreas
Islet of Langerhans
Blood vessels
β cell (insulin)
α cell (glucagon)
δ cell (somatostatin)
Exocrine cell

FIGURE 23-23 The endocrine system of the pancreas. The pancreas contains both exocrine cells (see Fig. 18-3b), which secrete digestive enzymes in the form of zymogens, and clusters of endocrine cells, the islets of Langerhans. The islets contain α, β, and δ cells (also known as A, B, and D cells, respectively), each cell type secreting a specific peptide hormone.

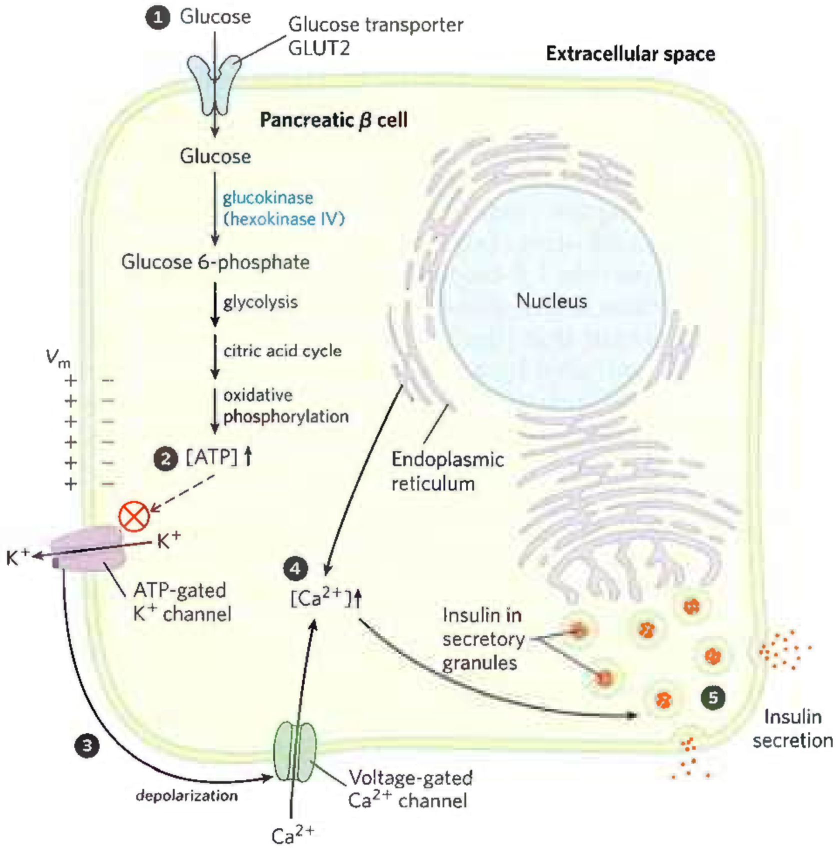

① Glucose
Glucose transporter GLUT2
Extracellular space
Pancreatic β cell
Glucose
glucokinase (hexokinase IV)
Glucose 6-phosphate
glycolysis
citric acid cycle
oxidative phosphorylation
V_m
+ + + + + +
− − − − − −
② [ATP]↑
K^+
K^+
ATP-gated K^+ channel
④ [Ca^{2+}]↑
Nucleus
Endoplasmic reticulum
Insulin in secretory granules
⑤
Insulin secretion
③
depolarization
Voltage-gated Ca^{2+} channel
Ca^{2+}

FIGURE 23-24 Glucose regulation of insulin secretion by pancreatic β cells. When the blood glucose level is high, active metabolism of glucose in the β cell raises intracellular [ATP], closing K^+ channels in the plasma membrane and thus depolarizing the membrane. In response to this membrane depolarization, voltage-gated Ca^{2+} channels open, allowing Ca^{2+} to flow into the cell. (Ca^{2+} is also released from the ER, in response to the initial elevation of [Ca^{2+}] in the cytosol.) Cytosolic [Ca^{2+}] is now high enough to trigger insulin release by exocytosis. The numbered processes are discussed in the text.

drugs, oral medications used in the treatment of type 2 diabetes mellitus, bind to the SUR1 (*s*ulfonyl*u*rea *r*eceptor) subunits of the K^+ channels, closing the channels and stimulating insulin release.

Mutations in the ATP-gated K^+ channels of β cells are, fortunately, rare. Mutations in Kir6.2 that result in constantly *open* K^+ channels (red residues in Fig. 23-25b) lead to neonatal diabetes mellitus, with severe hyperglycemia that requires insulin therapy. Other mutations in Kir6.2 or SUR1 (blue residues in Fig. 23-25b) produce permanently *closed* K^+ channels and continuous release of insulin. If untreated, individuals with these mutations develop a condition in which excessive insulin causes severe hypoglycemia (low blood glucose) leading to irreversible brain damage. One effective treatment is surgical removal of part of the pancreas to reduce insulin production. ■

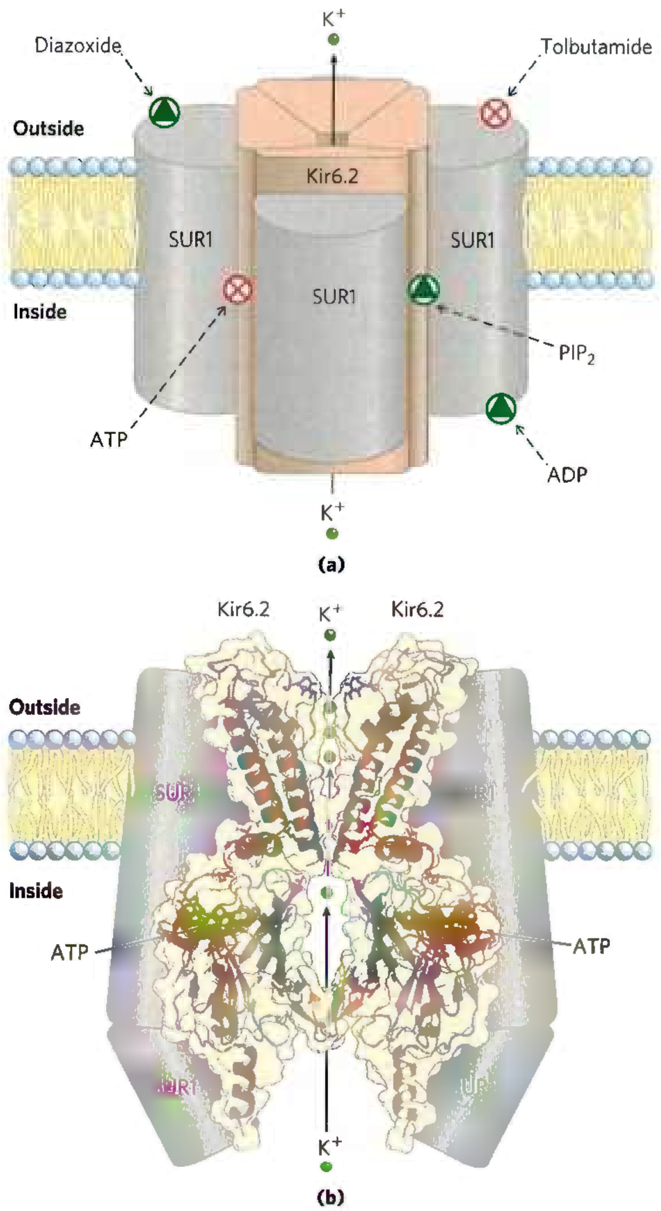


FIGURE 23-25 ATP-gated K^+ channels in β cells. (a) The ATP-gated channel, viewed in the plane of the membrane. The channel is formed by four identical Kir6.2 subunits, which are surrounded by four SUR1 (sulfonylurea receptor) subunits. The SUR1 subunits have binding sites for ADP and the drug diazoxide, both of which favor the open channel, and tolbutamide, a sulfonylurea drug that favors the closed channel. The Kir6.2 subunits constitute the channel, and they contain, on the cytosolic side, binding sites for ATP and phosphatidylinositol 4,5-bisphosphate (PIP_2), which favor the closed and the open channel, respectively. (b) The structure of the Kir6.2 portion of the channel, viewed in the plane of the membrane. For clarity, only two transmembrane domains and two cytosolic domains are shown. Three K^+ ions (green) are shown in the region of the selectivity filter. Mutation in certain amino acid residues (shown in red) leads to neonatal diabetes; mutation in others (shown in blue) leads to hyperinsulinism of infancy. This structure was obtained by mapping the known Kir6.2 sequence onto the crystal structures of a bacterial Kir channel (KirBac1.1) and the amino and carboxyl domains of another Kir protein, Kir3.1. [Data from (b) KirBac1.1: PDB ID 1P7B, A. Kuo et al., *Science* 300:1922, 2003; Kir3.1: PDB ID 1U4E, S. Pegan et al., *Nature Neurosci.* 8:279, 2005. Coordinates courtesy of Frances M. Ashcroft, Oxford University, used with permission of S. Haider and M. S. P. Sansom to re-create a model published in J. F. Antcliff et al., *EMBO J.* 24:229, 2005.]

Glucagon Counters Low Blood Glucose

P3 Several hours after the intake of dietary carbohydrate, blood glucose levels fall slightly because of the ongoing oxidation of glucose by the brain and other tissues. Lowered blood glucose triggers the pancreas to secrete **glucagon** and simultaneously decrease the release of insulin (**Fig. 23-26**).

Glucagon causes an *increase* in blood glucose concentration in several ways (**Table 23-4**). Like epinephrine, it stimulates the net breakdown of liver glycogen by activating glycogen phosphorylase and inactivating glycogen synthase; both effects are the result of phosphorylation of the regulated enzymes, triggered by cAMP. Glucagon inhibits glucose breakdown by glycolysis in the liver and stimulates glucose synthesis by gluconeogenesis. Both effects result from lowering the concentration of fructose 2,6-bisphosphate, an allosteric inhibitor of the gluconeogenic enzyme fructose 1,6-bisphosphatase (FBPase-1) and an activator of the glycolytic enzyme phosphofructokinase-1. Recall that [fructose 2,6-bisphosphate] is ultimately controlled by a cAMP-dependent protein phosphorylation reaction (see Fig. 14-25). Glucagon also inhibits the glycolytic enzyme pyruvate kinase, by promoting its cAMP-dependent phosphorylation, thus blocking the conversion of phosphoenolpyruvate to pyruvate and preventing oxidation of pyruvate via the citric acid cycle (see Fig. 14-26). The resulting accumulation of phosphoenolpyruvate favors gluconeogenesis. This effect is augmented by glucagon's stimulation of the synthesis of the gluconeogenic enzyme PEP carboxykinase. **P3** By stimulating glycogen breakdown, preventing glycolysis and promoting gluconeogenesis in hepatocytes, glucagon enables the liver to export glucose, restoring blood glucose to its normal level.

Although its primary target is the liver, glucagon (like epinephrine) also affects adipose tissue, activating TAG

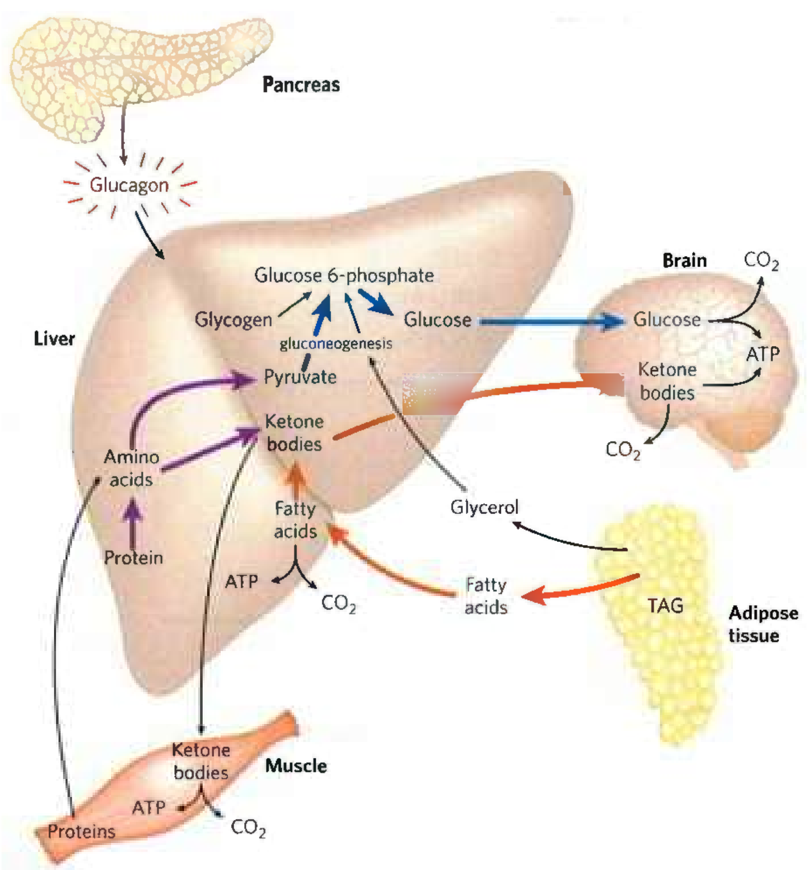


FIGURE 23-26 The fasting state: the glucogenic liver. After some hours without a meal, the liver becomes the principal source of glucose for the brain. Liver glycogen is broken down to glucose 1-phosphate, and this is converted to glucose 6-phosphate, then to free glucose, which is released into the bloodstream. Amino acids from the degradation of proteins in liver and muscle, and glycerol from the breakdown of TAGs in adipose tissue, are used for gluconeogenesis. The liver uses fatty acids as its principal fuel, and excess acetyl-CoA is converted to ketone bodies for export to other tissues; the brain is especially dependent on this fuel when glucose is in short supply (see Fig. 23-19). Blue arrows follow the path of glucose; orange arrows, the path of lipids; and purple arrows, the path of amino acids.

TABLE 23-4 Effects of Glucagon on Blood Glucose: Production and Release of Glucose by the Liver

Metabolic effect	Effect on glucose metabolism	Target enzyme
↑ Glycogen breakdown (liver)	Glycogen ⟶ glucose	↑ Glycogen phosphorylase
↓ Glycogen synthesis (liver)	Less glucose stored as glycogen	↓ Glycogen synthase
↓ Glycolysis (liver)	Less glucose used as fuel in liver	↓ PFK-1
↑ Gluconeogenesis (liver)	Amino acids Glycerol Oxaloacetate } ⟶ glucose	↑ FBPase-2 ↓ Pyruvate kinase ↑ PEP carboxykinase
↑ Fatty acid mobilization (adipose tissue)	Less glucose used as fuel by liver, muscle	↑ Hormone-sensitive lipase
		↑ PKA (perilipin–Ⓟ)
↑ Ketogenesis	Provides alternative to glucose as energy source for brain	↓ Acetyl-CoA carboxylase

breakdown by causing cAMP-dependent phosphorylation of perilipin and hormone-sensitive lipase. The activated lipase liberates free fatty acids, which are exported to the liver and other tissues as fuel, sparing glucose for the brain. The net effect of glucagon is therefore to stimulate glucose synthesis and release by the liver and to mobilize fatty acids from adipose tissue, to be used instead of glucose by tissues other than the brain. All these effects of glucagon are mediated by cAMP-dependent protein phosphorylation.

During Fasting and Starvation, Metabolism Shifts to Provide Fuel for the Brain

A healthy adult human has three types of fuel reserves: glycogen stored in the liver and, in smaller quantities, in muscles; large quantities of TAG in adipose tissues; and tissue proteins, which can be degraded when necessary to provide fuel (**Table 23-5**).

P3 Two hours after a meal, the blood glucose level is diminished slightly, and tissues receive glucose

TABLE 23-5 Available Metabolic Fuels in a Normal-Weight, 70 kg Man and in an Obese, 140 kg Man at the Beginning of a Fast

Type of fuel	Weight (kg)	Caloric equivalent (thousands of kcal (kJ))	Estimated survival (months)[a]
Normal-weight, 70 kg man			
Triacylglycerols (adipose tissue)	15	140 (590)	
Proteins (mainly muscle)	6	24 (100)	
Glycogen (muscle, liver)	0.23	0.90 (3.8)	
Circulating fuels (glucose, fatty acids, triacylglycerols, etc.)	0.023	0.10 (0.42)	
Total		165 (690)	3
Obese, 140 kg man			
Triacylglycerols (adipose tissue)	80	750 (3,100)	
Proteins (mainly muscle)	8	32 (130)	
Glycogen (muscle, liver)	0.23	0.92 (3.8)	
Circulating fuels	0.025	0.11 (0.46)	
Total		783 (3,200)	14

[a] Survival time is calculated on the assumption of a basal energy expenditure of 1,800 kcal/day.

released from liver glycogen. There is little or no synthesis of TAGs. By four hours after a meal, blood glucose has fallen further, insulin secretion has slowed, and glucagon secretion has increased. These hormonal signals mobilize TAGs from adipose tissue, which now become the primary fuel for muscle and liver. **Figure 23-27** shows the responses to prolonged fasting. ① To provide glucose for the brain, the liver degrades certain proteins—those most expendable in an organism not ingesting food. Their nonessential amino acids are transaminated or deaminated (Chapter 18), and ② the extra amino groups are converted to urea, which is exported via the bloodstream to the kidneys and excreted in the urine.

Also in the liver, and to some extent in the kidneys, the carbon skeletons of glucogenic amino acids are converted to pyruvate or intermediates of the citric acid cycle. ③ These intermediates (as well as the glycerol derived from TAGs in adipose tissue) provide the starting materials for gluconeogenesis in the liver, ④ yielding glucose for export to the brain. ⑤ Fatty acids released from adipose tissue are oxidized to acetyl-CoA in the liver, but as oxaloacetate is depleted by the use of citric acid cycle intermediates for gluconeogenesis, ⑥ entry of acetyl-CoA into the cycle is inhibited and acetyl-CoA accumulates. ⑦ This favors the formation of acetoacetyl-CoA and ketone bodies. After a few days of fasting, the levels of ketone bodies in the blood rise (**Fig. 23-28**) as they are exported from the liver to the heart, skeletal muscle, and brain, which use these fuels instead of glucose (Fig. 23-27, ⑧). When the concentration of ketone bodies in the blood exceeds the ability of the kidneys to reabsorb ketones ⑨, these compounds begin to appear in the urine.

Triacylglycerols stored in the adipose tissue of a normal-weight adult could provide enough fuel to maintain a basal rate of metabolism for about three months; a very obese adult has enough stored fuel to endure a fast of more than a year (Table 23-5). When fat reserves are gone, the degradation of *essential* proteins begins; this leads to loss of heart and liver function and, in prolonged starvation, to death. Stored fat can provide adequate energy (calories) during a fast or a rigid diet, but vitamins and minerals must be provided, and sufficient dietary glucogenic amino acids are needed to replace those being used for gluconeogenesis. Rations for those on a weight-reduction diet are commonly fortified with vitamins, minerals, and amino acids or proteins.

Epinephrine Signals Impending Activity

When an animal is confronted with a stressful situation that requires increased activity—fighting or fleeing, in the extreme case—neuronal signals from the brain trigger the release of epinephrine and norepinephrine from the adrenal medulla. Both hormones dilate the respiratory passages to facilitate the uptake of O_2, increase the rate and strength of the heartbeat, and raise the blood pressure, thereby promoting the flow of O_2 and fuels to the tissues (**Table 23-6**). This is the "fight-or-flight" response.

Epinephrine acts primarily on muscle, adipose, and liver tissues. It activates glycogen phosphorylase and inactivates glycogen synthase by cAMP-dependent phosphorylation of the enzymes, thus stimulating the

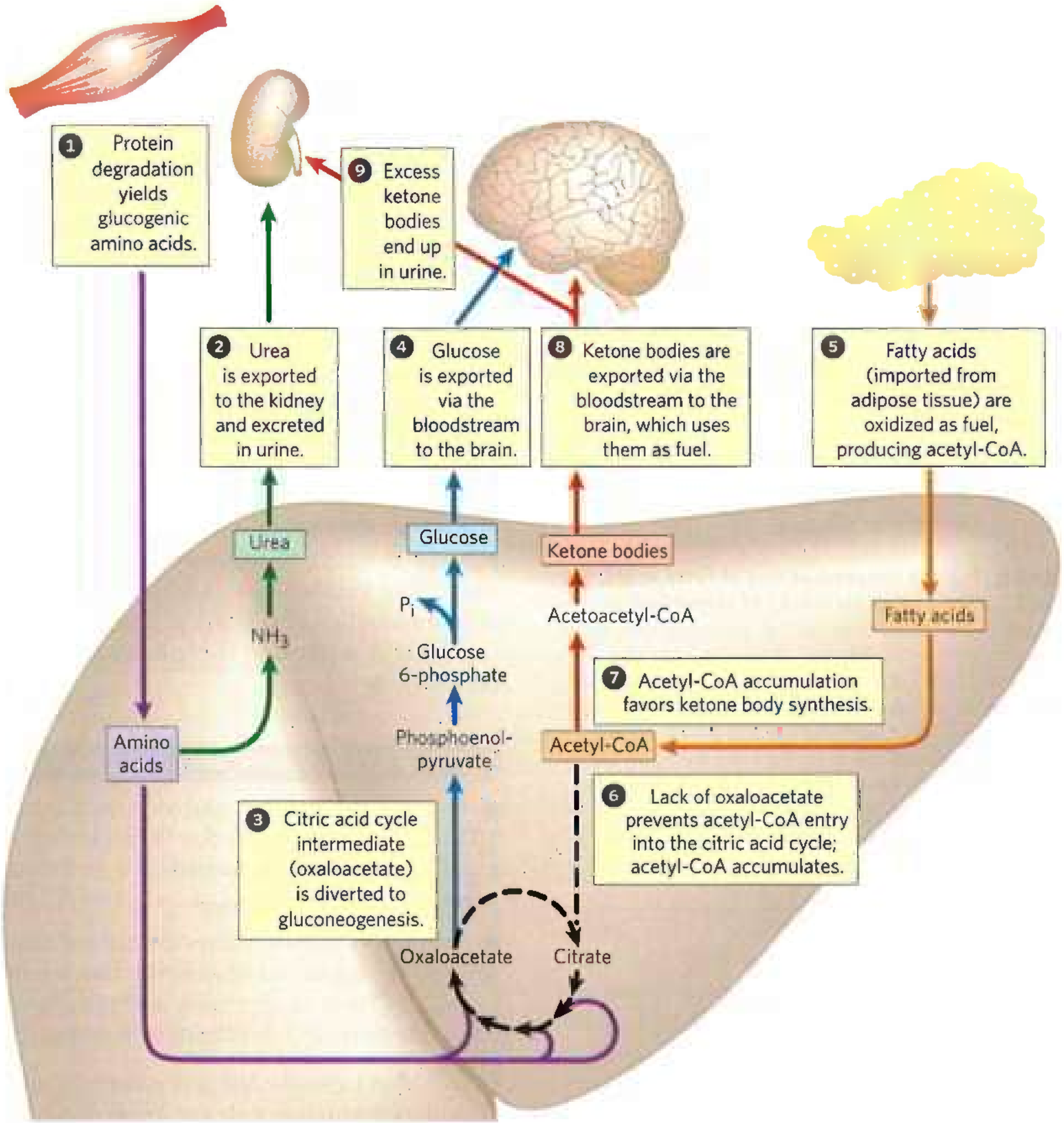

① Protein degradation yields glucogenic amino acids.
② Urea is exported to the kidney and excreted in urine.
③ Citric acid cycle intermediate (oxaloacetate) is diverted to gluconeogenesis.
④ Glucose is exported via the bloodstream to the brain.
⑤ Fatty acids (imported from adipose tissue) are oxidized as fuel, producing acetyl-CoA.
⑥ Lack of oxaloacetate prevents acetyl-CoA entry into the citric acid cycle; acetyl-CoA accumulates.
⑦ Acetyl-CoA accumulation favors ketone body synthesis.
⑧ Ketone bodies are exported via the bloodstream to the brain, which uses them as fuel.
⑨ Excess ketone bodies end up in urine.
Urea
NH_3
Amino acids
Glucose
P_i
Glucose 6-phosphate
Phosphoenol-pyruvate
Oxaloacetate
Citrate
Ketone bodies
Acetoacetyl-CoA
Acetyl-CoA
Fatty acids

FIGURE 23-27 Fuel metabolism in the liver during prolonged fasting or in uncontrolled diabetes mellitus. The numbered steps are described in the text. After depletion of stored carbohydrates (glycogen), gluconeogenesis in the liver becomes the main source of glucose for the brain (blue arrows). NH_3 from amino acid deamination is converted into urea and excreted (green arrows). Glucogenic amino acids from protein breakdown (purple arrows) provide substrates for gluconeogenesis, and glucose is exported to the brain. Fatty acids from adipose tissue are imported into the liver and oxidized to acetyl-CoA (orange arrows), and acetyl-CoA is the starting material for ketone body formation in the liver and for export to the brain to serve as an energy source (red arrows). Excess ketone bodies are excreted in the urine.

conversion of *liver* glycogen to blood glucose, the fuel for anaerobic muscular work. Epinephrine also promotes the anaerobic breakdown of *muscle* glycogen by lactic acid fermentation, stimulating glycolytic ATP formation. The stimulation of glycolysis is accomplished by raising the concentration of fructose 2,6-bisphosphate, a potent allosteric activator of the key glycolytic enzyme phosphofructokinase-1. Epinephrine also stimulates fat mobilization in adipose tissue, by activating hormone-sensitive lipase and moving aside perilipin (see Fig. 17-2). Finally, epinephrine stimulates glucagon secretion and inhibits insulin secretion, reinforcing its effect of mobilizing fuels and inhibiting fuel storage.

Cortisol Signals Stress, Including Low Blood Glucose

A variety of stressors (anxiety, fear, pain, hemorrhage, infection, low blood glucose, starvation) stimulate release of the glucocorticoid **cortisol** from the adrenal cortex (see Fig. 23-6). P3 Cortisol acts on muscle, liver, and adipose tissue to supply the organism with fuel to withstand the stress. Cortisol is a relatively slow-acting hormone that

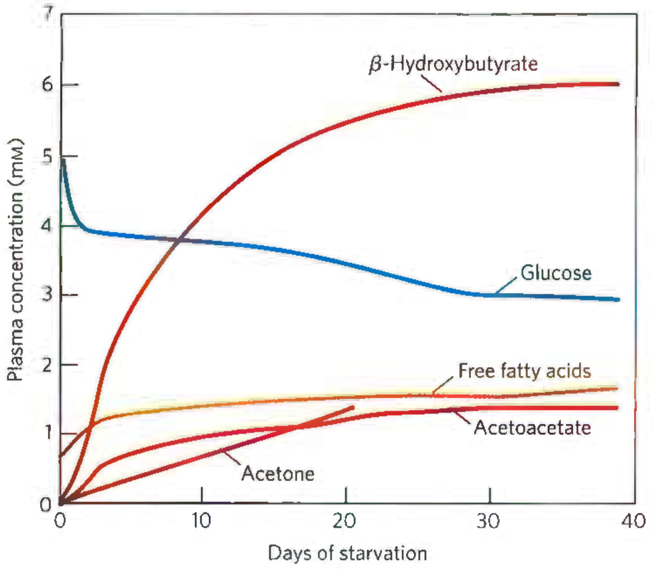


FIGURE 23-28 Plasma concentrations of fatty acids, glucose, and ketone bodies during six weeks of starvation. Despite the hormonal mechanisms for maintaining the glucose level in blood, glucose begins to diminish within 2 days of beginning a fast. The levels of ketone bodies, almost unmeasurable before the fast, rise dramatically after 2 to 4 days of fasting, with β-hydroxybutyrate as the major contributor. These water-soluble ketones, acetoacetate and β-hydroxybutyrate, supplement glucose as an energy source for the brain during a long fast. Acetone, a minor ketone body, is not metabolized but is eliminated in the breath. A much smaller rise in blood fatty acids also occurs, but this does not contribute to energy metabolism in the brain, as fatty acids do not cross the blood-brain barrier. [Data from G. F. Cahill, Jr., *Annu. Rev. Nutr.* 26:1, 2006, Fig. 2.]

alters metabolism by changing the kinds and amounts of certain enzymes synthesized in its target cells, rather than by regulating the activity of existing enzyme molecules.

In adipose tissue, cortisol leads to an increased release of fatty acids from stored TAGs. The exported fatty acids serve as fuel for other tissues, and the glycerol is used for gluconeogenesis in the liver. Cortisol stimulates the breakdown of nonessential muscle proteins and the export of amino acids to the liver, where they serve as precursors for gluconeogenesis. In the liver, cortisol promotes gluconeogenesis by stimulating synthesis of PEP carboxykinase; glucagon has the same effect, whereas insulin has the opposite effect. Glucose produced in this way is stored in the liver as glycogen or exported immediately to tissues that need glucose for fuel. The net effect of these metabolic changes is to restore blood glucose to its normal level and to increase glycogen stores, ready to support the fight-or-flight response commonly associated with stress. The effects of cortisol therefore counterbalance those of insulin. During extended periods of stress, the continued release of cortisol loses its positive adaptive value and begins to cause damage to muscle and bone and to impair endocrine and immune function.

Cushing disease is a medical condition in which a tumor on the pituitary gland causes the adrenal glands to overproduce cortisol. It is treated by surgery to remove the tumor, followed by chemotherapy to kill remaining tumor cells. Addison disease results from underproduction of cortisol, and is treated by administering hydrocortisone (the pharmaceutical name for cortisol). ■

SUMMARY 23.3 *Hormonal Regulation of Fuel Metabolism*

- Fluctuations in blood glucose (normally 70 to 100 mg/100 mL, or about 4.5 mM) due to dietary intake or vigorous exercise are counterbalanced by a variety of hormonally triggered changes in metabolism in several organs.
- High blood glucose elicits the release of insulin, which speeds the uptake of glucose by tissues and favors the storage of fuels as glycogen and triacylglycerols while inhibiting fatty acid mobilization in adipose tissue.
- Low blood glucose triggers release of glucagon, which stimulates glucose release from liver glycogen and shifts fuel metabolism in liver and muscle to fatty acid oxidation, sparing glucose for use by the brain. In prolonged fasting, TAGs become the principal fuel; the liver

TABLE 23-6 Physiological and Metabolic Effects of Epinephrine: Preparation for Action

Immediate effect	Overall effect
Physiological	
↑ Heart rate ↑ Blood pressure ↑ Dilation of respiratory passages	Increase delivery of O_2 to tissues (muscle)
Metabolic	
↑ Glycogen breakdown (muscle, liver) ↓ Glycogen synthesis (muscle, liver) ↑ Gluconeogenesis (liver)	Increase production of glucose for fuel
↑ Glycolysis (muscle)	Increases ATP production in muscle
↑ Fatty acid mobilization (adipose tissue)	Increases availability of fatty acids as fuel
↑ Glucagon secretion ↓ Insulin secretion	Reinforce metabolic effects of epinephrine

converts the fatty acids to ketone bodies for export to other tissues, including the brain.

- Epinephrine prepares the body for increased activity by mobilizing glucose from glycogen and other precursors, releasing the glucose into the blood.
- Cortisol, released in response to a variety of stressors (including low blood glucose), stimulates gluconeogenesis from amino acids and glycerol in the liver, thus raising blood glucose and counterbalancing the effects of insulin.

23.4 Obesity and the Regulation of Body Mass

In the U.S. population, more than 40% of adults are obese, including more than 10% who are severely obese, as defined in terms of **body mass index (BMI)**, calculated as (weight in kg)/(height in m)^2. A BMI below 25 is considered normal; an individual with a BMI of 25 to 30 is overweight; a BMI greater than 30 indicates **obesity**; a BMI greater than 40 indicates severe obesity. Obesity is life-threatening. It significantly increases the likelihood of developing type 2 diabetes, as well as heart attack, stroke, and cancers of the colon, breast, prostate, and endometrium. Consequently, there is great interest in understanding how body mass and the storage of fats in adipose tissue are regulated.

To a first approximation, obesity is the result of taking in more calories in the diet than are expended by the body's fuel-consuming activities. The body can deal with an excess of dietary calories in three ways: (1) convert excess fuel to fat and store it in adipose tissue, (2) burn excess fuel by extra exercise, and (3) "waste" fuel by diverting it to heat production (thermogenesis) by uncoupled mitochondria. P4 In mammals, a complex set of hormonal and neuronal signals acts to keep fuel intake and energy expenditure in balance so as to hold the amount of adipose tissue at a suitable level. Dealing effectively with obesity requires understanding how the various checks and balances work under normal conditions and how these homeostatic mechanisms can fail.

Adipose Tissue Has Important Endocrine Functions

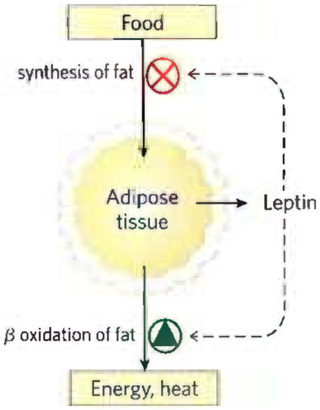


FIGURE 23-29 Set-point model for maintaining constant mass. When the mass of adipose tissue increases (dashed outline), released leptin inhibits feeding and fat synthesis and stimulates oxidation of fatty acids. When the mass of adipose tissue decreases (solid outline), lowered leptin production favors greater food intake and less fatty acid oxidation.

One early hypothesis to explain body-mass homeostasis, the "adiposity negative-feedback" model, postulated a mechanism that inhibits eating behavior and increases energy expenditure whenever body weight exceeds a certain value, called the set point; the inhibition is relieved when body weight drops below the set point (**Fig. 23-29**). This model predicts that a feedback signal originating in adipose tissue influences the brain centers that control eating behavior and metabolic and motor activity. The first such signal to be discovered was leptin, in 1994. Subsequent research revealed that P4 adipose tissue is an important endocrine organ that produces peptide hormones, known as **adipokines**. Adipokines may act locally (autocrine and paracrine action) or systemically (endocrine action), carrying information about the adequacy of the energy reserves (TAGs) stored in adipose tissue to other tissues and to the brain. Normally, adipokines produce changes in fuel metabolism and feeding behavior that reestablish adequate fuel reserves and maintain body mass. When adipokines are over- or underproduced, the resulting dysregulation may result in life-threatening disease.

Leptin (Greek *leptos*, "thin") is an adipokine (167 amino acid residues) that, on reaching the brain, acts on receptors in the hypothalamus to curtail appetite. Leptin was first identified as the product of a gene designated *OB* (obese) in laboratory mice. Mice with two defective copies of this gene (*ob/ob* genotype; lowercase letters signify a mutant form of the gene) show the behavior and physiology of animals in a constant state of starvation: their plasma cortisol levels are elevated; they exhibit unrestrained appetite, are unable to stay warm, grow abnormally large, and do not reproduce. As a consequence of unrestrained appetite, they become severely obese, weighing as much as three times more than normal mice (**Fig. 23-30**). They also have metabolic disturbances similar to those seen in diabetes, and they are insulin-resistant. When leptin is injected into *ob/ob* mice, they eat less, lose weight, and increase their locomotor activity and thermogenesis.

A second mouse gene, designated *DB* (diabetic), also has a role in appetite regulation. Mice with two defective copies (*db/db*) are obese and diabetic. The *DB* gene encodes the **leptin receptor**. When the receptor is defective, the signaling function of leptin is lost.

The leptin receptor is expressed primarily in regions of the brain known to regulate feeding behavior—neurons of

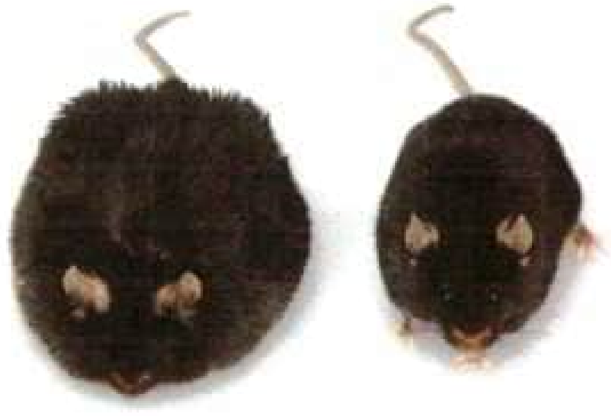


FIGURE 23-30 Obesity caused by defective leptin production. Both of these mice, which are the same age, have defects in the *OB* gene. The mouse on the right was injected daily with purified leptin and weighs 35 g. The mouse on the left got no leptin and consequently ate more food and was less active; it weighs 67 g. [The Rockefeller University/AP Photo.]

the **arcuate nucleus** of the hypothalamus (**Fig. 23-31a**). Leptin carries the message that fat reserves are sufficient, and it promotes reduction of fuel intake and increase in expenditure of energy. Leptin-receptor interaction in the hypothalamus alters the release of neuronal signals to the region of the brain that affects appetite. Leptin also stimulates the sympathetic nervous system, increasing blood pressure, heart rate, and thermogenesis by uncoupling the mitochondria of brown adipocytes (Fig. 23-31b). Recall that the uncoupling protein UCP1 forms a channel in the inner mitochondrial membrane that allows protons to reenter the mitochondrial matrix without passing through the ATP synthase complex. This permits constant oxidation of fuel (fatty acids in a brown or beige adipocyte) without ATP synthesis, dissipating energy as heat and consuming dietary calories or stored fats in potentially large amounts.

Leptin Stimulates Production of Anorexigenic Peptide Hormones

Two types of neurons in the arcuate nucleus control fuel intake and metabolism (**Fig. 23-32**). The **orexigenic** (appetite-stimulating) neurons stimulate eating by producing and releasing **neuropeptide Y (NPY)**, which causes the next neuron in the circuit to send the signal to the brain: Eat! The blood level of NPY rises during starvation and is elevated in mice with either two defective copies of the leptin gene (*ob/ob*) or two defective copies of the leptin receptor gene (*db/db*). The high NPY concentration presumably contributes to the obesity of these mice, which eat voraciously.

The **anorexigenic** (appetite-suppressing) neurons in the arcuate nucleus produce **α-melanocyte–stimulating hormone** (**α-MSH**; also known as melanocortin), formed from its polypeptide precursor pro-opiomelanocortin (POMC; Fig. 23-5). Release of α-MSH causes the next neuron in the circuit to send the signal to the brain: Stop eating!

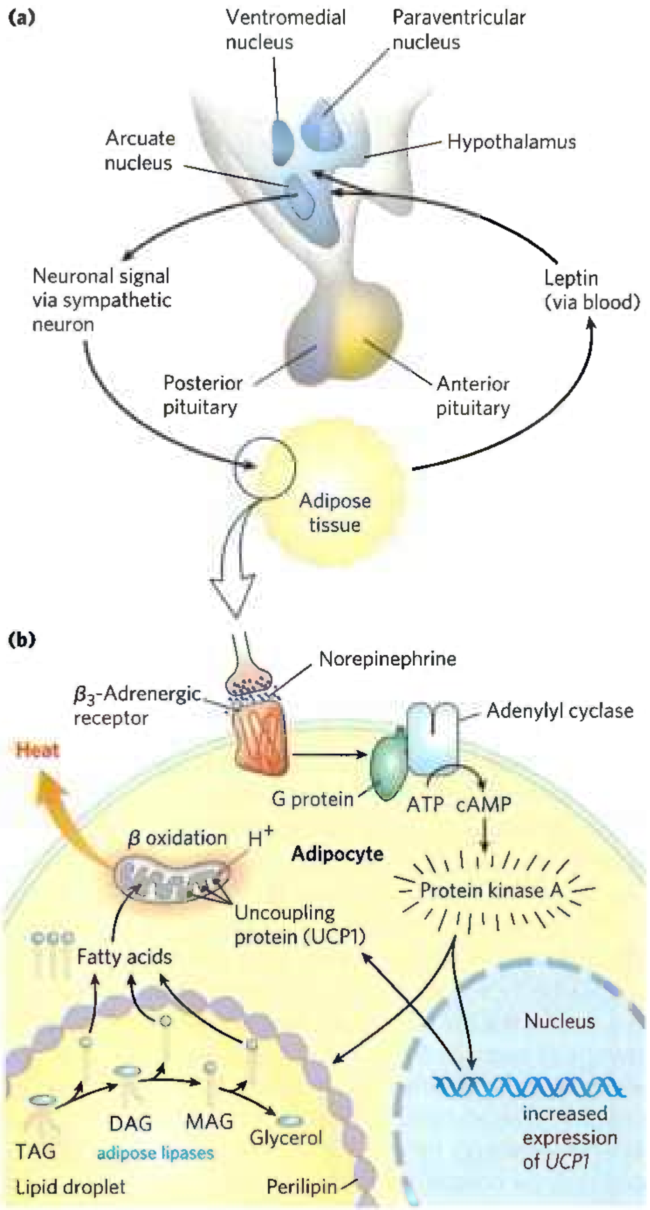

(a)
Ventromedial nucleus
Paraventricular nucleus
Arcuate nucleus
Hypothalamus
Neuronal signal via sympathetic neuron
Leptin (via blood)
Posterior pituitary
Anterior pituitary
Adipose tissue
(b)
Norepinephrine
β_3-Adrenergic receptor
Adenylyl cyclase
Heat
G protein
ATP
cAMP
β oxidation
H^+
Adipocyte
Protein kinase A
Uncoupling protein (UCP1)
Fatty acids
Nucleus
increased expression of *UCP1*
DAG
MAG
Glycerol
TAG
adipose lipases
Lipid droplet
Perilipin

FIGURE 23-31 Hypothalamic regulation of food intake and energy expenditure. (a) Role of the hypothalamus in its interaction with adipose tissue. The hypothalamus receives input (leptin) from adipose tissue and responds with neuronal signals to adipocytes. (b) This signal (norepinephrine) activates protein kinase A, which triggers mobilization of fatty acids from TAG and their uncoupled oxidation in mitochondria, generating heat but not ATP. DAG, diacylglycerol; MAG, monoacylglycerol.

The amount of leptin released by adipose tissue depends on both the number and the size of adipocytes. When weight loss decreases the mass of lipid tissue, leptin levels in the blood decrease, the production of NPY increases, and the processes in adipose tissue shown in Figure 23-31 are reversed. Uncoupling is diminished, slowing thermogenesis and saving fuel, and fat mobilization slows in response to reduced signaling by cAMP. Consumption of more food, combined with more efficient utilization of fuel, results in replenishment of the fat reserve in adipose tissue, bringing the system back into balance.

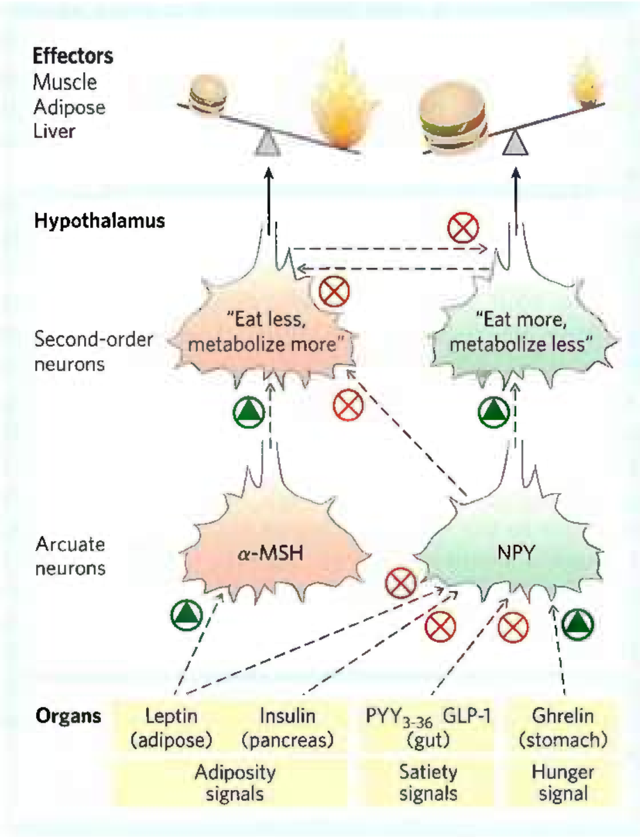


FIGURE 23-32 Hormones that control eating. In the arcuate nucleus of the hypothalamus, two sets of neurosecretory cells receive hormonal input and relay neuronal signals to the cells of muscle, adipose tissue, and liver. Leptin and insulin are released from adipose tissue and the pancreas, respectively, in proportion to the mass of body fat. The two hormones act on anorexigenic neurosecretory cells to trigger release of α-MSH (melanocyte-stimulating hormone); α-MSH carries the signal to second-order neurons in the hypothalamus, which puts out the signals to eat less and metabolize more fuel. Leptin and insulin also act on orexigenic neurosecretory cells to inhibit the release of NPY, reducing the "eat more" signal sent to the tissues. As described later in the text, the gastric hormone ghrelin *stimulates* appetite by activating the NPY-expressing cells; PYY_{3-36}, released from the colon, *inhibits* these neurons and decreases appetite. Each of the two types of neurosecretory cells inhibits hormone production by the other, so any stimulus that activates orexigenic cells inactivates anorexigenic cells, and vice versa. This strengthens the effect of stimulatory inputs.

Leptin Triggers a Signaling Cascade That Regulates Gene Expression

The leptin signal is transduced by a mechanism also used by receptors for some growth factors. The leptin receptor has a single transmembrane segment. When leptin binds to the extracellular domains of two monomers, they dimerize and undergo phosphorylation on several Tyr residues. This initiates a chain of events that ends in the nucleus with the increased synthesis of target genes including the gene for POMC, from which α-MSH is produced.

The increased catabolism and thermogenesis triggered by leptin are due in part to increased synthesis of the mitochondria in brown and beige adipocytes. Leptin stimulates UCP1 synthesis by altering synaptic transmissions from neurons of the arcuate nucleus to adipose and other tissues via the sympathetic nervous system. The consequent increased release of norepinephrine in these tissues acts through β_3-adrenergic receptors to stimulate transcription of the *UCP1* gene. The resulting uncoupling of electron transfer from oxidative phosphorylation consumes fat and is thermogenic (Fig. 23-31).

Might human obesity be the result of insufficient leptin production and therefore be treatable by the injection of leptin? Blood levels of leptin are, in fact, usually much *higher* in obese animals (including humans) than in animals of normal body mass (except, of course, in *ob/ob* mutants, which cannot make leptin). Some downstream element in the leptin response system must be defective in obese individuals, and the elevation in leptin is the result of an (unsuccessful) attempt to overcome the leptin resistance. In those very rare humans with extreme obesity who have a defective leptin gene (*OB*), leptin injection does result in dramatic weight loss. In the vast majority of obese individuals, however, the *OB* gene is intact. In clinical trials, the injection of leptin did not have the weight-reducing effect observed in obese *ob/ob* mice. Clearly, most cases of human obesity involve one or more factors in addition to leptin.

Adiponectin Acts through AMPK to Increase Insulin Sensitivity

Adiponectin is a peptide hormone produced almost exclusively in adipose tissue, an adipokine that sensitizes other organs to the effects of insulin. Adiponectin circulates in the blood and powerfully affects the metabolism of fatty acids and carbohydrates in liver and muscle. It increases the uptake of fatty acids from the blood by myocytes and the rate at which fatty acids undergo β oxidation in muscle. It also blocks fatty acid synthesis and gluconeogenesis in hepatocytes, and stimulates glucose uptake and catabolism in muscle and liver.

These effects of adiponectin are indirect and not fully understood, but the **AMP-activated protein kinase (AMPK)** mediates many of them. Acting through its GPCR, adiponectin triggers phosphorylation and activation of AMPK. Recall that AMPK is activated by factors that signal the need to shift metabolism toward energy generation and away from energy-requiring biosynthesis (**Fig. 23-33**; see also p. 503). When activated, AMPK profoundly affects the metabolism of individual cells and, through its actions in the brain, the metabolism of the whole animal.

AMPK Coordinates Catabolism and Anabolism in Response to Metabolic Stress

AMPK has emerged as a central player in the coordination of metabolic pathways, organism activity, and feeding behavior (**Fig. 23-34**). This heterotrimeric, AMP-activated protein kinase monitors the energy and nutrient status *in individual cells* and shifts metabolism toward energy generation when necessary to maintain metabolic homeostasis. Furthermore, by responding to a variety of hormone signals, AMPK in the hypothalamus acts to keep the *whole organism* in energetic balance (Fig. 23-8).

AMPK monitors the energy status of a cell through its response to increased [AMP]/[ATP]. Many of the energy-consuming reactions in cells convert ATP to ADP or AMP. Adenylate kinase catalyzes the reaction 2ADP → AMP + ATP, so [AMP] is a sensitive measure of the cell's energy status. AMPK is allosterically activated by AMP binding, and ATP prevents AMP binding, so the enzyme is activated when the cell is energetically depleted (high [AMP]) and inactivated when energy is plentiful (high [ATP], and high [ATP]/[AMP]). AMPK responds to the energetic needs of the whole organism through a second mode of regulation. The enzyme is activated 100-fold by phosphorylation of Thr^{172} by liver kinase B1 (LKB1), which is itself subject to regulation by upstream components, including adiponectin. When activated by phosphorylation and AMP binding, AMPK phosphorylates specific enzymes in metabolic pathways that are crucial to energy homeostasis (Fig. 23-34).

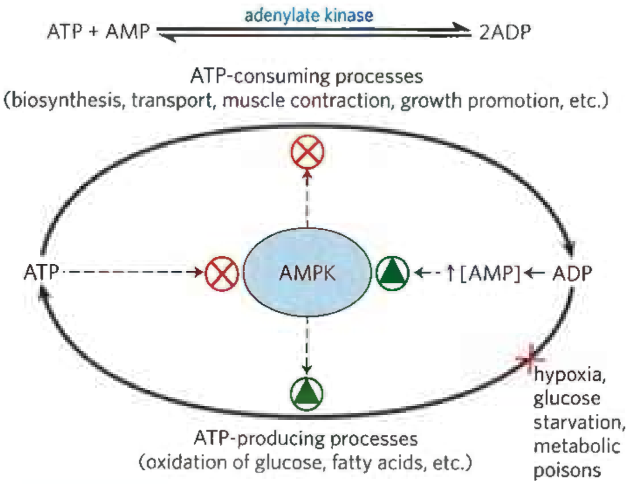

adenylate kinase
ATP + AMP
2ADP
ATP-consuming processes
(biosynthesis, transport, muscle contraction, growth promotion, etc.)
ATP
AMPK
↑[AMP]
ADP
hypoxia, glucose starvation, metabolic poisons
ATP-producing processes
(oxidation of glucose, fatty acids, etc.)

FIGURE 23-33 The role of AMP-activated protein kinase (AMPK) in maintaining energy homeostasis. ADP produced by synthetic reactions is converted to ATP and AMP by adenylate kinase. AMP activates AMPK, which reciprocally regulates ATP-consuming and ATP-generating pathways by phosphorylating key enzymes (see Fig. 23-34). Conditions or agents that inhibit ATP production by catabolic reactions (such as hypoxia, lack of glucose, or metabolic poisons) raise [AMP], activate AMPK, and stimulate catabolism. Cellular or organismal activities that consume ATP (muscle contraction, growth) increase [AMP] and stimulate catabolic reactions to replenish ATP. When [ATP] is high, ATP prevents AMP binding to AMPK, thus lowering AMPK activity and slowing catabolism.

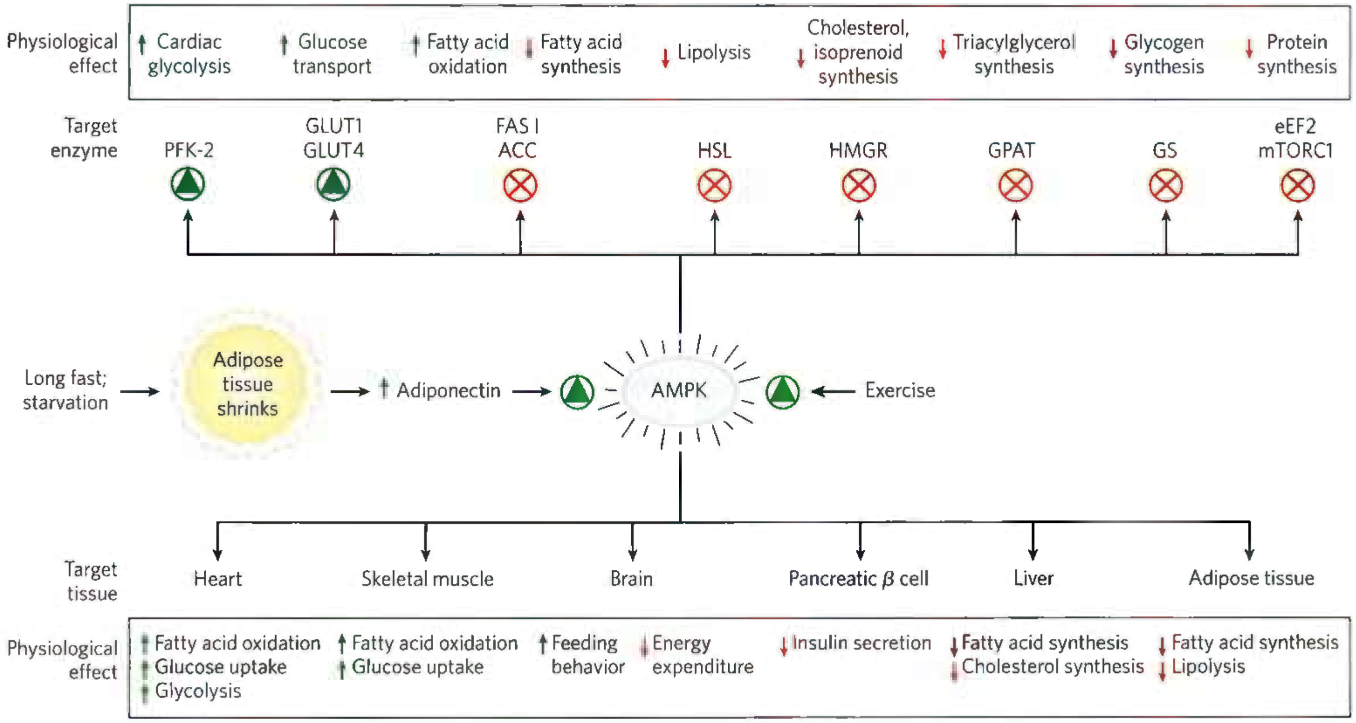

Physiological effect
↑ Cardiac glycolysis
↑ Glucose transport
↑ Fatty acid oxidation
↓ Fatty acid synthesis
↓ Lipolysis
↓ Cholesterol, isoprenoid synthesis
↓ Triacylglycerol synthesis
↓ Glycogen synthesis
↓ Protein synthesis
Target enzyme
PFK-2
GLUT1 GLUT4
FAS I ACC
HSL
HMGR
GPAT
GS
eEF2 mTORC1
Long fast; starvation
Adipose tissue shrinks
↑ Adiponectin
AMPK
Exercise
Target tissue
Heart
Skeletal muscle
Brain
Pancreatic β cell
Liver
Adipose tissue
Physiological effect
↑ Fatty acid oxidation
↑ Glucose uptake
↑ Glycolysis
↑ Fatty acid oxidation
↑ Glucose uptake
↑ Feeding behavior
↓ Energy expenditure
↓ Insulin secretion
↓ Fatty acid synthesis
↓ Cholesterol synthesis
↓ Fatty acid synthesis
↓ Lipolysis

FIGURE 23-34 Formation of adiponectin and its actions through AMPK. Extended fasting or starvation decreases triacylglycerol reserves in adipose tissue, which triggers adiponectin production and release from adipocytes. Adiponectin acts through its plasma membrane receptors in various cell types and organs to inhibit energy-consuming processes and stimulate energy-producing processes. It acts in the brain to stimulate feeding behavior and inhibit energy-consuming physical activity, and in brown fat to inhibit thermogenesis. Adiponectin exerts some of its metabolic effects by activating AMPK, which regulates (by phosphorylation) specific enzymes in key metabolic processes. PFK-2, phosphofructokinase-2; GLUT1 and GLUT4, glucose transporters; FAS I, fatty acid synthase I; ACC, acetyl-CoA carboxylase; HSL, hormone-sensitive lipase; HMGR, HMG-CoA reductase; GPAT, an acyl transferase; GS, glycogen synthase; eEF2, eukaryotic elongation factor 2 (required for protein synthesis; see Chapter 27); and mTORC1, mammalian target of rapamycin complex 1 (a protein kinase complex that regulates protein synthesis on the basis of nutrient availability). Exercise, through conversion of ATP to ADP and AMP, also stimulates AMPK.

When AMPK senses depletion of ATP in an individual cell, lipid synthesis is inhibited and use of lipid as fuel is stimulated. One enzyme regulated by AMPK in the liver and in white adipose tissue is acetyl-CoA carboxylase, which produces malonyl-CoA, the first intermediate committed to fatty acid synthesis. Malonyl-CoA is a powerful inhibitor of the enzyme carnitine acyltransferase 1, which starts the process of β oxidation by transporting fatty acids into the mitochondrion (see Fig. 17-6). By phosphorylating and inactivating acetyl-CoA carboxylase, AMPK inhibits fatty acid synthesis while relieving inhibition (by malonyl-CoA) of β oxidation (see Fig. 17-13). Cholesterol synthesis, a heavy energy consumer, is also inhibited by AMPK, which phosphorylates and inactivates HMG-CoA reductase, an enzyme central to sterol synthesis (see Fig. 21-34). Similarly, AMPK inhibits fatty acid synthase and acyl transferase, effectively blocking the synthesis of TAGs. In addition to its effects on lipid metabolism, AMPK inhibits the synthesis of glycogen and of protein (see Fig. 23-35). Inadequate supplies of oxygen (hypoxia) or blood glucose (hypoglycemia) are among the stressors that trigger AMPK activation.

In the hypothalamus, AMPK is positioned to receive a variety of signals from throughout the body (Fig. 23-8). Ghrelin and adiponectin signal "empty stomach" and "fat tissue depleted," and, like low blood glucose, they elicit hypothalamic signals, mediated by AMPK, that stimulate feeding and inhibit energy-requiring biosynthetic processes. Leptin, as we have seen, brings to the brain the signal "adipose tissue is full," slowing catabolic processes and favoring growth and biosynthesis.

At the cytoplasmic surface of lysosomes, AMPK interacts with a second central regulator of cellular activity, the protein kinase mTOR. This enzyme, at the center of an enormous complex, mTORC1, gauges whether sufficient nutrients and low molecular weight substrates are available to support cell growth and proliferation. Together, the two protein kinases, AMPK and mTOR, control major aspects of a cell's activity and fate.

The mTORC1 Pathway Coordinates Cell Growth with the Supply of Nutrients and Energy

The highly conserved Ser/Thr kinase **mTOR** forms a complex, **mTORC1**, with a scaffold protein, raptor, and other regulatory proteins. mTORC1 is recruited to the cytosolic surface of the lysosome through raptor by another complex, Ragulator, and a number of associated Rag G proteins that sense amino acid sufficiency in the cell. The Ragulator-Rag complex is tethered to the lysosomal membrane by covalently linked lipids. When mTORC1 is docked to the Ragulator-Rag complex, it is in contact with another G protein, **Rheb** (**Fig. 23-35**). This massive complex integrates signals from inside and outside of the lysosome about the energy status of the cell, the availability of critical amino acids needed for protein synthesis, and the presence of growth factors. When these signals indicate that the cell has what it needs to grow, GTP-bound Rheb activates the protein kinase activity of mTOR, which then phosphorylates many different proteins required for transcription, increased ribosome synthesis, and expression of genes encoding the enzymes of lipid synthesis and mitochondrial proliferation (**Fig. 23-36**). Because the lysosome is where the cell recycles defective or unneeded components, or extracellular substances brought into the cell through phagocytosis, it is, in effect, the warehouse of parts for cellular construction. The mTORC1-Ragulator-Rag complex is the supply chain manager determining whether the assembly line can operate.

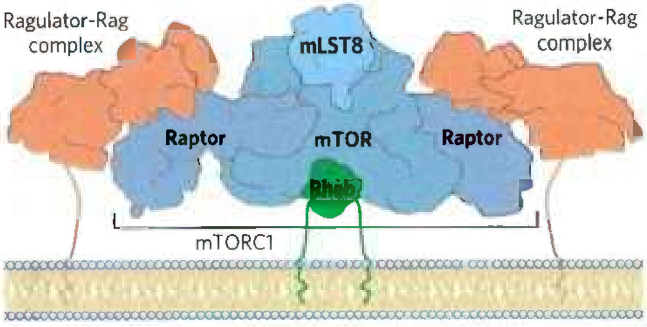


FIGURE 23-35 The mTORC1-Ragulator-Rag complex on the lysosomal surface. This model integrates the structures of mTORC1 and Ragulator-Rag, separately determined by cryo-EM. Raptor acts as a scaffold protein organizing the assembly, which puts the mTOR protein kinase in contact with the G protein Rheb for activation. [Information from J. H. Park et al., *Trends Biochem. Sci.* 45:367, 2019, Fig. 1; K. B. Rogala et al., *Science* 366:468, 2019, Fig. 5A.]

Fasting results in inactivation of mTORC1 by AMPK, leading to increased breakdown of protein and glycogen in the liver and muscle and mobilization of TAGs in adipose tissue. Chronic activation of mTORC1 by overeating results in excess deposition of TAGs in adipose tissue, as well as in liver and muscle, which may contribute to insulin insensitivity and type 2 diabetes. Mutations that produce constantly activated mTORC1 are commonly associated with human cancers.

Diet Regulates the Expression of Genes Central to Maintaining Body Mass

Proteins in a family of ligand-activated transcription factors known as **peroxisome proliferator-activated receptors (PPARs)** respond to changes in dietary lipid by altering the expression of genes involved in fat and carbohydrate metabolism. (These transcription factors were first recognized for their roles in peroxisome synthesis—thus their names.) Their normal ligands are fatty acids or fatty acid derivatives. PPARγ, PPARα, and PPARδ act in the nucleus by forming heterodimers with another nuclear receptor, RXR, then binding to regulatory regions of DNA near the genes under their control and changing the rate of transcription of those genes (**Fig. 23-37**).

PPARγ turns on genes that act in the differentiation of fibroblasts into adipocytes and genes that encode

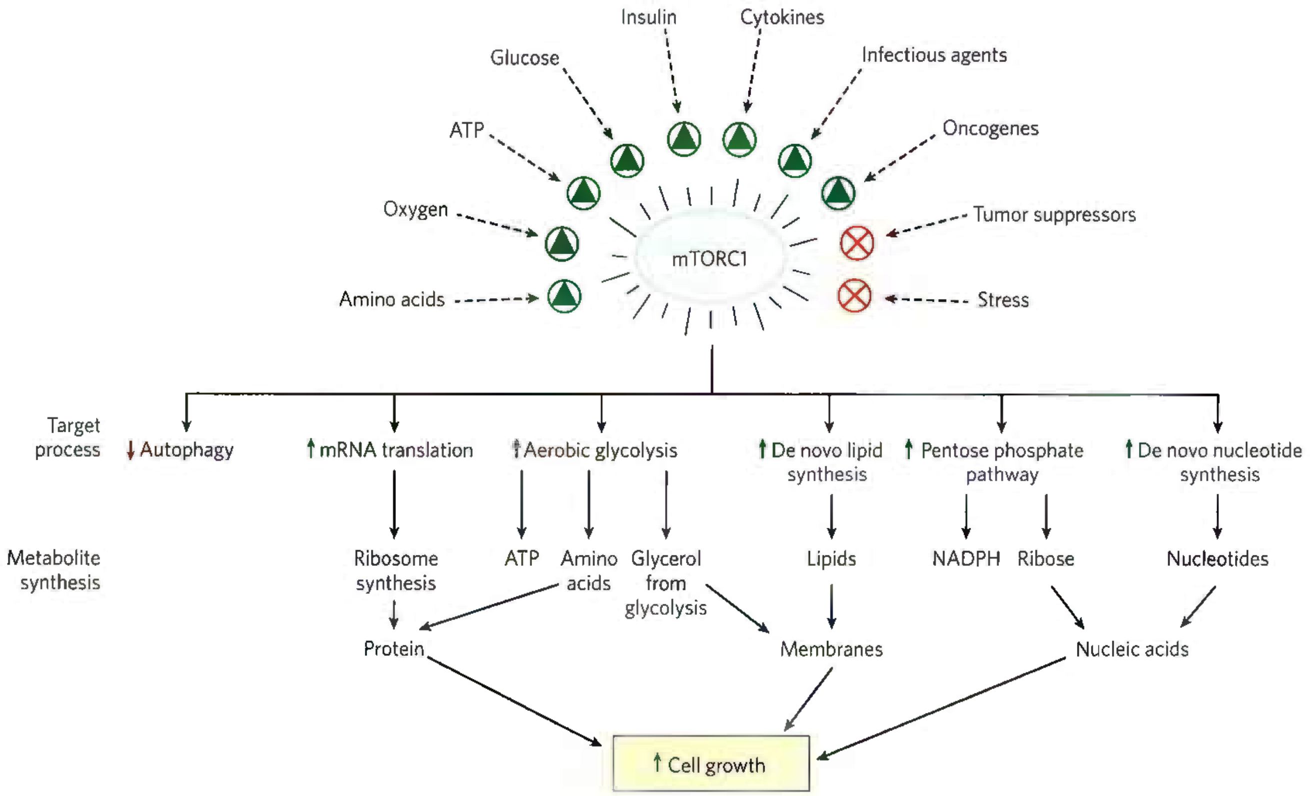

Insulin
Cytokines
Glucose
Infectious agents
ATP
Oncogenes
Oxygen
Tumor suppressors
mTORC1
Amino acids
Stress
Target process
↓ Autophagy
↑ mRNA translation
↑ Aerobic glycolysis
↑ De novo lipid synthesis
↑ Pentose phosphate pathway
↑ De novo nucleotide synthesis
Metabolite synthesis
Ribosome synthesis
ATP
Amino acids
Glycerol from glycolysis
Lipids
NADPH
Ribose
Nucleotides
Protein
Membranes
Nucleic acids
↑ Cell growth

FIGURE 23-36 A summary of mTORC1 activation signals and the cellular processes that active mTORC1 stimulates. The Ser/Thr protein kinase of mTORC1 is activated by the G protein Rheb, reflecting the integration of many signals that indicate that the cell is prepared for growth. By phosphorylating key target proteins, mTORC1 activates energy (ATP and NADPH) production for biosynthesis and stimulates the synthesis of proteins and lipids, allowing cell growth and proliferation.

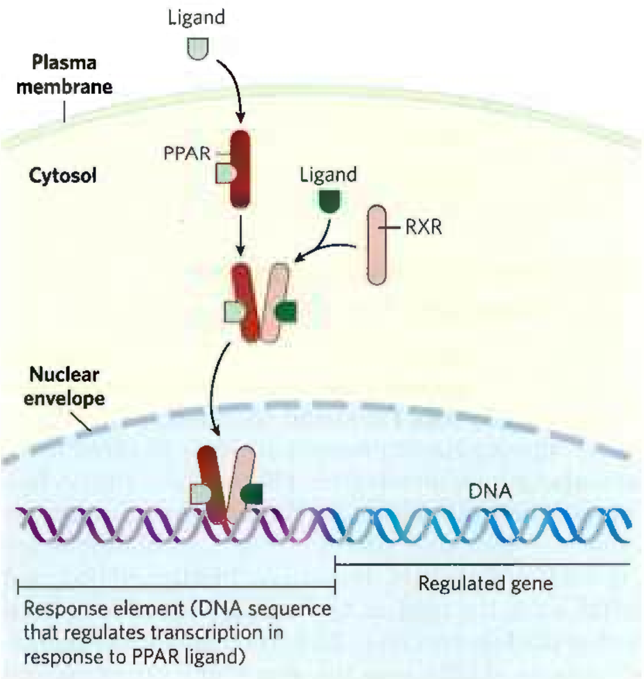

Ligand
Plasma membrane
PPAR
Cytosol
Ligand
RXR
Nuclear envelope
DNA
Regulated gene
Response element (DNA sequence that regulates transcription in response to PPAR ligand)

FIGURE 23-37 Mode of action of PPARs. PPARs are transcription factors that, when bound to their cognate ligand, form heterodimers with the nuclear receptor RXR. The dimer binds specific regions of DNA known as response elements, stimulating transcription of genes in those regions. [Information from R. M. Evans et al., *Nat. Med.* 10:355, 2004, Fig. 3.]

proteins required for lipid synthesis and storage in adipocytes (**Fig. 23-38**). PPARγ is activated by the thiazolidinedione drugs that are used to treat type 2 diabetes (discussed below).

PPARα is expressed in liver, kidney, heart, skeletal muscle, and brown adipose tissue. The ligands that activate this transcription factor include eicosanoids and free fatty acids. In hepatocytes, PPARα turns on the genes necessary for the uptake and β oxidation of fatty acids and for the formation of ketone bodies during fasting.

PPARδ is a key regulator of fat oxidation, which responds to changes in dietary lipid. PPARδ acts in liver and muscle, stimulating the transcription of at least nine genes encoding proteins for β oxidation and for energy dissipation through uncoupling of mitochondria. By stimulating fatty acid breakdown in uncoupled mitochondria, PPARδ causes fat depletion, weight loss, and thermogenesis.

Short-Term Eating Behavior Is Influenced by Ghrelin, PYY$_{3-36}$, and Cannabinoids

The peptide hormone **ghrelin** is produced in cells lining the stomach. It is a powerful appetite stimulant that works on a shorter time scale (between meals) than leptin and insulin. Ghrelin receptors are located in the hypothalamus, affecting appetite, as well as in heart muscle and adipose tissue. Ghrelin acts through a GPCR

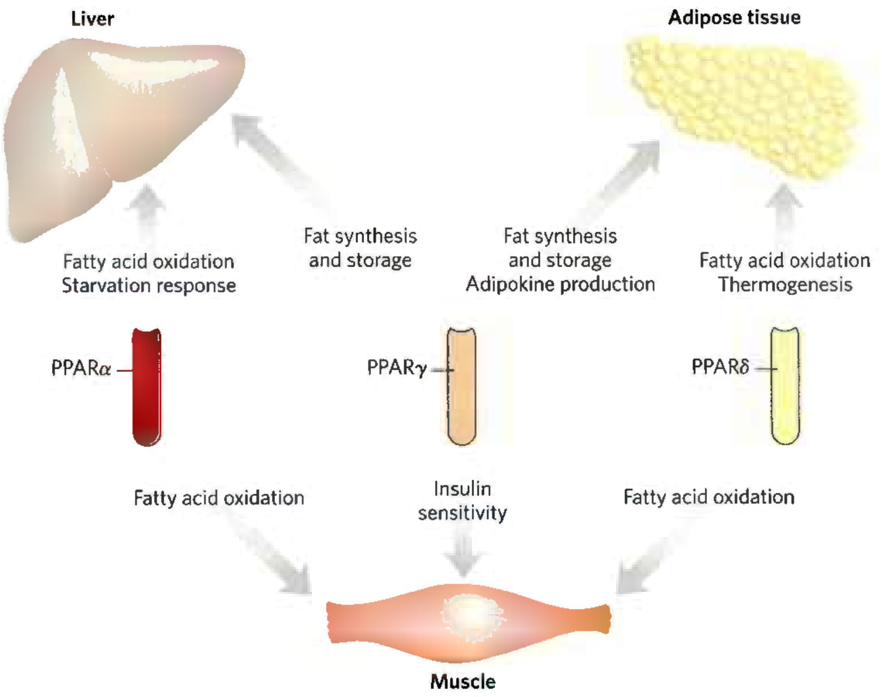

Liver
Adipose tissue
Fatty acid oxidation
Starvation response
Fat synthesis
and storage
Fat synthesis
and storage
Adipokine production
Fatty acid oxidation
Thermogenesis
PPARα
PPARγ
PPARδ
Fatty acid oxidation
Insulin
sensitivity
Fatty acid oxidation
Muscle

FIGURE 23-38 Metabolic integration by PPARs. The three PPAR isoforms regulate lipid and glucose homeostasis through their coordinated effects on gene expression in liver, muscle, and adipose tissue. PPARα and PPARδ regulate lipid utilization; PPARγ regulates lipid storage and the insulin sensitivity of various tissues.

to generate the second messenger IP_3, which mediates the hormone's action. The concentration of ghrelin in the blood fluctuates strikingly throughout the day, peaking just before a meal and dropping sharply just after a meal (**Fig. 23-39**). Injection of ghrelin into humans produces immediate sensations of intense hunger. Individuals with Prader-Willi syndrome, whose blood levels of ghrelin are exceptionally high, have an uncontrollable appetite, leading to extreme obesity that often results in death before the age of 30.

PYY_{3-36} is a peptide hormone (34 amino acid residues) secreted by endocrine cells in the lining of the small intestine and colon in response to food entering from the stomach. The level of PYY_{3-36} in the blood rises after a meal and remains high for some hours. The hormone is carried in the blood to the arcuate nucleus, where it acts on orexigenic neurons, inhibiting NPY release and reducing hunger (Fig. 23-32). Humans injected with PYY_{3-36} feel little hunger and eat less than normal amounts for about 12 hours.

Endocannabinoids (**Fig. 23-40**) are eicosanoid lipid messengers that act through specific GPCRs in the brain and peripheral nervous system to increase

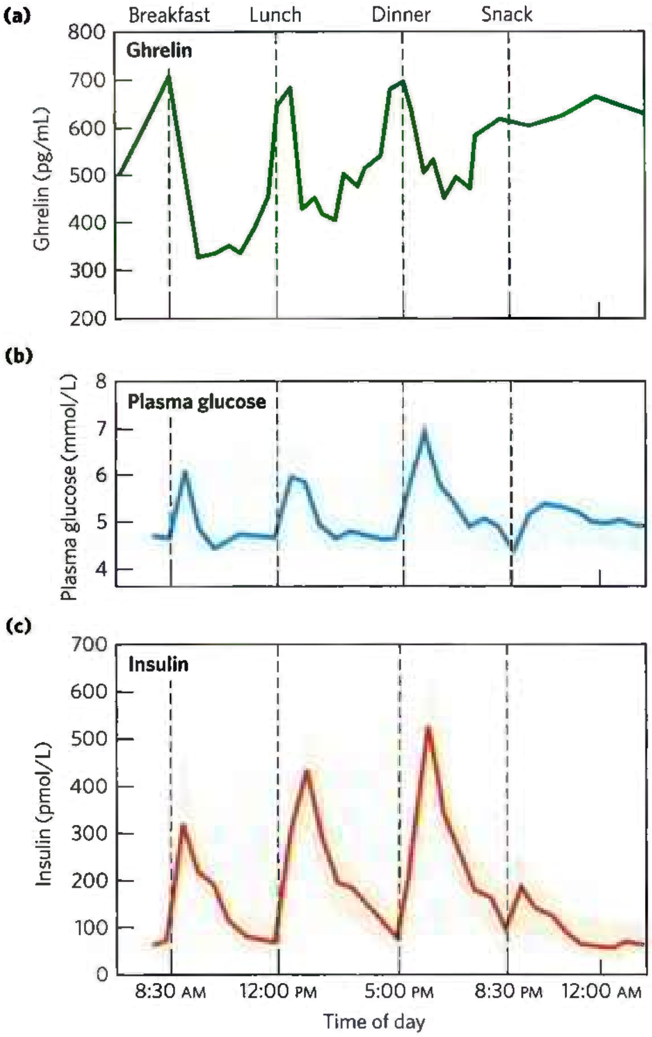
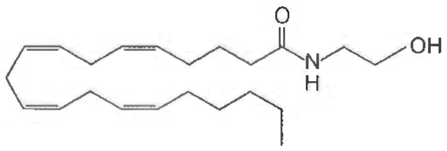
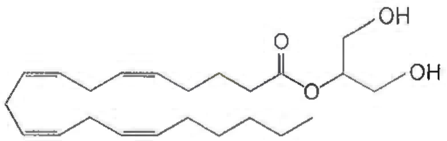


FIGURE 23-39 Variations in blood concentrations of glucose, ghrelin, and insulin relative to meal times. (a) Plasma levels of ghrelin rise sharply just *before* the usual time for meals (7 AM breakfast, 12 noon lunch, 5:30 PM dinner) and drop precipitously just after meals, paralleling subjective feelings of hunger. (b) Plasma glucose rises sharply *after* a meal, (c) followed immediately by a rise in insulin level in response to the increased blood glucose. [(a, c) Data from D. E. Cummings et al., *Diabetes* 50:1714, 2001, Fig. 1. (b) Data from M. D. Feher and C. J. Bailey, *Br. J. Diabet. Vasc. Dis.* 4:39, 2004.]

Endocannabinoids

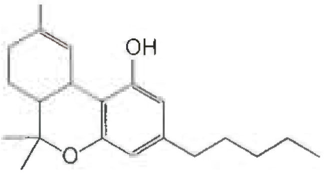


N-Arachidonoylethanolamide (NAE), anandamide

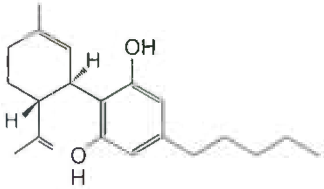


2-Arachidonoylglycerol (2-AG)

Exogenous cannabinoids



Tetrahydrocannabinol (THC)



Cannabidiol (CBD)

FIGURE 23-40 Cannabinoids. Two endocannabinoids produced by animals, and two products from the cannabis plant. Endocannabinoids carry retrograde signals: secreted by a postsynaptic cell, they diffuse across the synaptic cleft and activate GPCR receptors in the presynaptic cell. The receptors are of two types: CB_1 in the central nervous system, and CB_2 in the peripheral nervous system. Δ^9-Tetrahydrocannabinol (THC), the psychoactive compound in cannabis, is an agonist of both types of cannabinoid receptors in animals; cannabidiol (CBD) does not bind either.

appetite, heighten the sensory response to food (especially sweet and fatty foods), and elevate mood. When food enters the mouth, neuronal signals travel to the brain, and from there through the vagus nerve to the intestine, which then produces and releases endocannabinoids. The receptors for endocannabinoids control ion channels of sensory neurons, changing their membrane potentials and sending signals to the brain. Palatable food sensed in this way motivates further consumption of that food. The taste of fats (which are particularly high in caloric content) causes cannabinoid release, which effectively triggers further consumption. Well-conserved across vertebrate species, this system probably evolved to maximize the intake of food and to guard against starvation. In mammals, cannabinoid action stimulates an increase in fat mass and inhibits energy loss by motor activity or thermogenesis. Cannabinoid receptors also mediate the psychoactive effects of Δ^9-tetrahydrocannabinol (Fig. 23-40), the main active ingredient in marijuana, long known for its stimulating effect on appetite.

Microbial Symbionts in the Gut Influence Energy Metabolism and Adipogenesis

An adult human is host to about 10^{14} microbial cells that inhabit the gut. These microbes function as a major endocrine organ, producing a variety of metabolites with profound effects on host metabolism, feeding behavior, and body mass. Lean and obese individuals harbor different combinations of microbial symbionts in the gut. Investigation of this observation led to the discovery that gut microbes release fermentation products—the short-chain fatty acids acetate, propionate, butyrate, and lactate—that enter the bloodstream and trigger metabolic changes in adipose tissue (**Fig. 23-41**). Propionate, for example, drives the expansion of white adipose tissue by acting on GPCRs in the plasma membranes of several cell types, including adipocytes. These receptors trigger differentiation of precursor cells (preadipocytes) into adipocytes and inhibit lipolysis in existing adipocytes, leading to an increase in white adipose tissue mass—that is, obesity. Gut microbes also convert primary bile acids, synthesized in the liver, into the secondary bile acids deoxycholate and lithocholate, which enter the bloodstream and act through GPCRs and steroid receptors to activate beige adipocytes to produce UCP1 and increase energy expenditure.

These findings raise the possibility of preventing obesity by altering the makeup of the microbial community in the gut. Weight reduction might be accomplished either by adding, directly to the gut, microbial species (**probiotics**) that disfavor adipogenesis, or by adding to the diet nutrients (**prebiotics**) that favor the dominance of probiotic microbes. For example, experiments in mice have shown that fructans, polymers of fructose that are indigestible by animals, favor a specific microbial community. When this combination of microorganisms is present, fat storage in white adipose tissue and liver decreases, and there is none of the decrease in insulin sensitivity that is associated with obesity and lipid deposition in the liver (see below). Investigators have transplanted fecal material from lean mice to fat ones, and found that a new collection of microbes became established in the gut of the recipient animals, and the animals lost weight.

P4 Endocrine cells in the lining of the intestinal tract secrete peptides—the anorexigenic PYY_{3-36} and GLP-1 and the orexigenic ghrelin—that modulate food intake and energy expenditure. Interaction with specific microbes in the gut, or with their fermentation products, may trigger release of these peptides. Understanding how

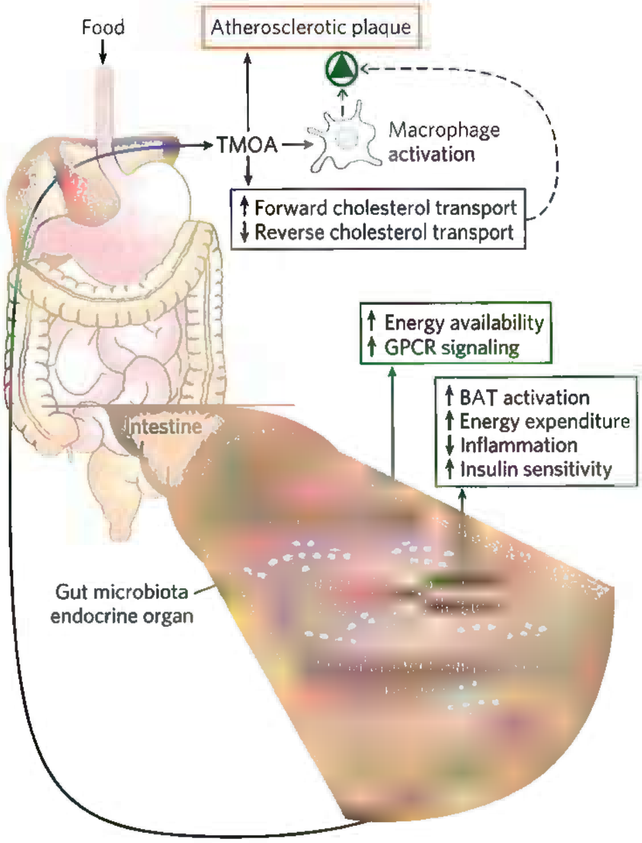


FIGURE 23-41 Effects of gut microbe metabolism on health. The enormous number and diversity of microorganisms (the microbiota) in the colon generate metabolic products that may have significant effects on health, both positive and negative. For example, the metabolism of primary bile acids by microbiota produces secondary products that act through nuclear receptors to stimulate thermogenesis in host brown adipose tissue (BAT) and increase both energy consumption and insulin sensitivity, while reducing inflammation. Metabolism of undigested carbohydrates by microbiota produces short-chain fatty acids (SCFAs) that signal expansion of the host's white adipose tissue (WAT), promoting obesity. SCFAs produced by microbiota are also a readily metabolizable energy source for the host. Microbial production of the SCFA propionate prevents lipogenesis in the liver and lowers blood cholesterol, both favorable to health. On the other hand, metabolic conversion of phosphatidylcholine and L-carnitine to trimethylamine (TMA), and its further conversion in liver to trimethylamine-*N*-oxide (TMAO), results in receptor-mediated changes in cholesterol transport and macrophage activity. The combination of altered sterol transport and increased macrophage activity can lead to formation of atherosclerotic plaque (see Fig. 21-46).

diet and microbial symbionts interact to affect energy metabolism and adipogenesis is an important key to understanding the development of obesity, metabolic syndrome, and type 2 diabetes and is a major challenge for the future.

The exquisitely interconnected system of neuroendocrine controls of food intake and metabolism presumably evolved to protect against starvation and to eliminate counterproductive accumulation of fat (extreme obesity). The difficulty most people face in trying to lose weight testifies to the remarkable effectiveness of these controls.

SUMMARY 23.4 *Obesity and the Regulation of Body Mass*

- Adipose tissue produces leptin, a hormone that regulates feeding behavior and energy expenditure so as to maintain adequate reserves of fat. Leptin production and release increase with the number and size of adipocytes.
- Leptin acts on receptors in the brain, causing the release of anorexigenic (appetite-suppressing) peptides, including α-MSH, that act in the brain to inhibit eating.
- Adiponectin stimulates fatty acid uptake and oxidation and inhibits fatty acid synthesis. It also sensitizes muscle and liver to insulin. At least some of the actions of adiponectin are mediated by AMPK, which is also activated by metabolic stress (low [AMP]), and by exercise.
- The protein kinase complex mTORC1 gauges the supply of essential amino acids and other metabolites, triggering cell growth if all required nutrients are available. It thus complements AMPK in determining the energetic status of a cell.
- Expression of the enzymes of lipid synthesis is under tight and complex regulation. PPARs are transcription factors that determine the rate of synthesis of many enzymes involved in lipid metabolism and adipocyte differentiation.
- Ghrelin, a hormone produced in the stomach, acts on orexigenic (appetite-stimulating) neurons in the arcuate nucleus to produce hunger before a meal. PYY_{3-36}, a peptide hormone of the intestine, acts at the same site to lessen hunger after a meal. Endocannabinoids signal the availability of sweet or fatty food and stimulate its consumption.
- Microbial symbionts in the gut produce fermentation products and secondary bile acids, which influence release of gut hormones that regulate body mass.

23.5 Diabetes Mellitus

Diabetes mellitus is a relatively common disease: about 9% of the U.S. population, and nearly 25% of the U.S. population over the age of 65, show some degree of abnormality in glucose metabolism that is indicative of diabetes or a tendency toward the condition. There are two major clinical classes of diabetes mellitus: **type 1 diabetes**, sometimes referred to as insulin-dependent diabetes mellitus (IDDM), and **type 2 diabetes**, or non-insulin-dependent diabetes mellitus (NIDDM), also called insulin-resistant diabetes. The discovery of insulin and its role in diabetes led to its development as a pharmaceutical, saving millions of lives (**Box 23-2**). ■

Diabetes Mellitus Arises from Defects in Insulin Production or Action

Type 1 diabetes usually begins early in life, and symptoms quickly become severe. This disease responds to insulin injection, because the metabolic defect

BOX 23-2 MEDICINE

The Arduous Path to Purified Insulin

Millions of people with type 1 diabetes mellitus inject themselves daily with pure insulin to compensate for the lack of production of this critical hormone by their own pancreatic β cells. Insulin injection is not a cure for diabetes, but it allows people who otherwise would have died young to lead long and productive lives. The discovery of insulin, which began with an accidental observation, illustrates the combination of serendipity and careful experimentation that led to the discovery of many hormones.

In 1889, Oskar Minkowski, a young assistant at the Medical College of Strasbourg, and Josef von Mering, at the Hoppe-Seyler Institute in Strasbourg, had a friendly disagreement about whether the pancreas, known to contain lipases, was important in fat digestion in dogs. To resolve the issue, they began an experiment on the digestion of fats. They surgically removed the pancreas from a dog, but before their experiment got any farther, Minkowski noticed that the dog was now producing far more urine than normal (a common symptom of untreated diabetes). Also, the dog's urine had glucose levels far above normal (another symptom of diabetes). These findings suggested that lack of some pancreatic product caused diabetes.

Minkowski tried unsuccessfully to prepare an extract of dog pancreas that would reverse the effect of removing the pancreas — that is, would lower the urinary or blood glucose levels. Despite considerable effort, no significant progress was made in the isolation or characterization of the "antidiabetic factor" until the summer of 1921, when Frederick G. Banting, a young scientist working in the laboratory of J. J. R. MacLeod at the University of Toronto, and a student assistant, Charles Best, took up the problem. By that time, several lines of evidence pointed to a group of specialized cells in the pancreas (the islets of Langerhans; see Fig. 23-23) as the source of the antidiabetic factor, which came to be called insulin (from Latin *insula*, "island").

Taking precautions to prevent proteolysis by the pancreatic proteases trypsin and chymotrypsin, Banting and Best (later aided by biochemist J. B. Collip) succeeded in December 1921 in preparing a purified pancreatic extract that cured the symptoms of experimentally induced diabetes in dogs. On January 25, 1922 (just one month later!), their insulin preparation was injected into Leonard Thompson, a 14-year-old boy severely ill with diabetes mellitus. Within days, the levels of ketone bodies and glucose in Thompson's urine dropped dramatically; the extract saved his life and the lives of other seriously ill children who also received these early preparations (Fig. 1). In 1923, Banting and MacLeod won the Nobel Prize for their isolation of insulin. Banting immediately announced that he would share his prize with Best; MacLeod shared his with Collip.

By 1923, pharmaceutical companies were supplying thousands of patients throughout the world with insulin extracted from porcine pancreas. With the development of genetic engineering techniques in the 1980s, it became possible to produce unlimited quantities of human insulin by inserting the cloned human gene for insulin into a microorganism, which was then cultured on an industrial scale. Many people with diabetes are now fitted with implanted insulin pumps, which release adjustable amounts of insulin on demand to meet changing needs at meal times and during exercise. There is a reasonable prospect that, in the future, transplanted pancreatic tissue will provide a source of insulin that responds as well as a normal pancreas, releasing insulin into the bloodstream only when blood glucose rises.

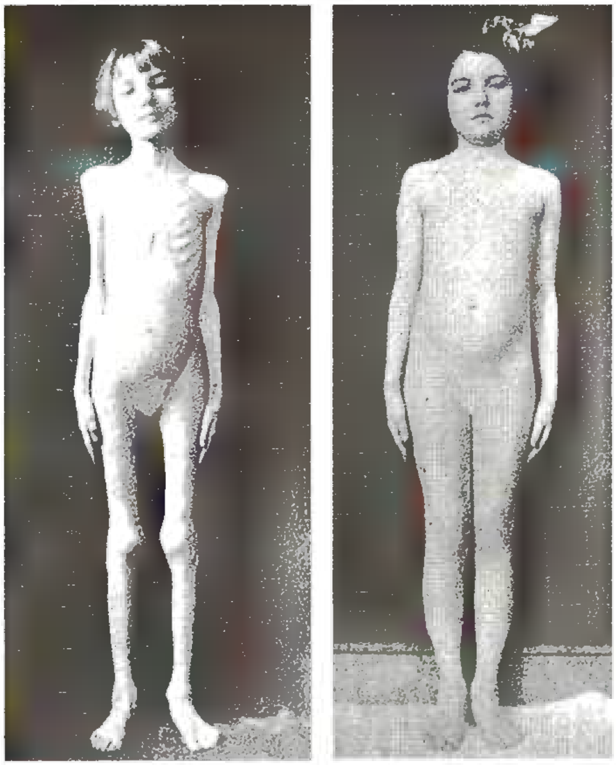


FIGURE 1 A child with type 1 diabetes, before (left) and after (right) three months of treatment with an early preparation of insulin. [H. R. Geyelin et al., *J. Metabol. Res.* 2:767, 1922.]

stems from an autoimmune destruction of pancreatic β cells and a consequent inability to produce sufficient insulin. Type 1 diabetes requires both insulin therapy and careful, lifelong control of the balance between dietary intake, activity, and insulin dose. Characteristic symptoms of untreated type 1 (and type 2) diabetes are excessive thirst and frequent urination, leading to the intake of large volumes of water. These symptoms are due to excretion of large amounts of glucose in the urine. ("Diabetes mellitus" means "excessive excretion of sweet urine.")

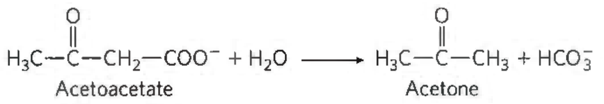
Type 2 diabetes is slower to develop (typically but not always in obese adults), and the symptoms are milder and often go unrecognized at first. This is really a group of diseases in which the regulatory activity of insulin is disordered: insulin is produced, but some feature of the insulin-response system is defective. Many individuals with this disorder are insulin-resistant. The connection between type 2 diabetes and obesity (discussed below) is an active area of research.

The pathology of diabetes includes cardiovascular disease, renal failure, blindness, and neuropathy. In 2019, the global mortality from diabetes was estimated at 4.2 million and rising. It is essential to understand diabetes and its relationship to obesity and to find countermeasures that prevent or reverse the damage done by this disease.

P5 Individuals with either type of diabetes are unable to take up glucose efficiently from the blood. Recall that insulin triggers the movement of GLUT4 glucose transporters to the plasma membrane in muscle and adipose tissue (see Fig. 12-23 and Box 11-1). Biochemical measurements on blood and urine samples are essential in the diagnosis and treatment of diabetes. A sensitive diagnostic criterion is the level of HbA1c, a glucose derivative of hemoglobin, which forms in the blood and reflects the average blood glucose level (see Box 7-2). Another measurement to confirm the diagnosis of diabetes is the **glucose-tolerance test**. The individual fasts overnight, then drinks a test dose of 100 g of glucose dissolved in a glass of water. The blood glucose concentration is measured before the test dose and at 30 min intervals for several hours thereafter. A healthy individual assimilates the glucose readily, the blood glucose rising to no more than about 9 or 10 mM; little or no glucose appears in the urine. In diabetes, individuals assimilate the test dose of glucose poorly; their blood glucose level rises dramatically and returns to the fasting level very slowly. Because the blood glucose levels exceed the kidney threshold (about 10 mM), glucose also appears in the urine. ■

Carboxylic Acids (Ketone Bodies) Accumulate in the Blood of Those with Untreated Diabetes

With glucose unavailable to cells, fatty acids become the principal fuel, which leads to another characteristic metabolic change in diabetes: excessive but incomplete oxidation of fatty acids in the liver. The acetyl-CoA produced by β oxidation cannot be completely oxidized by the citric acid cycle, because the high [NADH]/[NAD$^+$] ratio produced by β oxidation inhibits the cycle (recall that three steps of the cycle convert NAD$^+$ to NADH). Accumulation of acetyl-CoA leads to overproduction of the ketone bodies β-hydroxybutyrate and acetoacetate, which cannot be used by extrahepatic tissues as fast as they are made in the liver (see Figs. 17-15, 17-16). In addition to β-hydroxybutyrate and acetoacetate, the blood of individuals with diabetes contains small amounts of acetone, which results from the spontaneous decarboxylation of acetoacetate:



$$H_3C-\overset{\overset{\displaystyle O}{\|}}{C}-CH_2-COO^- + H_2O \longrightarrow H_3C-\overset{\overset{\displaystyle O}{\|}}{C}-CH_3 + HCO_3^-$$

Acetoacetate

Acetone

The overproduction of ketone bodies, called **ketosis**, results in greatly increased concentrations of ketone bodies in the blood (ketonemia) and urine (ketonuria). The ketone bodies are carboxylic acids, which ionize, releasing protons. In uncontrolled diabetes, this acid production can overwhelm the capacity of the blood's bicarbonate buffering system and produce a lowering of blood pH called **acidosis** or, in combination with ketosis, **ketoacidosis**, a potentially life-threatening condition. ■

In Type 2 Diabetes the Tissues Become Insensitive to Insulin

In the industrialized world, where the food supply is more than adequate, there is a growing epidemic of obesity and the type 2 diabetes associated with it. P5 The hallmark of **type 2 diabetes** is the development of insulin resistance, a state in which more insulin is needed to bring about the biological effects produced by a lower concentration of insulin in the normal, healthy state. In the early stages of the disease, pancreatic β cells secrete enough insulin to overcome the lower insulin sensitivity of muscle and liver. But the β cells eventually fail, and the lack of insulin becomes apparent in the body's inability to regulate blood glucose. The intermediate stage, preceding type 2 diabetes mellitus, is sometimes called **metabolic syndrome**. This is typified by obesity, especially in the abdomen; hypertension (high blood pressure); abnormal blood lipids (high TAG and LDL, low HDL); slightly high fasting blood glucose; and a reduced ability to clear glucose in the glucose-tolerance test. Individuals with metabolic syndrome often also show changes in blood proteins associated with abnormal clotting (high fibrinogen concentration) or inflammation (high concentration of the C-reactive peptide, not to be confused with the C peptide generated during the proteolytic maturation of insulin). About 30% of the adult population in the United States has these indicators of metabolic syndrome.

What predisposes individuals with metabolic syndrome to develop type 2 diabetes? According to the "lipid toxicity" hypothesis (**Fig. 23-42**), the action of PPARδ on adipocytes normally keeps the cells ready to synthesize and store triacylglycerols — the adipocytes are insulin-sensitive and produce leptin, which leads to their continued intracellular deposition of TAGs. However, excess caloric intake in obese individuals causes adipocytes to become filled with TAGs, leaving adipose tissue unable to meet any further demand for TAG storage. Lipid-filled adipose tissue releases protein factors, including MCP-1

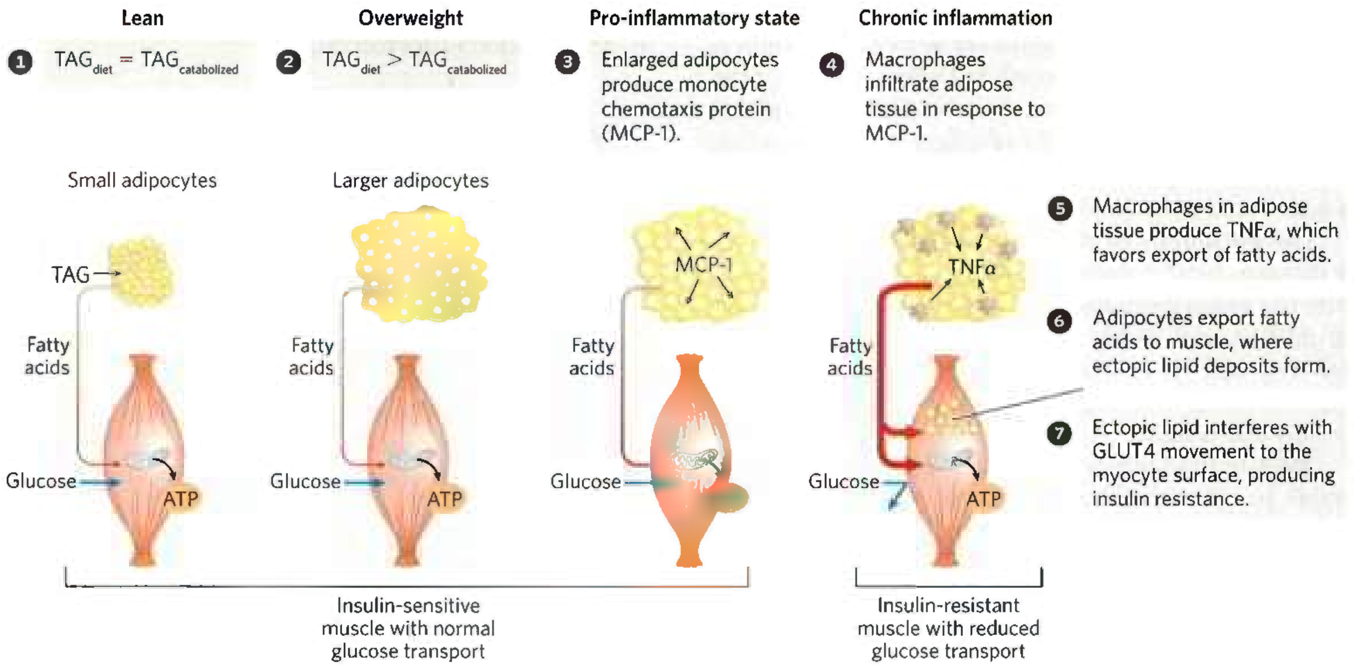

Lean
Overweight
Pro-inflammatory state
Chronic inflammation
① $TAG_{diet} = TAG_{catabolized}$
② $TAG_{diet} > TAG_{catabolized}$
③ Enlarged adipocytes produce monocyte chemotaxis protein (MCP-1).
④ Macrophages infiltrate adipose tissue in response to MCP-1.
Small adipocytes
Larger adipocytes
TAG
MCP-1
TNFα
⑤ Macrophages in adipose tissue produce TNFα, which favors export of fatty acids.
⑥ Adipocytes export fatty acids to muscle, where ectopic lipid deposits form.
⑦ Ectopic lipid interferes with GLUT4 movement to the myocyte surface, producing insulin resistance.
Fatty acids
Glucose
ATP
Insulin-sensitive muscle with normal glucose transport
Insulin-resistant muscle with reduced glucose transport

FIGURE 23-42 Overloading adipocytes with triacylglycerols triggers inflammation in fat tissue and ectopic lipid deposition and insulin resistance in muscle. In an individual of healthy body mass, dietary TAG uptake equals TAG oxidation for energy. In overweight individuals, excess caloric intake results in enlarged adipocytes, engorged with TAG and unable to store more. Enlarged adipocytes secrete MCP-1 (monocyte chemotaxis protein-1), attracting macrophages. Macrophages infiltrate the adipose tissue and produce TNFα (tumor necrosis factor α), which triggers lipid breakdown and release of fatty acids into the blood. The fatty acids enter myocytes, where they accumulate in small lipid droplets. This ectopic lipid storage in muscle somehow causes insulin resistance, perhaps by triggering lipid-activated protein kinases that inactivate some element in the insulin-signaling pathway. GLUT4 glucose transporters leave the myocyte surface, preventing glucose entry into muscle; the myocyte has now become insulin-resistant. It cannot use blood glucose for its fuel, so fatty acids are mobilized from adipose tissue and become the primary fuel. The increased influx of fatty acids into muscle leads to further deposition of ectopic lipids. In some individuals, insulin resistance develops into type 2 diabetes. [Information from A. Guilherme et al., *Mol. Cell Biol.* 9:367, 2008, Fig. 1.]

(monocyte chemotaxis protein-1), that attract macrophages, which infiltrate the tissue and may eventually represent as much as 50% of the adipose tissue by mass. Macrophages trigger the inflammatory response mediated by TNFα release, which impairs TAG deposition in adipocytes and favors release of free fatty acids into the blood. These excess fatty acids enter liver and muscle cells, where they are converted to TAGs that accumulate as lipid droplets. This ectopic (Greek *ektopos*, "out of place") deposition of TAGs inhibits GLUT4 transporter movement to the plasma membrane, which leads to insulin insensitivity in liver and muscle, the hallmark of type 2 diabetes.

According to this hypothesis, excess stored fatty acids and TAGs are toxic to liver and muscle. Some individuals are less well equipped genetically to handle this burden of ectopic lipids and are more susceptible to the cellular damage that leads to development of type 2 diabetes. Insulin resistance probably involves impairment of several of the mechanisms by which insulin acts on metabolism, which include changes in protein levels and changes in the activities of signaling enzymes and transcription factors. For example, both adiponectin synthesis in adipocytes and adiponectin levels in the blood decrease with obesity and increase with weight loss.

There are genetic factors that predispose individuals to type 2 diabetes. Although 80% of people with type 2 diabetes are obese, most obese individuals do not develop type 2 diabetes. Given the complexity of the regulatory mechanisms we have discussed in this chapter, it is not surprising that the genetics of diabetes is complex, involving interactions among variant genes and environmental factors, including diet and lifestyle. A growing number of genetic loci have been reliably linked to type 2 diabetes; variation in any of these "diabetogenes" alone would cause a relatively small increase in the likelihood of developing type 2 diabetes. ■

Type 2 Diabetes Is Managed with Diet, Exercise, Medication, and Surgery

Studies show that at least four factors improve the health of individuals with type 2 diabetes: dietary restriction, regular exercise, drugs that increase insulin sensitivity or insulin production, and surgery that reroutes food passage through the gastrointestinal tract. Dietary restriction (and accompanying weight loss) reduces the overall burden of handling fatty acids. The lipid composition of the diet influences, through

TABLE 23-7 Treatments for Type 2 Diabetes Mellitus

Intervention/treatment	Direct target	Effect of treatment
Weight loss	Adipose tissue; reduction in TAG content	Reduces lipid burden; increases capacity for lipid storage in adipose tissue; restores insulin sensitivity
Exercise	AMPK, activated by increasing [AMP]/[ATP]	Aids weight loss (see Fig. 23-34)
Bariatric surgery	Unknown	Leads to weight loss, better control of blood glucose
Sulfonylureas: glipizide (Glucotrol), glyburide (Micronase), glimepiride (Amaryl)	Pancreatic β cells; K^+ channels blocked	Stimulates insulin secretion by pancreas (see Fig. 23-24)
Biguanides: metformin (Glucophage)	AMPK, activated	Increases glucose uptake by muscle; decreases glucose production in liver
Thiazolidinediones: rosiglitazone (Avandia), pioglitazone (Actos)	PPARγ	Stimulates expression of genes potentiating the action of insulin in liver, muscle, adipose tissue; increases glucose uptake; decreases glucose synthesis in liver
GLP-1 modulators: exenatide (Byetta), sitagliptin (Januvia), dulaglutide (Trulicity)	Glucagon-like peptide-1, dipeptide protease IV	Enhances insulin secretion by pancreas

PPARs and other transcription factors, the expression of genes that encode proteins involved in burning fat.

Exercise contributes to weight loss directly by consuming calories. Exercise also increases release of **irisin** from muscle into the blood. Irisin increases the expression of *UCP1* genes in white adipose tissue and also stimulates the development of beige adipocytes, so that even after the exercise ends, energy continues to be used in thermogenesis. Exercise activates AMPK, as does adiponectin; AMPK shifts metabolism toward fat oxidation and inhibits fat synthesis.

Several classes of drugs are used in the management of type 2 diabetes (Table 23-7). Their targets include AMPK, the K^+ channels in β cells, PPARs, and GLP receptors.

In cases of extreme obesity, dramatic weight loss can be achieved by bariatric surgery, which reroutes food movement through the stomach and small intestine. In many cases, this procedure also moderates or even reverses type 2 diabetes. In Roux-en-Y gastric bypass (RYGBP, named for César Roux, the Swiss surgeon who developed the procedure), the stomach is reduced to a small pouch attached to the esophagus, and the middle region of the small intestine (the jejunum) is attached directly to the pouch. Food bypasses most of the stomach and the duodenum, and goes primarily to the "Roux limb" of the intestine. Stomach acid and digestive enzymes travel through bypassed portions of the gut to join the food in the common channel. People who undergo RYGBP surgery not only experience dramatic weight loss but also are less hungry. Remarkably, this surgery also reverses type 2 diabetes in many cases. The explanations for these effects are likely to lie in altered communication among the gut, the brain, and other organs. This may result from changes in the kind and amount of peptide hormones (such as GLP-1 and PYY$_{3-36}$) secreted in the intestine that signal satiety and inhibit feeding behavior. The last word has not been written on this issue. ■

SUMMARY 23.5 *Diabetes Mellitus*

- Metabolic syndrome, which includes obesity, hypertension, elevated blood lipids, and insulin resistance, is often the prelude to type 2 diabetes. Uncontrolled diabetes is characterized by high glucose levels in the blood and urine and the production and excretion of ketone bodies.
- In diabetes, insulin is either not produced or not recognized by the tissues, and the uptake of blood glucose is defective. Lacking access to glucose, cells rely on fatty acid oxidation, which results in ketone body formation, producing ketoacidosis.
- The insulin resistance that characterizes type 2 diabetes may be a consequence of abnormal lipid storage in muscle and liver, in response to a lipid intake that cannot be accommodated by adipose tissue.
- Effective treatments for type 2 diabetes include exercise, appropriate diet, and drugs that increase insulin sensitivity or insulin production. Surgical alteration of the digestive tract leads to weight loss and often reverses type 2 diabetes.

KEY TERMS

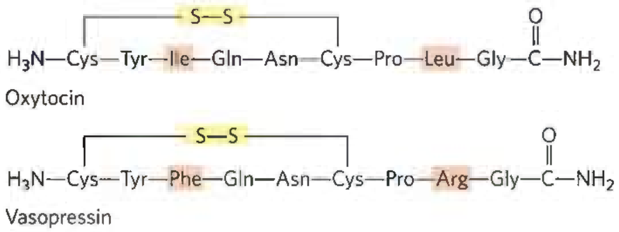
Terms in bold are defined in the glossary.

hormone	842	**endocrine**	843
neuroendocrine system	842	paracrine	843
metabotropic	843	autocrine	844
ionotropic	843	hypothalamus	845
		hepatocyte	849

white adipose tissue (WAT) 851
adipocyte 851
brown adipose tissue (BAT) 852
uncoupling protein 1 (UCP1) 852
thermogenesis 852
beige adipose tissue 852
myocyte 852
plasma proteins 858
ATP-gated K^+ channels 860
sulfonylurea drugs 861
glucagon 862
cortisol 865
body mass index (BMI) 867
adipokines 867
leptin 867
arcuate nucleus 868
orexigenic 868
anorexigenic 868
α-melanocyte–stimulating hormone (α-MSH) 868
adiponectin 869
AMP-activated protein kinase (AMPK) 869
mTORC1 (mechanistic target of rapamycin complex 1) 871
PPAR (peroxisome proliferator-activated receptor) 871
ghrelin 872
endocannabinoids 873
probiotics 874
prebiotics 874
diabetes mellitus 875
type 1 diabetes 875
type 2 diabetes 875
glucose-tolerance test 877
ketosis 877
acidosis 877
ketoacidosis 877
metabolic syndrome 877

PROBLEMS

1. **Peptide Hormone Activity** Explain how two peptide hormones as structurally similar as oxytocin and vasopressin can have such different effects (see Fig. 23-8).


S—S
H₃N—Cys—Tyr—Ile—Gln—Asn—Cys—Pro—Leu—Gly—C—NH₂
O
Oxytocin
S—S
H₃N—Cys—Tyr—Phe—Gln—Asn—Cys—Pro—Arg—Gly—C—NH₂
O
Vasopressin

2. **Metabolism of Glutamate in the Brain** Brain tissue takes up glutamate from the blood, transforms it into glutamine, and releases the glutamine into the blood. What does this metabolic conversion accomplish? How does this conversion take place? The amount of glutamine produced in the brain can exceed the amount of glutamate entering from the blood. How does this extra glutamine arise? (Hint: You may want to review amino acid catabolism in Chapter 18; recall that NH_4^+ is very toxic to the brain.)

3. **Proteins as Fuel during Fasting** When muscle proteins undergo catabolism in skeletal muscle during a fast, what are the fates of the amino acids?

4. **Absence of Glycerol Kinase in Adipose Tissue** The biosynthesis of triacylglycerols requires glycerol 3-phosphate. Adipocytes, specialized for the synthesis and degradation of TAGs, cannot use glycerol directly because they lack glycerol kinase, which catalyzes the reaction

$$\text{Glycerol} + \text{ATP} \longrightarrow \text{glycerol 3-phosphate} + \text{ADP}$$

How does adipose tissue obtain the glycerol 3-phosphate necessary for TAG synthesis?

5. **Oxygen Consumption during Exercise** A sedentary adult consumes about 0.05 L of O_2 in 10 seconds. A sprinter running a 100 m race consumes about 1 L of O_2 in 10 seconds. After finishing the race, the sprinter continues to breathe at an elevated (but declining) rate for some minutes, consuming an extra 4 L of O_2 above the amount consumed by the sedentary individual.
(a) Why does the need for O_2 increase dramatically during the sprint?
(b) Why does the demand for O_2 remain high after the sprinter finishes the race?

6. **Thiamine Deficiency and Brain Function** Individuals with thiamine deficiency show some characteristic neurological signs and symptoms, including loss of reflexes, anxiety, and mental confusion. Why might thiamine deficiency manifest as changes in brain function?

7. **Potency of Hormones** Under normal conditions, the human adrenal medulla secretes epinephrine ($C_9H_{13}NO_3$) at a rate sufficient to maintain a concentration of 10^{-10} M in circulating blood. To appreciate what that concentration means, calculate the volume of water that you would need to dissolve 1.0 g (about 1 teaspoon) of epinephrine to a concentration equal to that in blood.

8. **Regulation of Hormone Levels in the Blood** The half-life of most hormones in the blood is relatively short. For example, when researchers inject radioactively labeled insulin into an animal, half of the labeled hormone disappears from the blood within 30 min.
(a) What is the importance of the relatively rapid inactivation of circulating hormones?
(b) In what ways can the organism make rapid changes in the level of a circulating peptide hormone?

9. **Water-Soluble versus Lipid-Soluble Hormones** On the basis of their physical properties, hormones fall into one of two categories: those that are very soluble in water but relatively insoluble in lipids (e.g., epinephrine) and those that are relatively insoluble in water but highly soluble in lipids (e.g., steroid hormones). In their role as regulators of cellular activity, most water-soluble hormones do not enter their target cells. The lipid-soluble hormones, by contrast, do enter their target cells and ultimately act in the nucleus. What is the relationship between solubility, the location of receptors, and the mode of action of these two classes of hormones?

10. **Metabolic Differences between Muscle and Liver in a "Fight-or-Flight" Situation** When an animal confronts a "fight-or-flight" situation, the release of epinephrine promotes glycogen breakdown in the liver and skeletal muscle. The end product of glycogen breakdown in the liver is glucose; the end product in skeletal muscle is pyruvate.
(a) What is the reason for the different products of glycogen breakdown in the two tissues?
(b) What is the advantage of these specific glycogen breakdown routes to an animal that must fight or flee?

11. **Excessive Amounts of Insulin Secretion: Hyperinsulinism** Certain malignant tumors of the pancreas cause excessive production of insulin by β cells. Affected

individuals exhibit shaking and trembling, weakness and fatigue, sweating, and hunger.
(a) What is the effect of hyperinsulinism on the metabolism of carbohydrates, amino acids, and lipids by the liver?
(b) What are the causes of the observed symptoms? Suggest why this condition, if prolonged, leads to brain damage.

12. Thermogenesis Caused by Thyroid Hormones Thyroid hormones are intimately involved in regulating the basal metabolic rate. Liver tissue of animals given excess thyroxine shows an increased rate of O_2 consumption and increased heat output (thermogenesis), but the ATP concentration in the tissue is normal. Different explanations have been offered for the thermogenic effect of thyroxine. One is that excess thyroxine causes uncoupling of oxidative phosphorylation in mitochondria. How could such an effect account for the observations? Another explanation suggests that thermogenesis is due to an increased rate of ATP utilization by the thyroxine-stimulated tissue. Is this a reasonable explanation? Why or why not?

13. Function of Prohormones What are the possible advantages of synthesizing hormones as prohormones?

14. Sources of Glucose during Starvation The typical human adult uses about 160 g of glucose per day. Of this, the brain alone uses 120 g. The body's available reserve of glucose (~20 g of circulating glucose and ~190 g of glycogen) is adequate for about one day. After the glucose reserve has been depleted during starvation, how does the body obtain more glucose?

15. Parabiotic *ob/ob* Mice By careful surgery, researchers can connect the circulatory systems of two mice so that the same blood circulates through both animals. In these **parabiotic** mice, products released into the blood by one animal reach the other animal via the shared circulation. Both animals are free to eat independently. Suppose a researcher parabiotically joins a mutant *ob/ob* mouse (both copies of the *OB* gene are defective) and a normal *OB/OB* mouse (both copies of the *OB* gene are functional). What would happen to the weight of each mouse in the parabiotic pair?

16. Calculation of Body Mass Index A biochemistry professor weighs 260 lb (118 kg) and is 5 feet 8 inches (173 cm) tall. What is his body mass index (BMI)? How much weight would he have to lose to bring his BMI down to 25 (normal)?

17. Insulin Secretion Predict the effects on insulin secretion by pancreatic β cells of exposure to the potassium ionophore valinomycin. Explain your prediction.

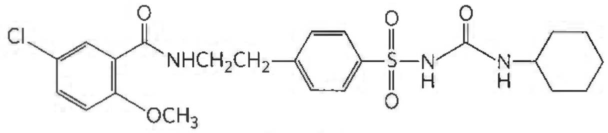
18. Effects of a Deleted Insulin Receptor A strain of mice specifically lacking the insulin receptor of liver is found to have mild fasting hyperglycemia (blood glucose = 132 mg/dL, vs. 101 mg/dL in controls) and a more striking hyperglycemia in the fed state (glucose = 363 mg/dL, vs. 135 mg/dL in controls). The mice have higher than normal levels of glucose 6-phosphatase in the liver and elevated levels of insulin in the blood. Explain these observations.

19. Decisions on Drug Safety The drug rosiglitazone (Avandia) is effective in lowering blood glucose levels in patients with type 2 diabetes, but a few years after rosiglitazone came into widespread use, it seemed that using the drug came with an increased risk of heart attack. In response, the U.S. Food and Drug Administration (FDA) severely restricted the conditions under which it could be prescribed. Two years later, after additional studies had been completed, the FDA lifted the restrictions, and today rosiglitazone is available by prescription in the United States, with no special limitations. Many other countries ban it completely. If it were your responsibility to decide whether this drug should remain on the market (labeled with suitable warnings about its side effects) or should be withdrawn from the market altogether, what factors would you weigh in making your decision?

20. Type 2 Diabetes Medication The drugs acarbose (Precose) and miglitol (Glyset), used in the treatment of type 2 diabetes mellitus, inhibit α-glucosidases in the brush border of the small intestine. These enzymes degrade oligosaccharides derived from glycogen or starch to monosaccharides. Suggest a possible mechanism for the salutary effect of these drugs for individuals with diabetes. What side effects, if any, would you expect from these drugs? Why? (Hint: Review lactose intolerance, p. 523.)

DATA ANALYSIS PROBLEM

21. Cloning the Sulfonylurea Receptor of the Pancreatic β Cell Glyburide, a member of the sulfonylurea family of drugs, is used to treat type 2 diabetes. It binds to and closes the ATP-gated K^+ channel shown in Figures 23-26 and 23-27.

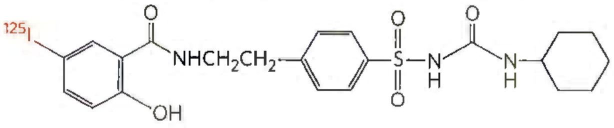


Glyburide

(a) Given the mechanism shown in Figure 23-27, would treatment with glyburide result in increased or decreased insulin secretion by pancreatic β cells? Explain your reasoning.
(b) How does treatment with glyburide help reduce the symptoms of type 2 diabetes?
(c) Would you expect glyburide to be useful for treating type 1 diabetes? Explain your answer.

Aguilar-Bryan and coauthors (1995) cloned the gene for the sulfonylurea receptor (SUR) portion of the ATP-gated K^+ channel from hamsters. The research team went to great lengths to ensure that the gene they cloned was, in fact, the SUR-encoding gene. Here we explore how it is possible for researchers to demonstrate that they have cloned the gene of interest rather than another gene.

The first step was to obtain pure SUR protein. As was already known, drugs such as glyburide bind SUR with very high affinity (K_d < 10 nM), and SUR has a molecular weight of 140 to 170 kDa. Aguilar-Bryan and coworkers made use of the high-affinity glyburide binding to tag the SUR protein with a radioactive label that would serve as a marker to purify the protein from a cell extract. First, they made a radiolabeled derivative of glyburide, using radioactive iodine (^{125}I):



[^{125}I]5-Iodo-2-hydroxyglyburide

(d) In preliminary studies, the ^{125}I-labeled glyburide derivative (hereafter, [^{125}I]glyburide) was shown to have the same K_d and binding characteristics as unaltered glyburide. Why was it necessary to demonstrate this? (What alternative possibilities did it rule out?)

Even though [^{125}I]glyburide bound to SUR with high affinity, a significant amount of the labeled drug would probably dissociate from the SUR protein during purification. To prevent this, [^{125}I]glyburide had to be covalently cross-linked to SUR. There are many methods for covalent cross-linking; Aguilar-Bryan and coworkers used UV light. When aromatic molecules are exposed to short-wave UV, they enter an excited state and readily form covalent bonds with nearby molecules. By cross-linking the radiolabeled glyburide to the SUR protein, the researchers could simply track the ^{125}I radioactivity to follow SUR through the purification procedure.

The research team treated hamster HIT cells (which express SUR) with [^{125}I]glyburide and UV light, purified the ^{125}I-labeled 140 kDa protein, and sequenced its 25 residue amino-terminal segment; they found the sequence PLAFCGTENHSAAYRVDQGVLNNGC. The investigators then generated antibodies that bound to two short peptides in this sequence, one binding to PLAFCGTE and the other to HSAAYRVDQGV, and showed that these antibodies bound the purified ^{125}I-labeled 140 kDa protein.

(e) Why was it necessary to include this antibody-binding step?

Next, the researchers designed PCR primers based on the sequences above, and then cloned a gene from a hamster cDNA library that encoded a protein with these sequences (see Chapter 9 on biotechnology methods). The cloned putative *SUR* cDNA hybridized to an mRNA of the appropriate length that was present in cells known to contain SUR. The putative *SUR* cDNA did not hybridize to any mRNA fraction of the mRNAs isolated from hepatocytes, which do not express SUR.

(f) Why was it necessary to include this putative *SUR* cDNA–mRNA hybridization step?

Finally, the cloned gene was inserted into and expressed in COS cells, which do not normally express the *SUR* gene. The investigators mixed these cells with [^{125}I]glyburide, with or without a large excess of unlabeled glyburide, exposed the cells to UV light, and measured the radioactivity of the 140 kDa protein produced. Their results are shown in the table.

Experiment	Cell type	Added putative *SUR* cDNA?	Added excess unlabeled glyburide?	^{125}I label in 140 kDa protein
1	HIT	No	No	+++
2	HIT	No	Yes	–
3	COS	No	No	–
4	COS	Yes	No	+++
5	COS	Yes	Yes	–

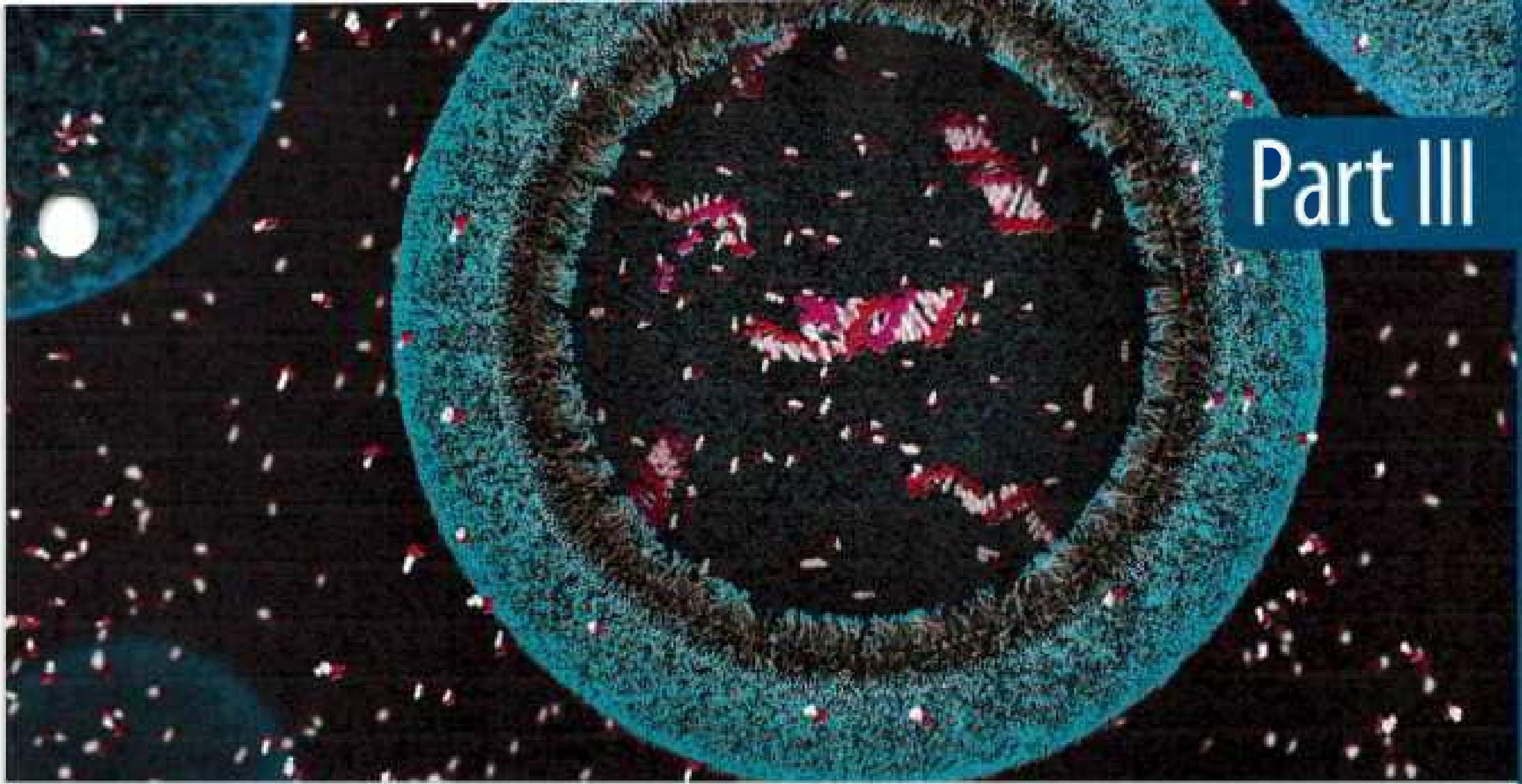
(g) Why was no ^{125}I-labeled 140 kDa protein found in experiment 2?

(h) How would you use the information in the table to argue that the cDNA encoded SUR?

(i) What other information would you want to collect to be more confident that you had cloned the *SUR* gene?

Reference

Aguilar-Bryan, L., C.G. Nichols, S.W. Wechsler, J.P. Clement, IV, A.E. Boyd, III, G. González, H. Herrera-Sosa, K. Nguy, J. Bryan, and D.A. Nelson. 1995. Cloning of the β cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* 268:423–426.



Part III

INFORMATION PATHWAYS

PART OUTLINE

24 **Genes and Chromosomes** *885*

25 **DNA Metabolism** *914*

26 **RNA Metabolism** *960*

27 **Protein Metabolism** *1005*

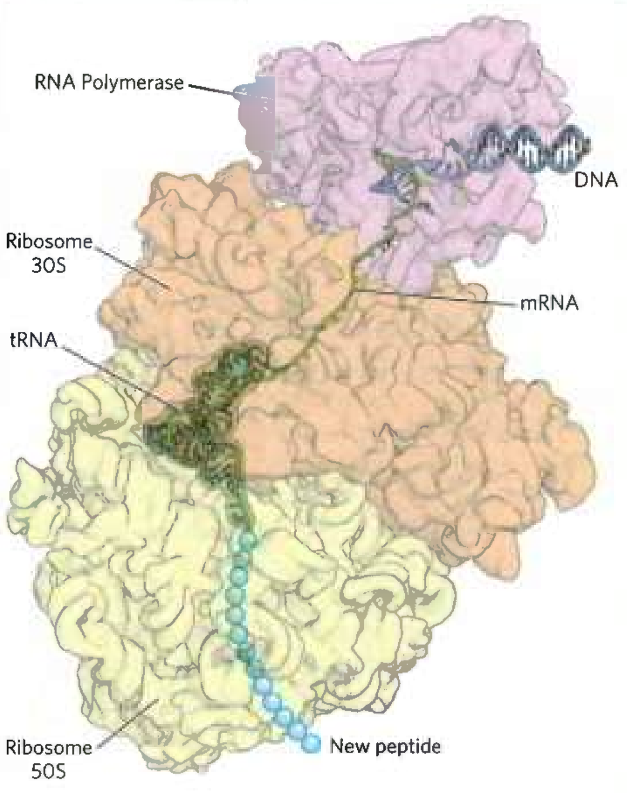
28 **Regulation of Gene Expression** *1054*

The third and final part of this book explores the biochemical mechanisms underlying the apparently contradictory requirements for both genetic continuity and the evolution of living organisms. What is the molecular nature of genetic material? How is genetic information transmitted from one generation to the next with high fidelity? How do the rare changes in genetic material that are the raw material of evolution arise? How is genetic information ultimately expressed in the amino acid sequences of the astonishing variety of protein molecules in a living cell?

Today's understanding of information pathways has arisen from the convergence of genetics, physics, and chemistry in modern biochemistry. This convergence was epitomized by the discovery of the double-helical structure of DNA, postulated by James Watson and Francis Crick in 1953. Genetic theory contributed the concept of coding by genes. Physics permitted the determination of molecular structure by x-ray diffraction analysis. Chemistry revealed the composition of DNA. The profound impact of the Watson-Crick hypothesis arose from its ability to account for a wide range of observations derived from studies in these diverse disciplines.

This discovery revolutionized our understanding of the structure of DNA and inevitably stimulated questions about its function. The double-helical structure itself clearly suggested how DNA might be copied so that the information it contains can be transmitted from one generation to the next. Clarification of how the information in DNA is converted into functional proteins came with the discoveries of messenger RNA and transfer RNA and with the deciphering of the genetic code.

These and other major advances gave rise to the central dogma of molecular biology, comprising the three major processes in the cellular utilization of genetic information. The first is replication, the copying of parental DNA to form daughter DNA molecules with identical nucleotide sequences. The second is transcription, the process by which parts of the genetic message encoded in DNA are copied precisely into RNA. The third is translation, whereby the genetic message encoded in messenger RNA is translated on the ribosomes into a polypeptide with a particular sequence of amino acids.



In bacteria, transcription and translation are tightly coupled such that ribosomes are in direct contact with RNA polymerases. The mRNA is translated as soon as it is synthesized. The overall complex has been given the name expressome.

Part III explores these and related processes. In Chapter 24 we examine the structure, topology, and packaging of chromosomes and genes. The processes underlying the central dogma are elaborated in Chapters 25 through 27. Finally, in Chapter 28, we turn to regulation, examining how the expression of genetic information is controlled.

A major theme running through these chapters is the added complexity inherent in the biosynthesis of macromolecules that contain information. Assembling nucleic acids and proteins with particular sequences of nucleotides and amino acids represents nothing less than preserving the faithful expression of the template upon which life itself is based. We might expect the formation of phosphodiester bonds in DNA or peptide bonds in proteins to be a trivial feat for cells, given the arsenal of enzymatic and chemical tools described in Part II. However, the framework of patterns and rules established in our examination of metabolic pathways thus far must be enlarged considerably to take into account molecular information. Bonds must be formed between *particular* subunits in informational biopolymers, avoiding either the occurrence or the persistence of sequence errors. This requirement has an enormous impact on the thermodynamics, chemistry, and enzymology of the biosynthetic processes. Formation of a peptide bond requires an energy input of only about 21 kJ per mole of bonds and can be catalyzed by relatively simple enzymes. But to synthesize a bond between two specific amino acids at a particular place in a polypeptide, the cell invests about 125 kJ/mol while making use of more than 200 enzymes, RNA molecules, and specialized proteins. The chemistry involved in peptide bond formation does not change because of this requirement, but additional processes are layered over the basic reaction to ensure that the peptide bond is formed between particular amino acids. Biological information is expensive.

The dynamic interaction between nucleic acids and proteins is another central theme of Part III. Regulatory and catalytic RNA molecules are gradually taking a more prominent place in our understanding of these pathways (discussed in Chapters 26 through 28). However, most of the processes that make up the pathways of cellular information flow are catalyzed and regulated by proteins. An understanding of these enzymes and other proteins can have practical as well as intellectual rewards, because they form the basis of recombinant DNA technology (introduced in Chapter 9).

Evolution again constitutes an overarching theme. Many of the processes outlined in Part III can be traced back billions of years, and a few can be traced to LUCA, the last universal common ancestor. The ribosome, most of the translational apparatus, and some parts of the transcriptional machinery are shared by every living organism on this planet. Genetic information is a kind of molecular clock that can help define ancestral relationships among species. Shared information pathways connect humans to every other species now living on Earth, and to all species that came before. Exploration of these pathways is allowing scientists to slowly open the curtain on the first act — the events that may have heralded the beginning of life on Earth.

Chapter 24

GENES AND CHROMOSOMES

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24.3 The Structure of Chromosomes 898

DNA molecules are typically the largest macromolecules in any cell, often many orders of magnitude longer than the cells or viral particles that contain them (**Fig. 24-1**). The extraordinary degree of organization required for the tertiary packaging of DNA into **chromosomes** — the repositories of genetic information, is the focus of this chapter. It also gives rise to the principles around which the chapter is organized:

P1 **Chromosomes include dedicated sequences that ensure their replication, transcription, packaging, and transmission from one generation to the next.** They are more than a long stretch of protein-encoding genes.

P2 **Chromosomes are large.** To constrain them in a small space can require multiple layers and multiple modes of tertiary structure.

P3 **Chromosomes in all cells are maintained in a state of torsional stress.** DNA is underwound relative to the stable B-form structure, facilitating both the packaging of DNA and access to the genetic information contained within it.

P4 **Specialized proteins and RNAs maintain chromosome structure.** Topoisomerases control DNA underwinding. Histones, condensins, cohesins, and other DNA-binding proteins provide scaffolds to organize chromosome structure. Certain long, noncoding RNAs also play important roles in chromosome structure and function.

The chapter begins with an examination of the elements that make up viral and cellular chromosomes, and then considers chromosomal size and organization. We then discuss DNA topology, describing the coiling and supercoiling of DNA molecules. Finally, we consider the protein-DNA interactions that organize chromosomes into compact structures.

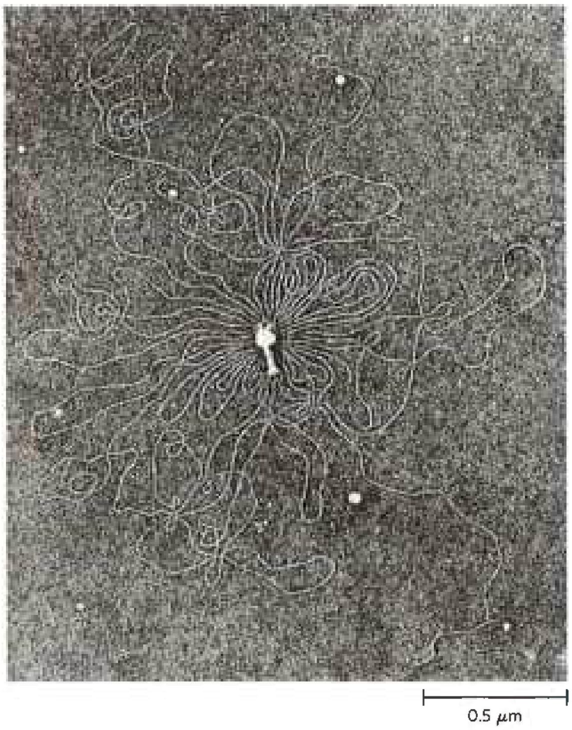


FIGURE 24-1 Bacteriophage T2 protein coat surrounded by its single, linear molecule of DNA. The DNA was released by lysing the bacteriophage particle in distilled water and allowing the DNA to spread on the water surface. An undamaged T2 bacteriophage particle consists of a head structure that tapers to a tail by which the bacteriophage attaches itself to the outer surface of a bacterial cell. All the DNA shown in this electron micrograph is normally packaged inside the phage head. [Republished with permission of Elsevier, from A. K. Kleinschmidt et al. (1962), "Preparation and length measurements of the total deoxyribonucleic acid content of T2 bacteriophages," *Biochim. Biophys. Acta* 61:857–864; permission conveyed through Copyright Clearance Center, Inc.]

24.1 Chromosomal Elements

Cellular DNA contains genes and intergenic regions, both of which may serve functions vital to the cell. The more complex genomes, such as those of eukaryotic cells, demand increased levels of chromosomal organization, and this is reflected in the structural features of the chromosomes. We begin by considering the different types of DNA sequences and structural elements within chromosomes.

Genes Are Segments of DNA That Code for Polypeptide Chains and RNAs

Our understanding of genes has evolved tremendously over the past century. Classically, a gene was defined as a portion of a chromosome that determines or affects a single character or **phenotype** (visible property), such as eye color. George Beadle and Edward Tatum proposed a molecular definition of a gene in 1940. After exposing spores of the fungus *Neurospora crassa* to x-rays and other agents now known to damage DNA and cause alterations in DNA sequence **(mutations)**, they detected mutant fungal strains that lacked one or another specific enzyme, sometimes resulting in the failure of an entire metabolic pathway. Beadle and Tatum concluded that a gene is a segment of genetic material that determines, or codes for, one enzyme: the **one gene–one enzyme** hypothesis. Later this concept was broadened to **one gene–one polypeptide**, because many genes code for a protein that is not an enzyme or for one polypeptide of a multisubunit protein.

The modern biochemical definition of a gene is even more precise. A **gene** is all the DNA that encodes the primary sequence of some final gene product, which can be either a polypeptide or an RNA with a structural or catalytic function. DNA also contains other segments or sequences that have a purely regulatory function. **Regulatory sequences** provide signals that may denote the beginning or the end of genes, or influence the transcription of genes, or function as initiation points for replication or recombination (Chapter 28). Some genes can be expressed in different ways to generate multiple gene products from a single segment of DNA; the special transcriptional and translational mechanisms that allow this are described in Chapters 26 through 28.

We can estimate directly the minimum overall size of genes that encode proteins. As described in detail in Chapter 27, each amino acid of a polypeptide chain is coded for by a sequence of three consecutive nucleotides in a single strand of DNA (**Fig. 24-2**), with these "codons" arranged in a sequence that corresponds to the sequence of amino acids in the polypeptide that the gene encodes. A polypeptide chain of 350 amino acid residues (an average-size chain) corresponds to 1,050 base pairs (bp) of coding DNA. Many genes in eukaryotes and a few in bacteria and archaea are interrupted by noncoding DNA segments and are therefore considerably longer than this simple calculation would suggest.

How many genes are in a single chromosome? The *Escherichia coli* chromosome is a circular DNA molecule (in the sense of an endless loop rather than a perfect circle) with 4,641,652 bp. These base pairs encode about 4,300 genes for proteins and more than 200 genes for structural or catalytic RNA molecules. Among eukaryotes, the approximately 3.1 billion base pairs of the human genome include approximately 20,000 genes on the 24 different chromosomes.

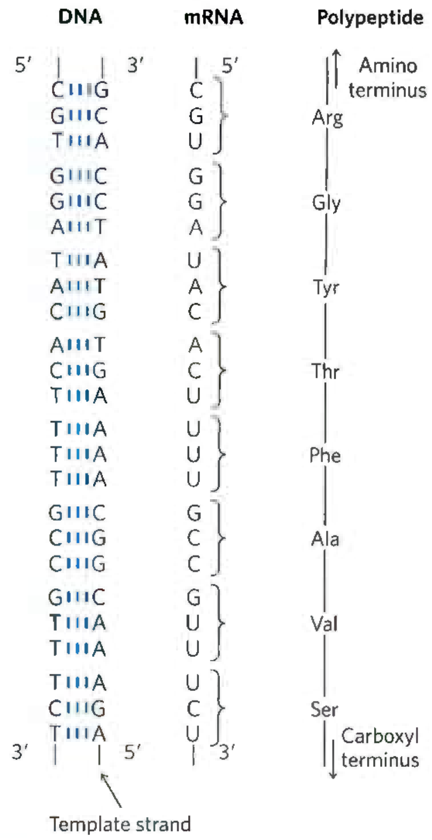


FIGURE 24-2 Colinearity of the coding nucleotide sequences of DNA and mRNA and the amino acid sequence of a polypeptide chain. The triplets of nucleotide units in DNA determine the amino acids in a protein through the intermediary mRNA. One of the DNA strands serves as a template for synthesis of mRNA, which has nucleotide triplets (codons) complementary to those of the DNA. In some bacterial and many eukaryotic genes, coding sequences are interrupted at intervals by regions of noncoding sequences (called introns).

DNA Molecules Are Much Longer than the Cellular or Viral Packages That Contain Them

Chromosomal DNAs are often many orders of magnitude longer than the cells or viruses in which they are located (Fig. 24-1; **Table 24-1**). This is true of every class of organism or viral parasite.

Viruses Viruses are not free-living organisms; rather, they are infectious parasites that use the resources of a host cell to carry out many of the processes they require to propagate. Many viral particles consist of no more than a genome (usually a single RNA or DNA molecule) surrounded by a protein coat.

Almost all plant viruses and some bacterial and animal viruses have RNA genomes. These genomes tend to be particularly small. For example, the genomes of mammalian retroviruses such as HIV consist of 9,000 nucleotides of single-stranded RNA.

The genomes of DNA viruses vary greatly in size. Many viral DNAs are circular for at least part of their life

TABLE 24-1 The Sizes of DNA and Viral Particles for Some Bacterial Viruses (Bacteriophages)

Virus	Size of viral DNA (bp)	Length of viral DNA (nm)	Long dimension of viral particles (nm)
ϕX174	5,386	1,939	25
T7	39,936	14,377	78
λ (lambda)	48,502	17,460	190
T4	168,889	60,800	210

Note: Data on size of DNA are for the replicative form (double-stranded). The contour length is calculated assuming that each base pair occupies a length of 3.4 Å (see Fig. 8-13).

cycle. During viral replication within a host cell, specific types of viral DNA called **replicative forms** may appear; for example, many linear DNAs become circular and all single-stranded DNAs become double-stranded. A typical medium-size DNA virus is bacteriophage λ (lambda), which infects *E. coli*. In its replicative form inside cells, λ DNA is a circular double helix. This double-stranded DNA contains 48,502 bp and has a contour length of 17.5 μm. Bacteriophage ϕX174 is much smaller; the DNA in the viral particle is a single-stranded circle, and the double-stranded replicative form contains 5,386 bp. **P2** Although viral genomes are small, the contour lengths of their DNAs are typically hundreds of times longer than the long dimensions of the viral particles that contain them (Table 24-1).

Bacteria A single *E. coli* cell contains almost 100 times as much DNA as a bacteriophage λ particle. The chromosome of an *E. coli* cell is a single, double-stranded circular DNA molecule. Its 4,641,652 bp have a contour length of about 1.7 mm, some 850 times the length of the *E. coli* cell (**Fig. 24-3**). In addition to the very large, circular DNA chromosome in their nucleoid, many bacteria contain one or more small circular DNA molecules that are free in the cytosol. These extrachromosomal elements are called **plasmids** (**Fig. 24-4**; see also p. 305). Most plasmids are only a few thousand base pairs long, but some contain up to 400,000 bp. They carry genetic information and undergo replication to yield daughter plasmids, which pass into the daughter cells at cell division. Plasmids have been found in yeast and other fungi as well as in bacteria.

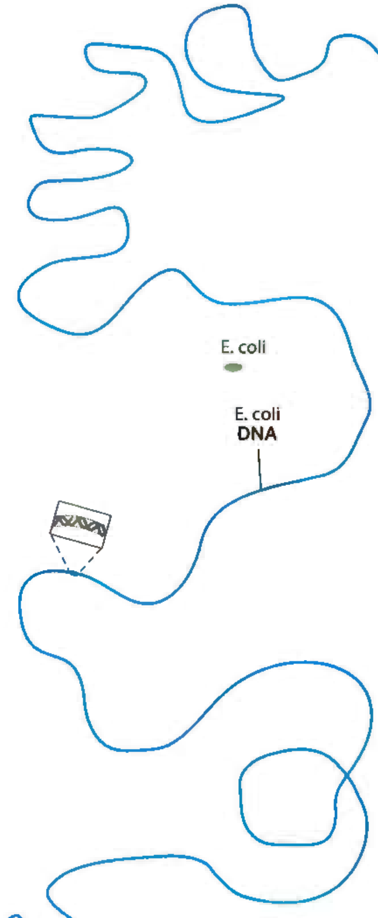
In many cases plasmids confer no obvious advantage on their host, and their sole function seems to be self-propagation. However, some plasmids carry genes that are useful to the host bacterium. For example, some plasmid genes make a host bacterium resistant to antibacterial agents. Plasmids carrying the gene for the enzyme β-lactamase confer resistance to β-lactam antibiotics such as penicillin, ampicillin, and amoxicillin (see Fig. 6-34). These and similar plasmids may pass from an antibiotic-resistant cell to an antibiotic-sensitive cell of the same or another bacterial species, making the recipient cell antibiotic-resistant. The extensive use of antibiotics in some human populations and in animal feeds has served as a strong selective force, encouraging the spread of antibiotic resistance–coding plasmids (as well as transposable elements, described below, that harbor similar genes) in disease-causing bacteria.

Eukaryotes A yeast cell, one of the simplest eukaryotes, has 2.6 times more DNA in its genome than an *E. coli* cell (**Table 24-2**). Cells of *Drosophila*, the fruit fly used in classical genetic studies, contain more than 35 times as much DNA as *E. coli* cells, and human cells have almost 700 times as much. The cells of many plants and amphibians contain even more. The genetic material of eukaryotic cells is apportioned into chromosomes, the diploid ($2n$) number depending on the species. A human somatic cell, for example, has 46 chromosomes (**Fig. 24-5**). Each chromosome of a eukaryotic cell, such as that shown in Figure 24-5a, contains a single, very large, duplex DNA molecule. The DNA molecules in the 24 different types of human chromosomes (22 matching pairs of autosomes plus the X and Y sex chromosomes) vary in length over a 25-fold range. Each type of chromosome in eukaryotes carries a characteristic set of genes.

P2 DNA molecules of one human genome (22 chromosomes plus X and Y), placed end to end, would extend for about a meter. Most human cells are diploid, so each cell contains a total of 2 m of DNA. An adult human body contains approximately 10^{14} cells and thus a total DNA length of 2×10^{11} km. Compare this with the circumference of the earth (4×10^4 km) or the distance between the earth and the sun (1.5×10^8 km)—a dramatic illustration of the extraordinary degree of DNA compaction in our cells.

Eukaryotic cells also have organelles, mitochondria and chloroplasts, that contain DNA. Mitochondrial DNA (mtDNA) molecules are much smaller than the nuclear chromosomes. In animal cells, mtDNA contains fewer than 20,000 bp (16,569 bp in human mtDNA) and is a circular duplex. Each mitochondrion typically has 2 to 10 copies of this mtDNA molecule, and the number can rise to hundreds in certain cells of an embryo that is undergoing cell differentiation. Plant cell mtDNA ranges in size from 200,000 to 2,500,000 bp. Chloroplast DNA (cpDNA) also exists as circular duplexes and ranges in size from 120,000 to 160,000 bp. Mitochondrial and chloroplast DNAs have an evolutionary origin in the chromosomes of ancient bacteria that gained

FIGURE 24-3 A bacterial cell and its DNA. The length of the *E. coli* chromosome (1.7 mm), depicted in linear form, relative to the length of a typical *E. coli* cell (2 μm).



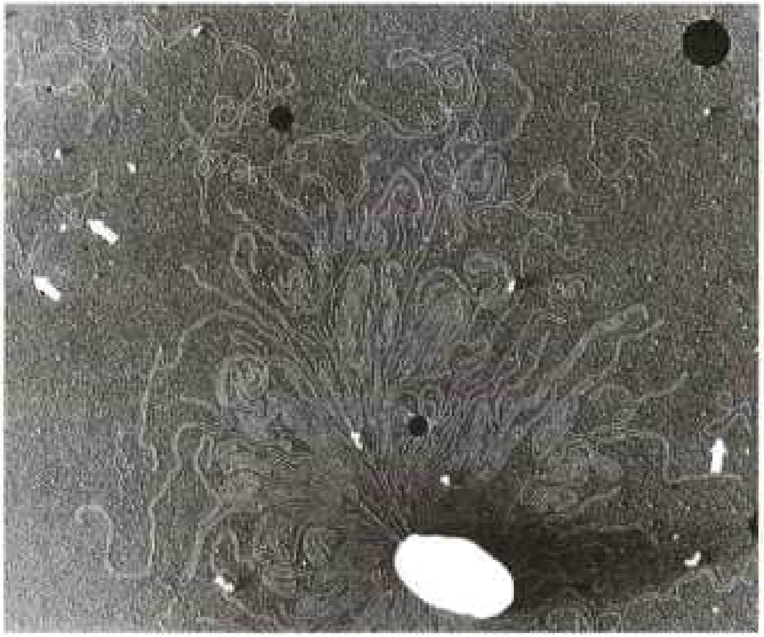


FIGURE 24-4 DNA from a lysed *E. coli* cell. In this electron micrograph, several small, circular plasmid DNAs are indicated by white arrows. The black spots and white specks are artifacts of the preparation. [Huntington Potter, University of Colorado School of Medicine, and David Dressler, Balliol College, Oxford University.]

TABLE 24-2 DNA, Gene, and Chromosome Content in Some Genomes

	Total DNA (bp)	Number of chromosomes[a]	Approximate number of protein-coding genes
Escherichia coli K12 (bacterium)	4,641,652	1	4,494[b]
Saccharomyces cerevisiae (yeast)	12,157,105	16[c]	6,600
Caenorhabditis elegans (nematode)	100,286,401	12[d]	20,191
Arabidopsis thaliana (plant)	119,667,750	10	27,655
Drosophila melanogaster (fruit fly)	143,726,002	18	13,931
Oryza sativa (rice)	375,049,285	24	37,849
Mus musculus (mouse)	2,730,871,774	40	22,480
Homo sapiens (human)	3,096,649,726	46	20,454[e]

Note: This information is constantly being refined. For the most current information, consult the websites for the individual genome projects. [Data from ensembl.org. Accessed April 21, 2020.]

[a] The diploid chromosome number is given for all eukaryotes except yeast.

[b] Includes known RNA-coding genes.

[c] Haploid chromosomes number. Wild yeast strains generally have eight (octoploid) or more sets of these chromosomes.

[d] Number for females, with two X chromosomes. Males have an X but no Y, thus 11 chromosomes in all.

[e] When known genes encoding functional RNAs are included, this number rises to more than 43,000.

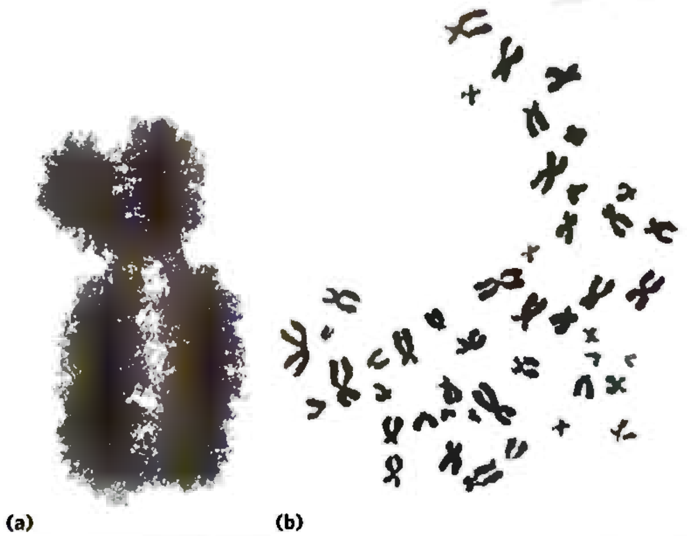


FIGURE 24-5 Eukaryotic chromosomes. (a) A pair of linked and condensed sister chromatids from a Chinese hamster ovary cell. Eukaryotic chromosomes are in this state after replication at metaphase during mitosis. (b) A complete set of chromosomes from a leukocyte from one of the authors. There are 46 chromosomes in every normal human somatic cell. [(a) Don W. Fawcett/ Science Source. (b) © Michael M. Cox.]

access to the cytoplasm of host cells and became the precursors of these organelles (see Fig. 1-37). Mitochondrial DNA codes for the mitochondrial tRNAs and rRNAs and for a few mitochondrial proteins. More than 95% of mitochondrial proteins are encoded by nuclear DNA. Mitochondria and chloroplasts divide when the cell divides. Their DNA is replicated before and during division, and the daughter DNA molecules pass into the daughter organelles.

Eukaryotic Genes and Chromosomes Are Very Complex

Many bacterial species have only one chromosome per cell and, in nearly all cases, each chromosome contains only one copy of each gene. A very few genes, such as those for rRNAs, are repeated several times. Genes and regulatory sequences account for almost all the DNA in bacteria. Moreover, almost every gene is precisely colinear with the amino acid sequence (or RNA sequence) it encodes throughout its entire length (Fig. 24-2).

The organization of genes in eukaryotic DNA is structurally and functionally much more complex. Studies of eukaryotic chromosome structure and the sequencing of entire eukaryotic genomes have yielded many surprises.

P1 Many, if not most, eukaryotic genes have a distinctive structural feature: the colinearity of the DNA and amino acid sequence is periodically broken by intervening segments of DNA that do not code for the amino acid sequence of the polypeptide product. Such nontranslated DNA segments in genes are called **introns**, and the coding segments are called **exons**. Few bacterial genes contain introns. In higher eukaryotes, the typical gene has much more intron sequence than sequences devoted to exons. For example, in the gene coding for the single polypeptide chain of ovalbumin, an avian egg protein (**Fig. 24-6**), the introns are much longer than the exons; altogether, the seven introns make up 85% of the gene's DNA. The gene encoding the hemoglobin β subunit has only two introns, but again they are larger than the exons. Genes for histones seem to have no introns. In many cases the function of introns is not clear. In total, only about 1.5% of human DNA is "coding" or exon DNA, carrying sequence information for protein products. However, when the much larger introns are included as functional elements in the gene and their length is included, as much as 30% of the human genome consists of protein-coding genes.

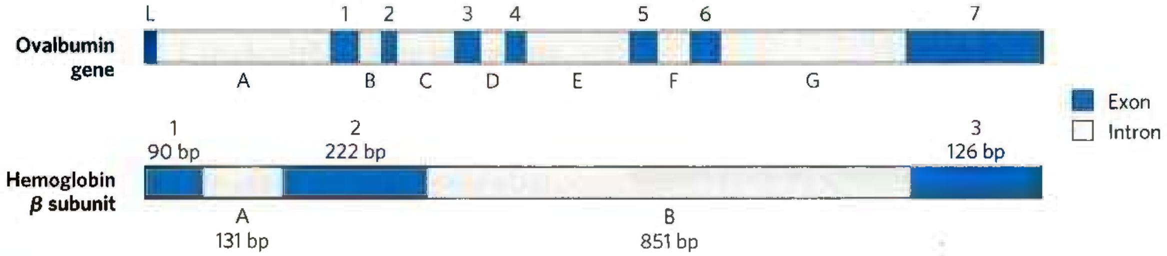


FIGURE 24-6 Introns in two eukaryotic genes. The gene for ovalbumin has seven introns (A to G), splitting the coding sequences into eight exons (L, and 1 to 7). The gene for the β subunit of hemoglobin has two introns and three exons, including one intron that alone contains more than half the base pairs of the gene.

A great deal of work remains to be done to understand the genomic sequences that do not correspond to protein-encoding genes. Much of the DNA that is not within genes is made up of repeated sequences of several kinds. These include transposable elements (transposons), molecular parasites that account for nearly half of the DNA in the human genome (see Fig. 9-25a and Chapters 25 and 26), and genes encoding functional RNA molecules of many types.

Approximately 3% of the human genome consists of **highly repetitive sequences**, also referred to as **simple-sequence DNA** or **simple sequence repeats (SSR)**. These short sequences, generally less than 10 bp long, are sometimes repeated millions of times per cell. The simple-sequence DNA is also called **satellite DNA**, so named because its unusual base composition often causes it to migrate as "satellite" bands (separated from the rest of the DNA) when fragmented cellular DNA samples are centrifuged in a cesium chloride density gradient. Studies suggest that simple-sequence DNA does not encode proteins or RNAs. The functional importance of the highly repetitive DNA has been defined in at least some cases. Much of it is associated with two crucial features of eukaryotic chromosomes: centromeres and telomeres.

P1 The **centromere** (Fig. 24-7) is a sequence of DNA that functions during cell division as an attachment point for proteins that link the chromosome to the mitotic spindle. This attachment is essential for the equal and orderly segregation of chromosome sets to daughter cells. The centromeres of *Saccharomyces cerevisiae* have been isolated and studied. The sequences essential to centromere function are about 130 bp long and are very rich in A=T pairs. The centromeric sequences of higher eukaryotes are much longer and, unlike those of yeast, generally consist of thousands of tandem copies of one or several sequences of 5 to 10 bp, in the same orientation.

P1 **Telomeres** (Greek *telos*, "end") are sequences at the ends of eukaryotic chromosomes that help stabilize the chromosome. Telomeres end with multiple repeated sequences of the form

$$(5')(T_xG_y)_n$$
$$(3')(A_xC_y)_n$$

where x and y are generally between 1 and 4 (Table 24-3). The number of telomere repeats, n, is in the range of 20 to 100 for most single-celled eukaryotes and is generally more than 1,500 in mammals. The ends of a linear DNA molecule cannot be routinely replicated by the cellular replication machinery (which may be one reason why bacterial DNA molecules are circular). Repeated telomeric sequences are added to eukaryotic chromosome ends primarily by the enzyme telomerase (see Fig. 26-35).

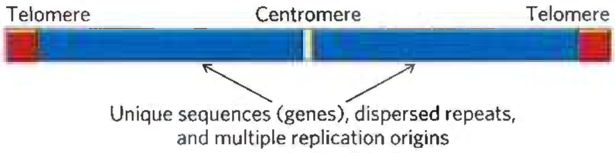

Telomere
Centromere
Telomere
Unique sequences (genes), dispersed repeats, and multiple replication origins

FIGURE 24-7 Important structural elements of a yeast chromosome.

TABLE 24-3 Telomere Sequences

Organism	Telomere repeat sequence
Homo sapiens (human)	$(TTAGGG)_n$
Tetrahymena thermophila (ciliated protozoan)	$(TTGGGG)_n$
Saccharomyces cerevisiae (yeast)	$(T(G)_{1-3}(TG)_{2-3})_n$
Arabidopsis thaliana (plant)	$(TTTAGGG)_n$

Artificial chromosomes (Chapter 9) have been constructed as a means of better understanding the functional significance of many structural features of eukaryotic chromosomes. A reasonably stable artificial linear chromosome requires only three components: a centromere, a telomere at each end, and sequences that allow the initiation of DNA replication. Yeast artificial chromosomes (YACs; see Fig. 9-6) have been developed as a research tool in biotechnology. Similarly, human artificial chromosomes (HACs) are being developed for the treatment of genetic diseases. These may eventually provide a new path to the intracellular replacement of missing or defective gene products, or somatic gene therapy.

SUMMARY 24.1 *Chromosomal Elements*

- Genes are segments of a chromosome that contain the information for a functional polypeptide or RNA molecule. In addition to genes, chromosomes contain a variety of regulatory sequences involved in replication, transcription, and other processes.
- Genomic DNA and RNA molecules are generally orders of magnitude longer than the viral particles or cells that contain them.
- Many genes in eukaryotic cells (but few in bacteria and archaea) are interrupted by noncoding sequences, or introns. The coding segments separated by introns are called exons. Only about 1.5% of human genomic DNA encodes proteins; even when introns are included, less than one-third of human genomic DNA consists of genes. Much of the remainder consists of repeated sequences of various types. Nucleic acid parasites known as transposons account for about half of the human genome. Eukaryotic chromosomes have two important special-function repetitive DNA sequences: centromeres, which are attachment points for the mitotic spindle, and telomeres, located at the ends of chromosomes.

24.2 DNA Supercoiling

How is cellular or viral DNA compacted into the cells or viral coats that contain it in a way that still permits access to the information in the DNA? The extreme compaction implies a high degree of structural organization. First, the many negative charges of the phosphoryl groups in the DNA backbone must be neutralized. Cations, particularly Mg^{2+} ions, and a class of molecules called polyamines provide multiple positive counterions to permit DNA compaction. Polyamines are derived from the amino acid ornithine (see Box 6-1). The second key to DNA compaction is a DNA structural alteration known as **supercoiling**. All cells maintain their DNA in a state that is **underwound**—having fewer right-handed helical turns per given length of DNA—than B-form DNA. The underwinding places structural strain on the DNA, causing it to twist upon itself. Supercoiling affects and is affected by processes such as replication and transcription. We introduce it here as a prelude to a broader discussion of DNA metabolism.

P3 "Supercoiling" means the coiling of a coil. An old-fashioned telephone cord, for example, was typically a coiled wire. The path taken by the wire between the base of the phone and the receiver often included one or more supercoils (**Fig. 24-8**). DNA is coiled in the form of a double helix, with both strands of the DNA coiling around an axis. The further coiling of that axis upon itself (**Fig. 24-9**) produces DNA supercoiling. As detailed below, DNA supercoiling is generally a manifestation of structural strain. When there is no net bending of the DNA axis upon itself, the DNA is said to be in a **relaxed** state.

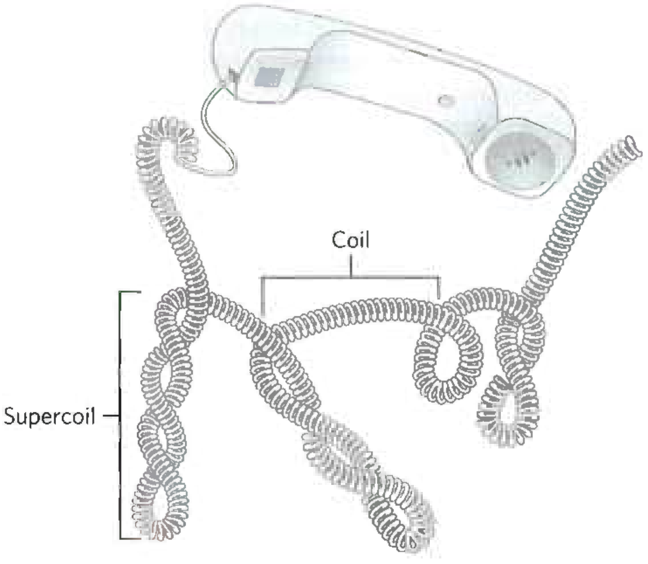


FIGURE 24-8 Supercoils. An old-fashioned phone cord is coiled like a DNA helix, and the coiled cord can itself coil in a supercoil. Examining phone cords helped lead Jerome Vinograd and his colleagues to the insight that many properties of small circular DNAs can be explained by supercoiling. They first detected DNA supercoiling—in small circular viral DNAs—in 1965.

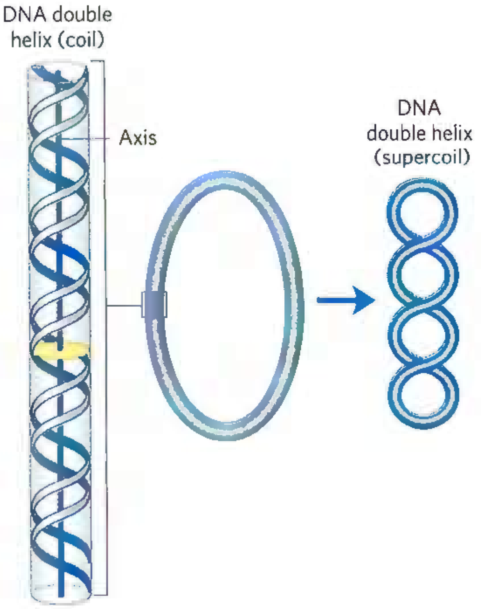


FIGURE 24-9 Supercoiling of DNA. When the axis of the DNA double helix is coiled on itself, it forms a new helix (superhelix). The DNA superhelix is usually called a supercoil.

As we shall see, the supercoiling that occurs in cells reflects underwinding of the DNA, facilitating the separation of strands required for the processes of replication and transcription (**Fig. 24-10**).

That a DNA molecule would bend on itself and become supercoiled in tightly packaged cellular DNA would seem logical, and perhaps even trivial, were it not for one additional fact: many circular DNA molecules remain highly supercoiled even after they are extracted and purified, freed from protein and other cellular components. P3 This indicates that supercoiling is an intrinsic property of DNA tertiary structure. It occurs in all cellular DNAs and is highly regulated by each cell.

Several measurable properties of supercoiling have been established, and the study of supercoiling has provided many insights into DNA structure and function. This work has drawn heavily on concepts derived from a branch of mathematics called **topology**, the study of the properties of an object that do not change under continuous deformations. For DNA, continuous deformations include conformational changes due to thermal motion or due to interaction with proteins or other molecules; discontinuous deformations involve DNA strand breakage. For circular DNA molecules, a topological property is one that is unaffected by deformations of the DNA strands as long as no breaks are introduced. Topological properties are changed only by breakage and rejoining of the backbone of one or both DNA strands.

We now examine the fundamental properties and physical basis of supercoiling.

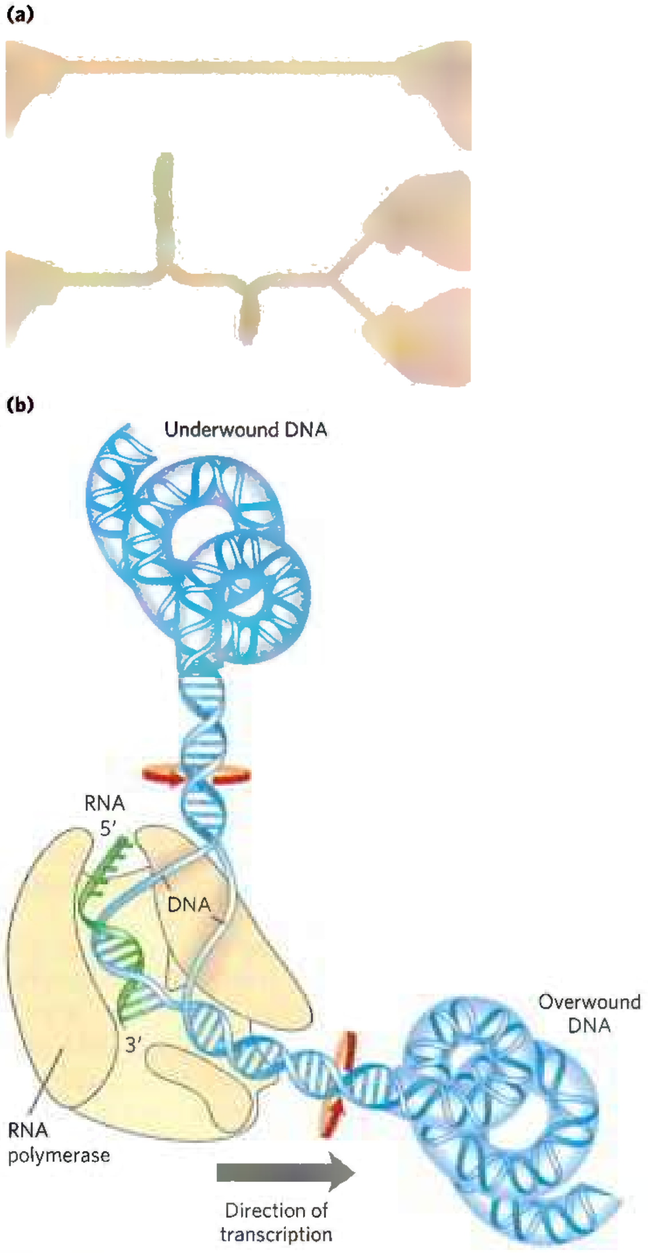


FIGURE 24-10 The effects of replication and transcription on DNA supercoiling. Because DNA is a double-helical structure, strand separation leads to added stress and supercoiling if the DNA is constrained (not free to rotate) ahead of the strand separation. (a) The general effect can be illustrated by twisting two strands of a rubber band about each other to form a double helix. If one end is constrained, separating the two strands at the other end will lead to twisting. (b) In a DNA molecule, the progress of a DNA polymerase or RNA polymerase (as shown here) along the DNA involves separation of the strands. As a result, the DNA becomes overwound ahead of the enzyme (upstream) and underwound behind it (downstream). Red arrows indicate the direction of winding.

Most Cellular DNA Is Underwound

To understand supercoiling, we must first focus on the properties of small circular DNAs such as plasmids and small viral DNAs. When these DNAs have no breaks in either strand, they are referred to as **closed-circular DNAs**. In aqueous solutions, DNA is most stable—that is, it is in its lowest free-energy form—in the B-form structure. If the DNA of a closed-circular molecule conforms closely to the B-form structure (Watson-Crick structure; see Fig. 8-13), with one turn of the double helix per 10.5 bp, the DNA is relaxed rather than supercoiled (**Fig. 24-11**). Supercoiling results when DNA is subject to some form of structural strain such that it is overwound or underwound. Purified closed-circular DNA is rarely relaxed, regardless of its biological origin. **P3** Furthermore, DNAs derived from a given cellular source have a characteristic degree of supercoiling. DNA structure is therefore strained in a manner that is regulated by the cell to induce the supercoiling.

In almost every instance, the strain is a result of underwinding of the DNA double helix in the closed circle. In other words, the DNA has *fewer* helical turns than would be expected for the B-form structure. The effects of underwinding are summarized in **Figure 24-12**. An 84 bp segment of a circular DNA in the relaxed state would contain eight double-helical turns, one for every 10.5 bp. If one of these turns were removed, there would be (84 bp)/7 = 12.0 bp per turn, rather than the 10.5 found in B-DNA (Fig. 24-12b). This is a deviation from the most stable DNA form, and the molecule is thermodynamically strained as a result. Generally, much of this strain would be accommodated by coiling the axis of the DNA on itself to form a supercoil (Fig. 24-12c; some of the strain in this 84 bp segment would simply become dispersed in the untwisted structure of the larger DNA molecule). In principle, the strain could also be accommodated by separating the two DNA strands over a distance of about 10 bp (Fig. 24-12d). In isolated closed-circular DNA, strain introduced by underwinding is generally accommodated by supercoiling rather than strand separation, because coiling the axis of the DNA usually requires less energy than breaking the hydrogen bonds and disrupting the base stacking that stabilizes paired bases. Note, however, that the underwinding of DNA in vivo makes separation of the DNA strands easier, facilitating access to the information they contain.

Every cell actively underwinds its DNA with the aid of enzymatic processes (described below), and the resulting strained state represents a form of stored energy. Cells maintain DNA in an underwound state to facilitate its compaction by coiling. The underwinding of DNA is also important to enzymes of DNA metabolism that must bring about strand separation as part of their function.

The underwound state can be maintained only if the DNA is a closed circle or if it is bound and stabilized by proteins so that the strands are not free to rotate about each other. If there is a break in one strand of an isolated, protein-free circular DNA, free rotation at that point will cause the underwound DNA to revert spontaneously to the relaxed state. In a closed-circular DNA molecule, however, the number of helical turns cannot be changed without at least transiently breaking one of the DNA

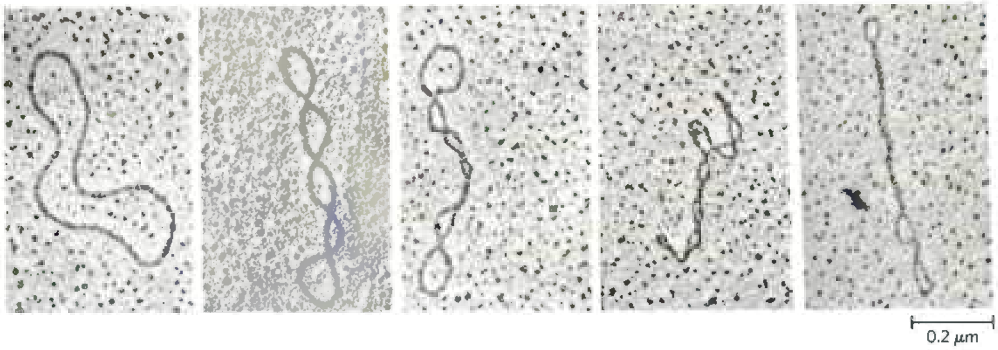
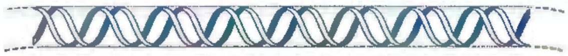
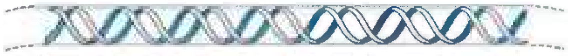


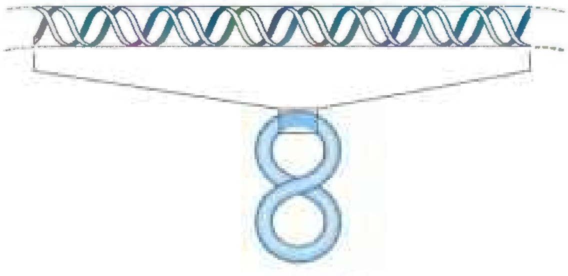
FIGURE 24-11 Relaxed and supercoiled plasmid DNAs. The molecule in the leftmost electron micrograph is relaxed; the degree of supercoiling increases from left to right. [Laurien Polder, from A. Kornberg, *DNA Replication*, p. 29, W. H. Freeman, 1980.]



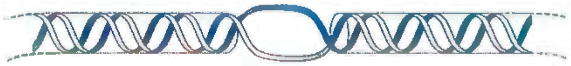
(a) Relaxed (8 turns)



(b) Strained (7 turns)

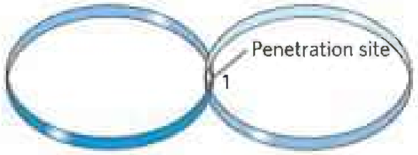


(c) Supercoil

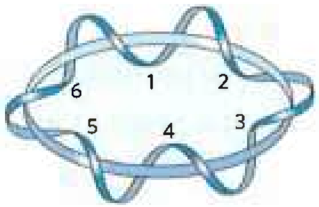


(d) Strand separation

FIGURE 24-12 Effects of DNA underwinding. (a) A segment of DNA in a closed-circular molecule, 84 bp long, in its relaxed form with eight helical turns. (b) Removal of one turn induces structural strain. (c) The strain is generally accommodated by formation of a supercoil. (d) DNA underwinding also makes the separation of strands somewhat easier. In principle, each turn of underwinding should facilitate strand separation over about 10 bp, as shown here. However, the hydrogen-bonded base pairs would generally preclude strand separation over such a short distance, and the effect becomes important only for longer DNAs and higher levels of DNA underwinding.



(a) *Lk* = 1



(b) *Lk* = 6

FIGURE 24-13 Linking number, *Lk*. Here, as usual, each blue ribbon represents one strand of a double-stranded DNA molecule. For the molecule in (a), *Lk* = 1. For the molecule in (b), *Lk* = 6. One of the strands in (b) is kept untwisted for illustrative purposes, to define the border of an imaginary surface (shaded blue). The number of times the twisting strand penetrates this surface provides a rigorous definition of linking number.

strands. The number of helical turns in a DNA molecule therefore provides a precise description of supercoiling.

DNA Underwinding Is Defined by Topological Linking Number

The field of topology provides some ideas that are useful to the discussion of DNA supercoiling, particularly the concept of **linking number**. Linking number is a topological property of double-stranded DNA, because it does not vary when the DNA is bent or deformed, as long as both DNA strands remain intact. Linking number (*Lk*) is illustrated in **Figure 24-13**. As we shall see, all cells have enzymes called topoisomerases that catalyze changes in the linking number. Because of this critical role, topoisomerases are the targets of many antibiotics and cancer chemotherapy agents.

Let's begin by visualizing the separation of the two strands of a double-stranded circular DNA. If the two strands are linked as shown in Figure 24-13a, they are effectively joined by what can be described as a topological bond. Even if all hydrogen bonds and base-stacking interactions were abolished such that the strands were not in physical contact, this topological bond would still link the two strands. Visualize one of the circular strands as the boundary of a surface (such as the soap film framed by the loop of a bubble wand before you blow a bubble). The linking number can be defined as the number of times the second strand pierces this surface. For the molecule in Figure 24-13a, *Lk* = 1; for that in Figure 24-13b, *Lk* = 6. The linking number for a closed-circular DNA is always an integer. By convention, if the links between two DNA strands are arranged so that the strands are interwound in a right-handed helix, the linking number is defined as positive (+); for strands interwound in a left-handed helix, the linking number is negative (−). Negative linking numbers are, for all practical purposes, not encountered in DNA.

We can now extend these ideas to a closed-circular DNA with 2,100 bp (**Fig. 24-14a**). When the molecule is relaxed, the linking number is simply the number of base pairs divided by the number of base pairs per turn, which is close to 10.5; so in this case, $Lk = 200$. The linking number in relaxed DNA is designated Lk_0. For a circular DNA molecule to have a topological property such as linking number, both strands must be intact, without a break. If there is a break in either strand, the two strands can, in principle, be unraveled and separated completely. In this case, no topological bond exists and Lk is undefined (Fig. 24-14b).

We can now describe DNA underwinding in terms of changes in the linking number. For the molecule shown in Figure 24-14a, $Lk_0 = 200$; if two turns are removed from this molecule, $Lk = 198$. The change can be described by the equation

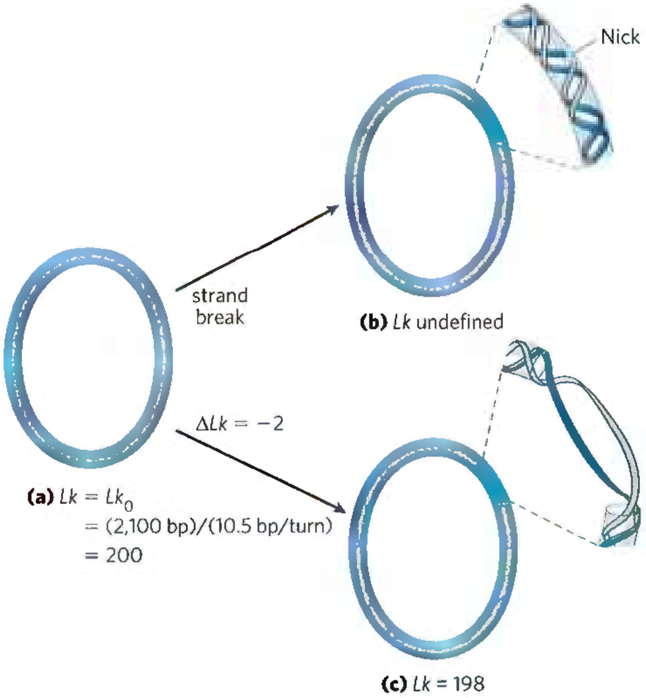
$$\begin{aligned}\Delta Lk &= Lk - Lk_0 \\ &= 198 - 200 = -2\end{aligned} \qquad (24\text{-}1)$$

It is often convenient to express the change in linking number in terms of a quantity that is independent of the length of the DNA molecule. This quantity, called the **specific linking difference** or **superhelical density (σ)**, is a measure of the number of turns removed relative to the number present in relaxed DNA:

$$\sigma = \frac{\Delta Lk}{Lk_0} \qquad (24\text{-}2)$$

In the example in Figure 24-14c, $\sigma = 0.01$, which means that 1% (2 of 200) of the helical turns present in the DNA (in its B form) have been removed. The degree of underwinding in cellular DNAs generally falls in the range of 5% to 7%; that is, $\sigma = -0.05$ to -0.07. The negative sign indicates that the change in linking number is due to underwinding of the DNA. The supercoiling induced by underwinding is therefore defined as negative supercoiling. Conversely, under some conditions DNA can be overwound, resulting in positive supercoiling. Note that the twisting path taken by the axis of the DNA helix when the DNA is underwound (negative supercoiling) is the mirror image of that taken when the DNA is overwound (positive supercoiling) (**Fig. 24-15**). Supercoiling is not a random process; the path of the supercoiling is largely prescribed by the torsional strain imparted to the DNA by decreasing or increasing the linking number relative to B-DNA. **P2** **P3** An increase in superhelical density brings about an increase in DNA compaction.

Linking number can be changed by ±1 by breaking one DNA strand, rotating one of the ends 360° about the unbroken strand, and rejoining the broken ends. This change has no effect on the number of base pairs or the number of atoms in the circular DNA molecule. Two forms of a circular DNA that differ only in a topological property such as linking number are referred to as **topoisomers**.



(a) $Lk = Lk_0$
$= (2{,}100 \text{ bp})/(10.5 \text{ bp/turn})$
$= 200$

(b) Lk undefined

(c) $Lk = 198$

FIGURE 24-14 Linking number applied to closed-circular DNA molecules. A 2,100 bp circular DNA is shown in three forms: (a) relaxed, $Lk = 200$; (b) relaxed with a nick (break) in one strand, Lk undefined; and (c) underwound by two turns, $Lk = 198$. The underwound molecule generally exists as a supercoiled molecule, but underwinding also facilitates the separation of DNA strands.

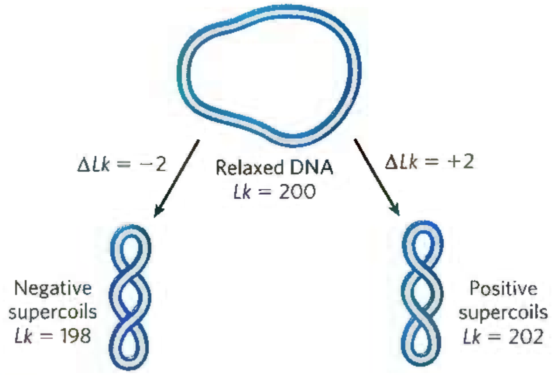


FIGURE 24-15 Negative and positive supercoils. For the relaxed DNA molecule of Figure 24-14a, underwinding or overwinding by two helical turns ($Lk = 198$ or 202) will produce negative or positive supercoiling, respectively. Notice that the DNA axis twists in opposite directions in the two cases.

WORKED EXAMPLE 24-1 *Calculation of Superhelical Density*

What is the superhelical density (σ) of a closed-circular DNA with a length of 4,200 bp and a linking number (Lk) of 374? What is the superhelical density of the same DNA when $Lk = 412$? Are these molecules negatively or positively supercoiled?

SOLUTION: First, calculate Lk_0 by dividing the length of the closed-circular DNA (in bp) by 10.5 bp/turn: (4,200 bp)/(10.5 bp/turn) = 400. We can now calculate ΔLk from Equation 24-1: $\Delta Lk = Lk - Lk_0 = 374 - 400 = -26$. Substituting the values for ΔLk and Lk_0 into Equation 24-2: $\sigma = \Delta Lk / Lk_0 = -26/400 = -0.065$. Because the superhelical density is negative, this DNA molecule is negatively supercoiled.

When the same DNA molecule has an Lk of 412, $\Delta Lk = 412 - 400 = 12$, and $\sigma = 12/400 = 0.03$. The superhelical density is positive, and the molecule is positively supercoiled.

In addition to causing supercoiling and making strand separation somewhat easier, the underwinding of DNA facilitates structural changes in the molecule. These are of less physiological importance but they help illustrate the effects of underwinding. Recall that a cruciform (see Fig. 8-19) generally contains a few unpaired bases; DNA underwinding helps to maintain the required strand separation (**Fig. 24-16**). Underwinding of a right-handed DNA helix also enables the formation of short stretches of left-handed Z-DNA in regions where the base sequence is consistent with the Z form (see Chapter 8).

Topoisomerases Catalyze Changes in the Linking Number of DNA

DNA supercoiling is a precisely regulated process that influences many aspects of DNA metabolism. Every cell has enzymes with the sole function of underwinding and/or relaxing DNA. The enzymes that increase or decrease the extent of DNA underwinding are **topoisomerases**; the property of DNA that they change is the linking number. These enzymes play an especially important role in processes such as replication and DNA packaging. There are two classes of topoisomerases. **Type I topoisomerases** act by transiently breaking one of the two DNA strands, passing the unbroken strand through the break, and rejoining the broken ends; they change Lk in increments of 1. **Type II topoisomerases** break both DNA strands and change Lk in increments of 2.

P2 When a circular DNA is supercoiled, it is twisted upon itself and therefore is more compact than when it is relaxed. The supercoiled molecule will thus migrate faster in a gel matrix (**Fig. 24-17**). A population of identical plasmid DNAs with the same linking number migrates as a discrete band during agarose gel electrophoresis. Topoisomers with Lk values differing by as little as 1 can be separated by this method, so changes in linking number induced by topoisomerases are readily detected.

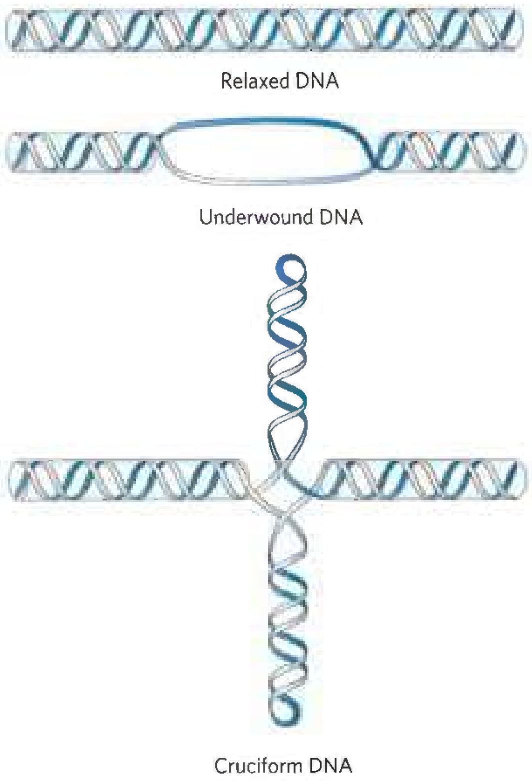


FIGURE 24-16 Promotion of cruciform structures by DNA underwinding. In principle, cruciforms can form at palindromic sequences (see Fig. 8-19), but they seldom occur in relaxed DNA because the linear DNA accommodates more paired bases than does the cruciform structure. Underwinding of the DNA facilitates the partial strand separation needed to promote cruciform formation at appropriate sequences.

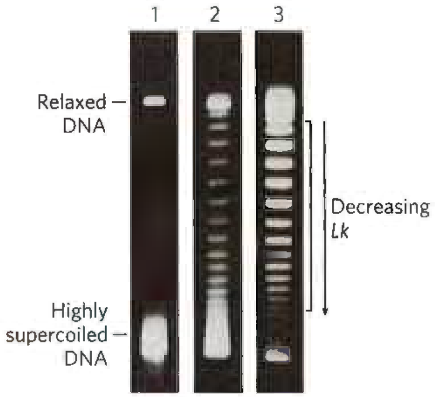


FIGURE 24-17 Visualization of topoisomers. In this experiment, all DNA molecules have the same number of base pairs but exhibit some range in the degree of supercoiling. Because supercoiled DNA molecules are more compact than relaxed molecules, they migrate more rapidly during gel electrophoresis. The gels shown here separate topoisomers (moving from top to bottom) over a limited range of superhelical density. In lane 1, highly supercoiled DNA migrates in a single band, even though different topoisomers are probably present. Lanes 2 and 3 illustrate the effect of treating the supercoiled DNA with a type I topoisomerase; the DNA in lane 3 was treated for a longer time than that in lane 2. As the superhelical density of the DNA is reduced to the point where it corresponds to the range in which the gel can resolve individual topoisomers, distinct bands appear. Individual bands in the region indicated by the bracket next to lane 3 each contain DNA circles with the same linking number; Lk changes by 1 from one band to the next. (Courtesy of Michael Cox Lab.)

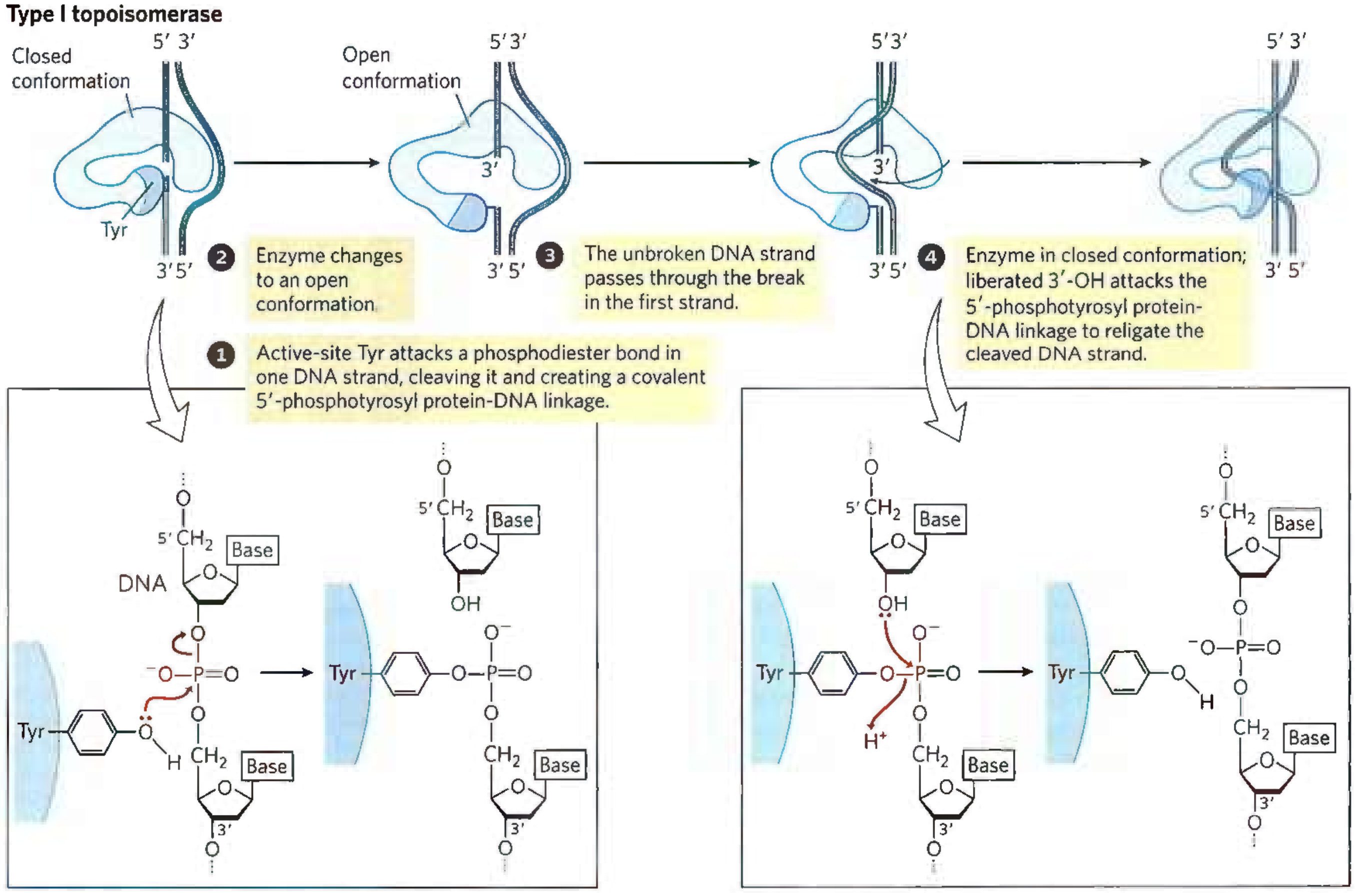
E. coli has at least four individual topoisomerases (I through IV). Those of type I (topoisomerases I and III) generally relax DNA by removing negative supercoils (increasing *Lk*). The way in which bacterial type I topoisomerases change linking number is illustrated in **Figure 24-18**. A bacterial type II enzyme, called either topoisomerase II or DNA gyrase, can introduce negative supercoils (decrease *Lk*). It uses the energy of ATP to accomplish this. To alter DNA linking number, type II topoisomerases cleave both strands of a DNA molecule and pass another duplex through the break. The overall degree of supercoiling of bacterial DNA is maintained by regulation of the net activity of topoisomerases I and II. Topoisomerases III and IV have more-specialized roles in DNA metabolism.

Eukaryotic cells also have type I and type II topoisomerases. The type I enzymes are topoisomerases I and III. The single type II enzyme has two isoforms in vertebrates, called IIα and IIβ. Most type II enzymes, including a DNA gyrase in archaea, are related and define a family called type IIA. The eukaryotic type II topoisomerases cannot underwind DNA (introduce negative supercoils), but they can relax both positive and negative supercoils (**Fig. 24-19a**). The capacity of type II topoisomerases to pass one duplex DNA segment through a double-strand break in another duplex allows these enzymes to untangle **catenanes**, DNA circles that are topologically linked (Fig. 24-19b). Some topoisomerases are specialized for decatenation functions. For example, the bacterial type II enzyme called topoisomerase IV is involved in chromosome untangling during cell division (Chapter 25).

Archaea have an unusual enzyme, topoisomerase VI, which alone defines the type IIB family. The full diversity of DNA topoisomerases is illustrated in **Table 24-4**. As we shall show in the next few chapters, topoisomerases play a critical role in every aspect of DNA metabolism, making them important drug targets for the treatment of bacterial infections and cancer.

DNA Compaction Requires a Special Form of Supercoiling

Supercoiled DNA molecules are uniform in several respects. The supercoils are right-handed in a negatively supercoiled DNA molecule (Fig. 24-15), and they tend to be extended and narrow rather than compacted, often with multiple


Type I topoisomerase

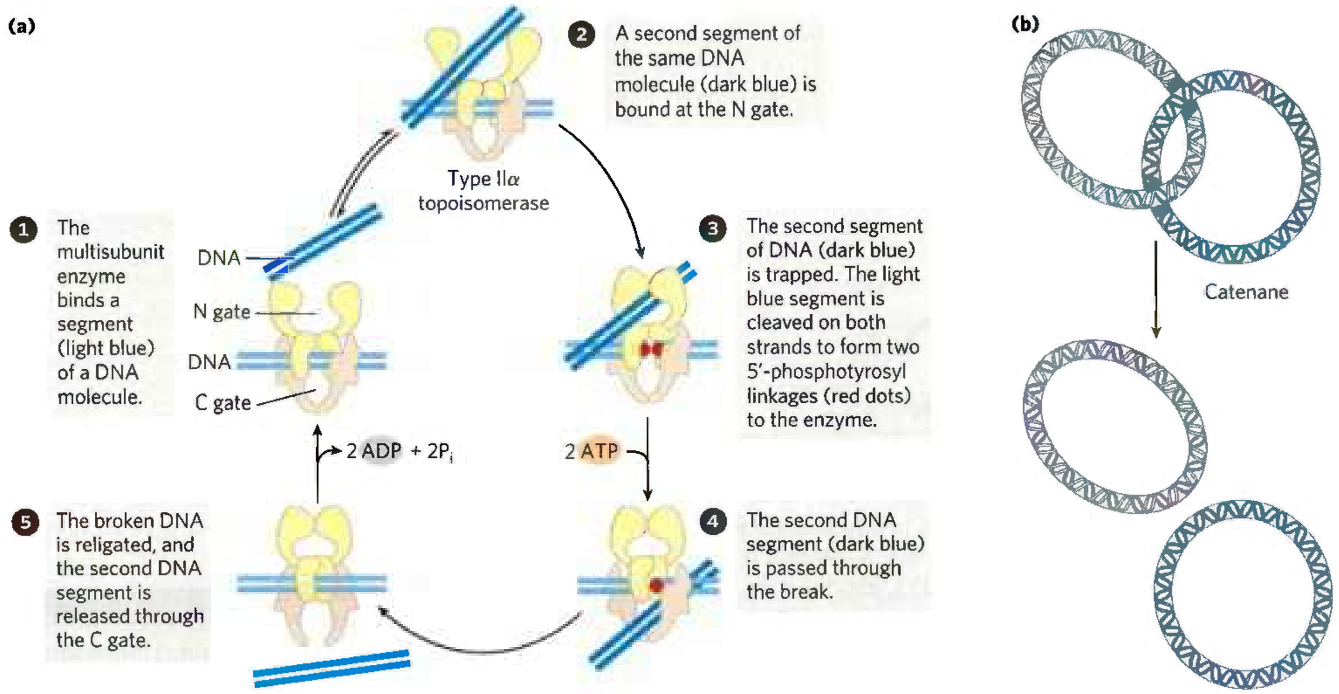
❷ Enzyme changes to an open conformation.

❸ The unbroken DNA strand passes through the break in the first strand.

❹ Enzyme in closed conformation; liberated 3′-OH attacks the 5′-phosphotyrosyl protein-DNA linkage to religate the cleaved DNA strand.

❶ Active-site Tyr attacks a phosphodiester bond in one DNA strand, cleaving it and creating a covalent 5′-phosphotyrosyl protein-DNA linkage.

MECHANISM FIGURE 24-18 The type I topoisomerase reaction. Bacterial topoisomerase I increases *Lk* by breaking one DNA strand, passing the unbroken strand through the break, then resealing the break. Nucleophilic attack by the active-site Tyr residue breaks one DNA strand. The ends are ligated by a second nucleophilic attack. At each step, one high-energy bond replaces another. [Information from J. J. Champoux, *Annu. Rev. Biochem.* 70:369, 2001, Fig. 3.]



① The multisubunit enzyme binds a segment (light blue) of a DNA molecule.

② A second segment of the same DNA molecule (dark blue) is bound at the N gate.

③ The second segment of DNA (dark blue) is trapped. The light blue segment is cleaved on both strands to form two 5′-phosphotyrosyl linkages (red dots) to the enzyme.

④ The second DNA segment (dark blue) is passed through the break.

⑤ The broken DNA is religated, and the second DNA segment is released through the C gate.

FIGURE 24-19 Alteration of the linking number by a eukaryotic type IIα topoisomerase. (a) The general mechanism features passage of one intact duplex DNA segment through a transient double-strand break in another segment. The DNA segment enters and leaves the topoisomerase through gated cavities, called the N gate and the C gate, above and below the bound DNA. Two ATPs are bound and hydrolyzed during this cycle. The enzyme structure and use of ATP are specific to this reaction. (b) When topologically linked as shown, two DNA circles are referred to as a catenane. By cleaving both strands of one circle and passing a segment of the second circle through the break, a type II topoisomerase can decatenate the circles. [(a) Information from J. J. Champoux, *Annu. Rev. Biochem.* 70:369, 2001, Fig. 11.]

TABLE Diversity in DNA Topoisomerases

Type	Mechanism	Family (defined by structural class)	Domain(s)	Notes
IA	Strand passage[a]	Topoisomerase I	Bacteria, Eukarya	Relaxes (−)
		Topoisomerase III	Bacteria, Eukarya	Relaxes (−)
		Reverse gyrase	Archaea, Bacteria	Uses ATP to introduce positive supercoils; thermophilic bacteria and archaea only
IB	Swivelase[b]	Topoisomerase IB	Bacteria, Eukarya	A few bacteria; all eukaryotes
IC	Swivelase	Topoisomerase V	Archaea	*Methanopyrus* only
IIA	Strand passage[c]	Topoisomerase II (DNA gyrase)	Archaea, Bacteria	Introduces negative supercoils (ATPase)
		Topoisomerase IIα	Eukarya	Relaxes (+ or −)
		Topoisomerase IIβ	Eukarya	Relaxes (+ or −)
		Topoisomerase IV	Bacteria	Decatenase[d]
IIB	Strand passage	Topoisomerase VI	Archaea, Bacteria, Eukarya	Among eukaryotes, plants, algae, and protists only

[a] See Figure 24-18.

[b] A nick is made in one strand, and the other strand is allowed to rotate to relieve topological strain.

[c] See Figure 24-19a.

[d] See Figure 24-19b.

branches (**Fig. 24-20**). At the superhelical densities normally encountered in cells, the length of the supercoil axis, including branches, is about 40% of the length of the DNA. This type of supercoiling is referred to as **plectonemic** (from the Greek *plektos*, "twisted," and *nema*, "thread"). The term can be applied to any structure with strands intertwined in some simple and regular way, and it is a good description of the general structure of supercoiled DNA in solution.

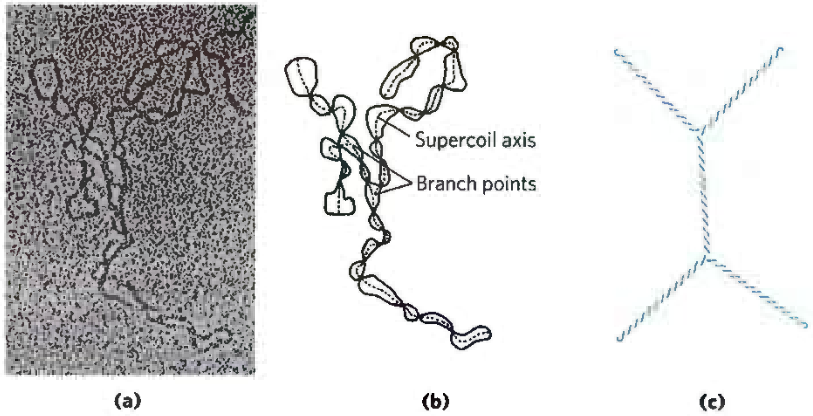


FIGURE 24-20 Plectonemic supercoiling. (a) Electron micrograph of plectonemically supercoiled plasmid DNA and (b) an interpretation of the observed structure. The dotted lines show the axis of the supercoil; notice the branching of the supercoil. (c) An idealized representation of this structure. [(a, b) Republished with permission of Elsevier, from T. C. Boles et al. (1990), "Structure of plectonemically supercoiled DNA," *J. Mol. Biol.* 213:931–951, Fig. 2; permission conveyed through Copyright Clearance Center, Inc.]

Plectonemic supercoiling, the form observed in isolated DNAs in the laboratory, does not produce sufficient compaction to package DNA in the cell. A second form of supercoiling, **solenoidal** (**Fig. 24-21**), can be adopted by an underwound DNA. Instead of the extended right-handed supercoils characteristic of the plectonemic form, solenoidal supercoiling involves tight left-handed turns, similar to the shape taken up by a garden hose neatly wrapped on a reel. Although their structures are dramatically different, plectonemic and solenoidal supercoiling are two forms of negative supercoiling that can be taken up by the *same* segment of underwound DNA. The two forms are readily interconvertible. Although the plectonemic form is more stable in solution, the solenoidal form can be stabilized by protein binding, as it is in eukaryotic chromosomes; it provides a much greater degree of compaction. Solenoidal supercoiling is a primary mechanism by which underwinding contributes to DNA compaction in cells.

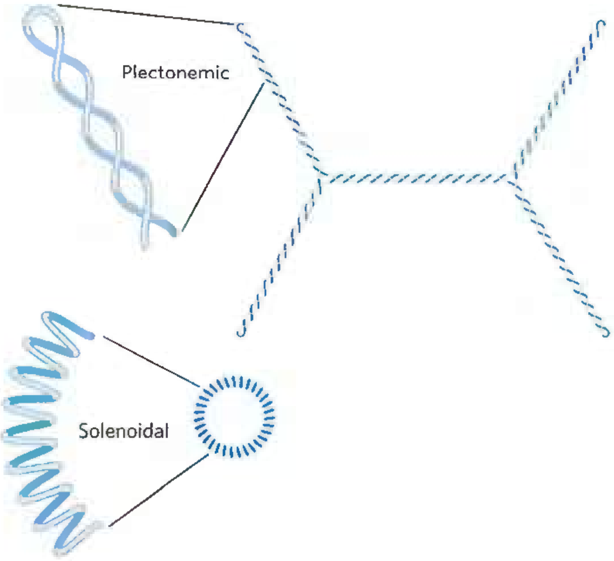


FIGURE 24-21 Plectonemic and solenoidal supercoiling of the same DNA molecule, drawn to scale. Plectonemic supercoiling takes the form of extended right-handed coils. Solenoidal negative supercoiling takes the form of tight left-handed turns about an imaginary tubelike structure. The two forms are readily interconverted, although the solenoidal form is generally not observed unless certain proteins are bound to the DNA. Solenoidal supercoiling provides a much greater degree of compaction.

SUMMARY 24.2 *DNA Supercoiling*

- Most cellular DNAs are supercoiled. Underwinding decreases the total number of helical turns in DNA relative to the relaxed, B form. To maintain an underwound state, DNA must be either a closed circle or bound to protein.
- Underwinding is quantified by a topological parameter called linking number, *Lk*. Underwinding is measured in terms of specific linking difference, or superhelical density, σ, which is $(Lk - Lk_0)/Lk_0$. For cellular DNAs, σ is typically –0.05 to –0.07, which means that approximately 5% to 7% of the helical turns in the DNA have been removed. DNA underwinding facilitates strand separation by enzymes of DNA metabolism.
- DNAs that differ only in linking number are called topoisomers. Topoisomerases, enzymes that underwind and/or relax DNA, catalyze changes in linking number. The two classes of topoisomerases, type I and type II, change *Lk* in increments of 1 or 2, respectively, per catalytic event.
- Supercoiled DNA in solution, unconstrained by proteins, takes on a plectonemic supercoiling structure. When supercoiled DNA is wrapped around specialized DNA-binding proteins, it forms solenoidal supercoils.

24.3 The Structure of Chromosomes

The term "chromosome" is used to refer to a nucleic acid molecule that is the repository of genetic information in a virus, a bacterium, an archaeon, a eukaryotic cell, or an organelle. It also refers to the densely colored bodies seen in the nuclei of dye-stained eukaryotic cells undergoing mitosis, as visualized using a light microscope.

Chromatin Consists of DNA, Proteins, and RNA

The eukaryotic cell cycle produces remarkable changes in the structure of chromosomes (**Fig. 24-22**). In nondividing eukaryotic cells (in the G0 phase) and those in interphase (G1, S, and G2), the chromosomal material,

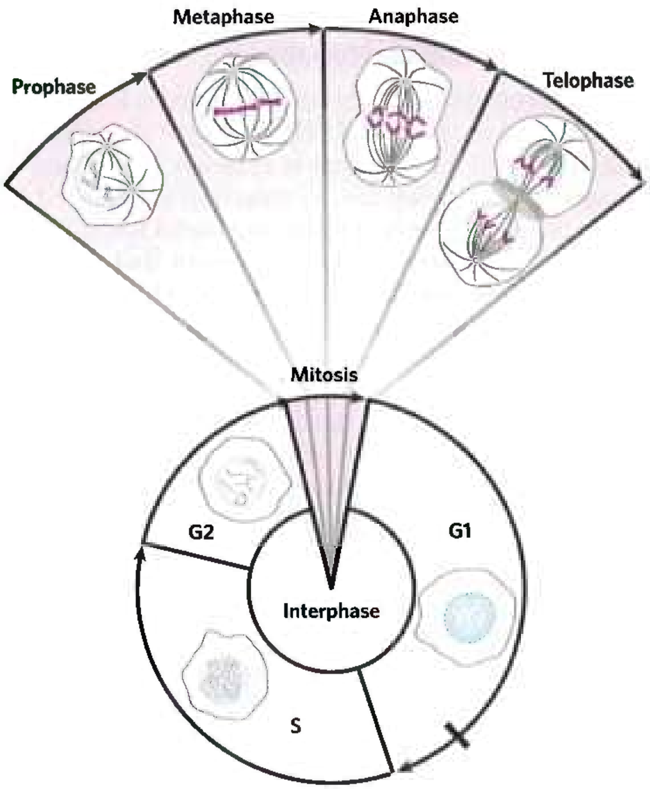


FIGURE 24-22 Changes in chromosome structure during the eukaryotic cell cycle. The relative lengths of the phases shown here are arbitrary. The duration of each phase varies with cell type and with growth conditions (for single-celled organisms) or metabolic state (for multicellular organisms); mitosis is typically the shortest phase. Cellular DNA is uncondensed throughout interphase, as shown in the cartoons of the nucleus. The interphase period can be divided into the G1 (gap) phase; the S (synthesis) phase, when the DNA is replicated; and the G2 phase, throughout which the replicated chromosomes (chromatids) cohere to one another. Mitosis can be divided into four stages. The DNA undergoes condensation in prophase. During metaphase, the condensed chromosomes line up in pairs along the plane halfway between the spindle poles. The two chromosomes of each pair are linked to different spindle poles via microtubules that extend between the spindle and the centromere. The sister chromatids separate at anaphase, each drawn toward the spindle pole to which it is connected. The process is completed in telophase. After cell division, the chromosomes decondense and the cycle begins anew.

chromatin, is amorphous. In the S phase of interphase, the DNA in this amorphous state replicates, each chromosome producing two sister chromosomes (called sister chromatids) that remain associated with each other after replication is complete. The chromosomes become much more condensed during prophase of mitosis, taking the form of a species-specific number of well-defined pairs of sister chromatids (Fig. 24-5).

P4 Chromatin consists of fibers containing protein and DNA in approximately equal proportions (by mass), along with a significant amount of associated RNA. The DNA in the chromatin is very tightly associated with proteins called **histones**, which package and order the DNA into structural units called **nucleosomes** (Fig. 24-23). Also found in chromatin are many nonhistone proteins, some of which help maintain chromosome structure and others that regulate the expression of specific genes (Chapter 28).

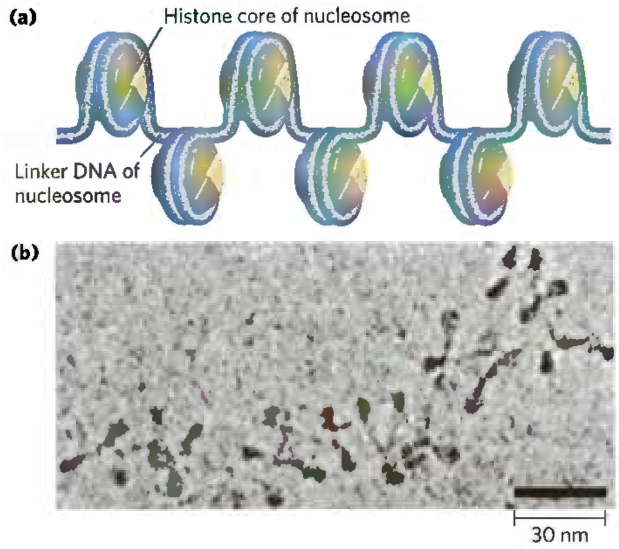


FIGURE 24-23 Nucleosomes. (a) Regularly spaced nucleosomes consist of core histone proteins bound to DNA. (b) In this electron micrograph, the DNA-wrapped histone octamer structures are clearly visible. [(b) J. Bednar et al., Nucleosomes, linker DNA, and linker histone form a unique structural motif that directs the higher-order folding and compaction of chromatin, *Proc. Natl. Acad. Sci. USA* vol. 95 no. 24: 14173–14178, November 1998 Cell Biology Fig. 1. © 1998 National Academy of Sciences, U.S.A.]

Beginning with nucleosomes, eukaryotic chromosomal DNA is packaged into a succession of higher-order structures that ultimately yield the compact chromosome seen with the light microscope. We now turn to a description of this structure in eukaryotes and compare it with the packaging of DNA in bacterial cells.

Histones Are Small, Basic Proteins

Found in the chromatin of all eukaryotic cells, histones have molecular weights between 11,000 and 21,000 and are very rich in the basic amino acids arginine and lysine (together these make up about one-fourth of the amino acid residues). All eukaryotic cells have five major classes of histones, differing in molecular weight and amino acid composition (Table 24-5). The H3 histones are nearly identical in amino acid sequence in all eukaryotes, as are the H4 histones, suggesting strict conservation of their functions. For example, only 2 of 102 amino acid residues differ between the H4 histone molecules of peas and cows, and only 8 differ between the H4 histones of humans and yeast. Histones H1, H2A, and H2B show less sequence similarity across eukaryotic species.

Each type of histone is subject to enzymatic modification by methylation, acetylation, ADP-ribosylation, phosphorylation, glycosylation, SUMOylation, or ubiquitination (p. 216 and Fig. 6-38). Such modifications affect the net electric charge, shape, and other properties of histones, as well as the structural and functional properties of the chromatin. The modifications play a role in the regulation of transcription and in chromatin structure at different stages of the cell cycle.

In addition, eukaryotes generally have several variant forms of certain histones, most notably histones

TABLE 24-5 Types and Properties of the Common Histones

Histones	Molecular weight	Number of amino acid residues	Content of basic amino acids (% of total)	
			Lys	Arg
H1[a]	21,130	223	29.5	11.3
H2A[a]	13,960	129	10.9	19.3
H2B[a]	13,774	125	16.0	16.4
H3	15,273	135	19.6	13.3
H4	11,236	102	10.8	13.7

[a] The sizes of these histones vary somewhat from species to species. The numbers given here are for bovine histones.

H2A and H3, described in more detail below. The variant forms, along with their modifications, have specialized roles in DNA metabolism.

Nucleosomes Are the Fundamental Organizational Units of Chromatin

P4 The eukaryotic chromosome depicted in Figure 24-5 represents the compaction of a DNA molecule about 10^5 μm long into a cell nucleus that is typically 5 to 10 μm in diameter. This 10,000-fold compaction is achieved by means of several levels of highly organized folding. Subjection of chromosomes to treatments that partially unfold them reveals a structure in which the DNA is bound tightly to beads of protein, often regularly spaced. The beads in this "beads-on-a-string" arrangement are complexes of histones and DNA. The bead plus the connecting DNA that leads to the next bead form the nucleosome, the fundamental unit of organization on which the higher-order packing of chromatin is built (**Fig. 24-24**). The bead of each nucleosome contains eight histone molecules: two copies each of H2A, H2B, H3, and H4.

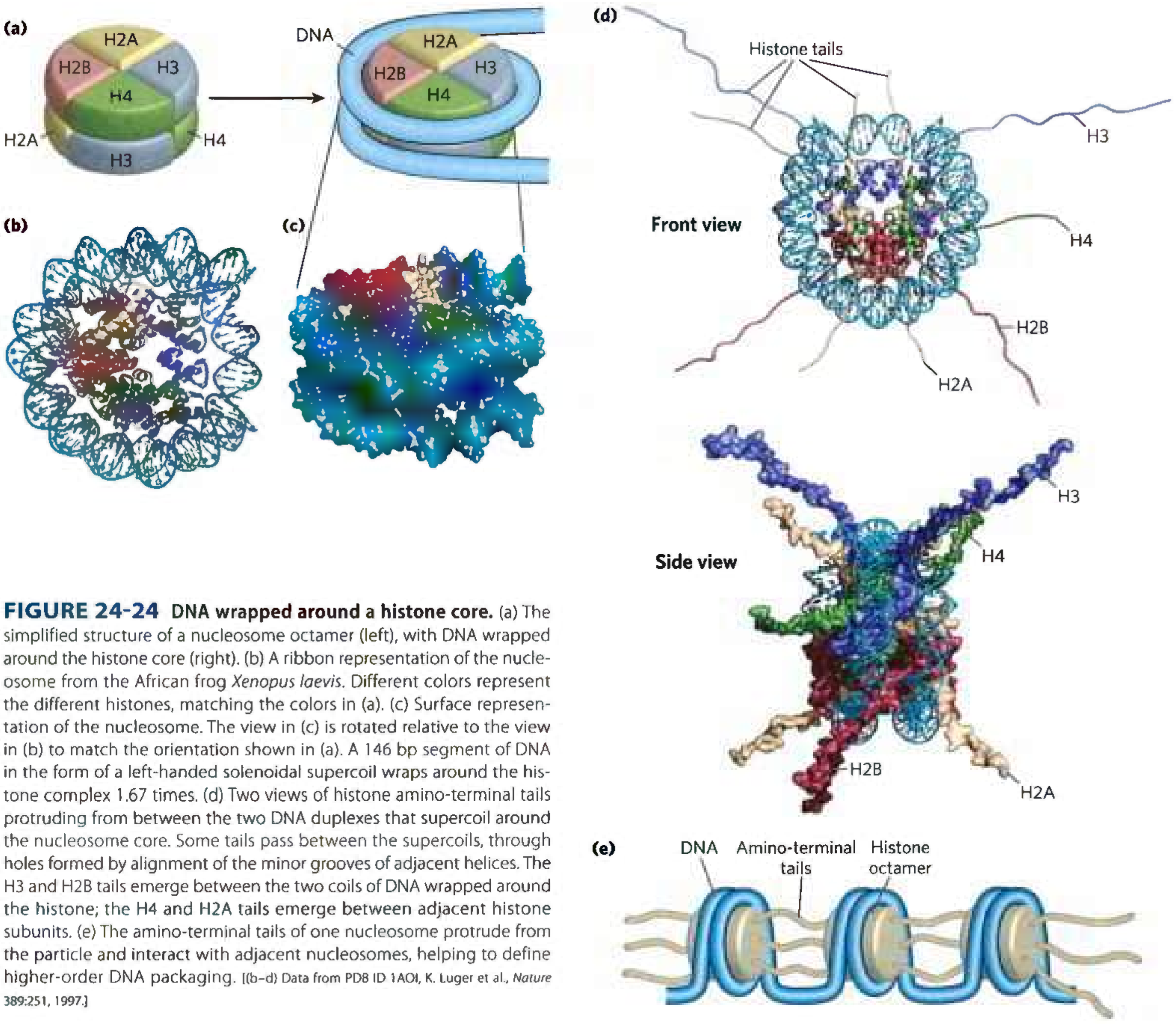


FIGURE 24-24 DNA wrapped around a histone core. (a) The simplified structure of a nucleosome octamer (left), with DNA wrapped around the histone core (right). (b) A ribbon representation of the nucleosome from the African frog *Xenopus laevis*. Different colors represent the different histones, matching the colors in (a). (c) Surface representation of the nucleosome. The view in (c) is rotated relative to the view in (b) to match the orientation shown in (a). A 146 bp segment of DNA in the form of a left-handed solenoidal supercoil wraps around the histone complex 1.67 times. (d) Two views of histone amino-terminal tails protruding from between the two DNA duplexes that supercoil around the nucleosome core. Some tails pass between the supercoils, through holes formed by alignment of the minor grooves of adjacent helices. The H3 and H2B tails emerge between the two coils of DNA wrapped around the histone; the H4 and H2A tails emerge between adjacent histone subunits. (e) The amino-terminal tails of one nucleosome protrude from the particle and interact with adjacent nucleosomes, helping to define higher-order DNA packaging. [(b–d) Data from PDB ID 1AOI, K. Luger et al., *Nature* 389:251, 1997.]

The spacing of the nucleosome beads provides a repeating unit typically of about 200 bp, of which 146 bp are bound tightly around the eight-part histone core and the remainder serve as linker DNA between nucleosome beads. Histone H1 binds to the linker DNA. Brief treatment of chromatin with enzymes that digest DNA causes the linker DNA to degrade preferentially, releasing histone particles containing 146 bp of bound DNA that is protected from digestion.

Researchers have crystallized nucleosome cores obtained in this way, and x-ray diffraction analysis reveals a particle made up of the eight histone molecules with the DNA wrapped around the core in the form of a left-handed solenoidal supercoil (Fig. 24-24; see also Fig. 24-21). Extending out from the nucleosome core are the amino-terminal tails of the histones, which are intrinsically disordered (Fig. 24-24d). Most of the histone modifications occur in these tails. The tails play a key role in forming contacts between nucleosomes in the chromatin (Fig. 24-24e). As the nucleosomes are approximately 10 to 11 nm in diameter, this simple beads-on-a-string structure is sometimes called the 10 nm fiber.

A close inspection of the nucleosome structure reveals why eukaryotic DNA is underwound even though eukaryotic cells lack enzymes that underwind DNA. Recall that the solenoidal wrapping of DNA in nucleosomes is but one form of supercoiling that can be taken up by underwound (negatively supercoiled) DNA. The tight wrapping of DNA around the histone core requires the removal of about one helical turn in the DNA. When the protein core of a nucleosome binds in vitro to a relaxed closed-circular DNA, the binding introduces a negative supercoil. Because this binding process does not break the DNA or change the linking number, the formation of a negative solenoidal supercoil must be accompanied by a compensatory positive supercoil in the unbound region of the DNA (**Fig. 24-25**). As mentioned earlier, eukaryotic topoisomerases can relax positive supercoils. Relaxing the unbound positive supercoil leaves the negative supercoil fixed (through its binding to the nucleosome's histone core) and results in an overall decrease in linking number. Indeed, topoisomerases have proved necessary for assembling chromatin from purified histones and closed-circular DNA in vitro.

Another factor that affects the binding of DNA to histones in nucleosome cores is the sequence of the bound DNA. Histone cores do not bind at random positions on the DNA; rather, some locations are more likely to be bound than others. This positioning is not fully understood, but in some cases it seems to depend on a local abundance of A=T base pairs in the DNA helix where it is in contact with the histones (**Fig. 24-26**). A cluster of two or three A=T base pairs facilitates the compression of the minor groove that is needed for the DNA to wrap tightly around the nucleosome's histone core. Nucleosomes bind particularly well to sequences where AA or AT or TT dinucleotides are staggered at 10 bp intervals, an arrangement that can account for up to 50% of the positions of bound histones in vivo.

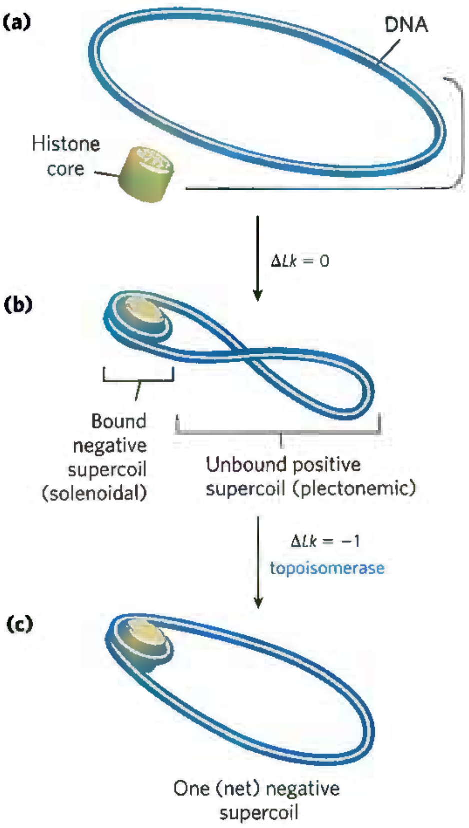
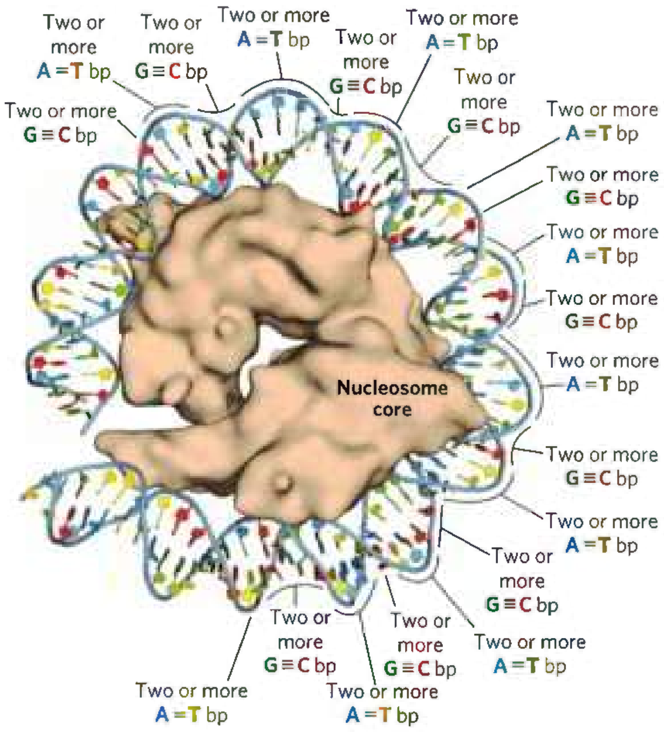

(a)
DNA
Histone core
ΔLk = 0
(b)
Bound negative supercoil (solenoidal)
Unbound positive supercoil (plectonemic)
ΔLk = −1
topoisomerase
(c)
One (net) negative supercoil

FIGURE 24-25 Chromatin assembly. (a) Relaxed closed-circular DNA. (b) Binding of a histone core to form a nucleosome induces one negative supercoil. In the absence of any strand breaks, a positive supercoil must form elsewhere in the DNA ($\Delta Lk = 0$). (c) Relaxation of this positive supercoil by a topoisomerase leaves one net negative supercoil ($\Delta Lk = -1$).

Nucleosome cores are deposited on DNA during replication or following other processes that require a transient displacement of nucleosomes. Other, nonhistone proteins are required for the positioning of some nucleosome cores. In several organisms, certain proteins bind to a specific DNA sequence and facilitate the formation of a nucleosome core nearby. Nucleosome cores seem to be deposited stepwise. A tetramer of two H3 and two H4 histones binds first, followed by two H2A–H2B dimers. The incorporation of nucleosomes into chromosomes after chromosomal replication is mediated by a complex of **histone chaperones** conserved in all eukaryotes and described here for the yeast system. These include the proteins CAF1 (chromatin assembly factor 1), RTT106 and RTT109 (regulation of Ty1 transposition), and ASF1 (anti-silencing factor 1). ASF1 binds to newly synthesized H3–H4 dimers and facilitates RTT109-mediated acetylation at Lys^{56} (K56) of histone H3 (H3K56). The H3K56 modification increases the affinity of H3 for CAF1 and RTT, which in turn promote the deposition of H3-containing histone complexes on the DNA after replication. CAF1 binds directly to a key component of the


Two or more A=T bp
Two or more G≡C bp
Two or more A=T bp
Two or more G≡C bp
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Two or more A=T bp
Two or more G≡C bp
Two or more A=T bp
Two or more G≡C bp
Two or more A=T bp
Nucleosome core
Two or more G≡C bp
Two or more A=T bp
Two or more G≡C bp
Two or more A=T bp
Two or more G≡C bp
Two or more G≡C bp
Two or more A=T bp
Two or more A=T bp

FIGURE 24-26 **The effect of DNA sequence on nucleosome binding.** Runs of two or more A=T base pairs facilitate the bending of DNA, whereas runs of two or more G≡C base pairs have the opposite effect. When spaced at about 10 bp intervals, consecutive A=T base pairs help bend DNA into a circle. When consecutive G≡C base pairs are spaced 10 bp apart, offset by 5 bp from runs of A=T base pairs, DNA binding to the nucleosome core is facilitated. [Data from PDB ID 1AOI, K. Luger et al., *Nature* 389:251, 1997.]

replication complex called PCNA (see Chapter 25), so that nucleosome deposition is closely coordinated with replication. Some of the same histone chaperones, or different ones, may help assemble nucleosomes after DNA repair, transcription, or other processes.

Histone exchange factors permit the substitution of histone variants for core histones in contexts other than postreplication. Proper placement of these variant histones is important. Studies show that mice lacking one of the variant histones die as early embryos (**Box 24-1**). Precise positioning of nucleosome cores also plays a role in the expression of some eukaryotic genes (Chapter 28).

Nucleosomes Are Packed into Highly Condensed Chromosome Structures

Wrapping of DNA around a nucleosome core compacts the DNA length about sevenfold. The overall compaction in a chromosome, however, is greater than 10,000-fold—ample evidence for even higher orders of structural organization. The condensation does not follow a rigid organization, but it is also not random and occurs so as to avoid the formation of knots.

P4 The higher levels of folding are not yet fully understood, but certain regions of DNA seem to associate with a chromosomal scaffold (**Fig. 24-27**) that contains many proteins. Given the need to fold and compact the chromosome without creating knots, topoisomerase II is one of the most abundant proteins in the chromosome, further emphasizing the relationship between DNA underwinding and chromatin structure. **P3** Topoisomerase II is so important to the maintenance of chromatin structure that inhibitors of this enzyme can kill rapidly dividing cells. Several drugs used in cancer chemotherapy are topoisomerase II inhibitors that allow the enzyme to promote strand breakage but not the resealing of the breaks (**Box 24-2**, p. 906).

There are additional layers of organization in the eukaryotic nucleus. Just before cell division during mitosis, chromosomes can be seen as highly condensed and organized structures (**Figure 24-28a**). During interphase, chromosomes appear dispersed (Fig. 24-28b,

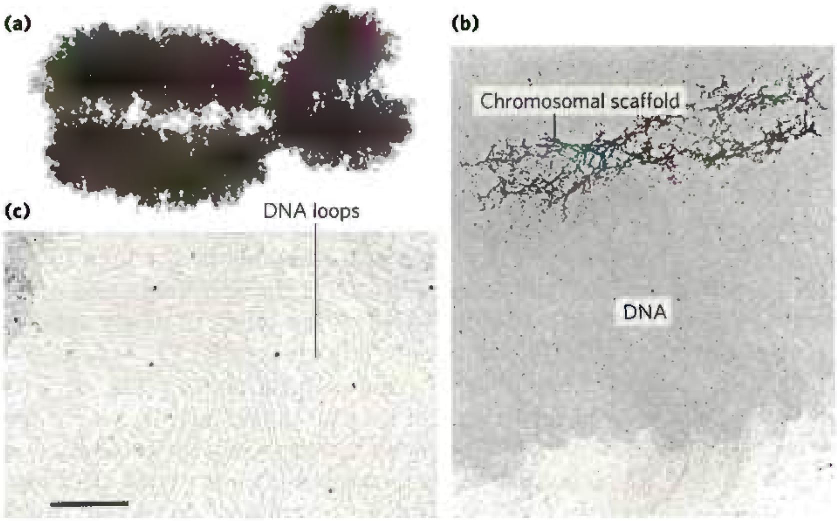
(a)
(b)
Chromosomal scaffold
(c)
DNA loops
DNA

FIGURE 24-27 **Loops of DNA attached to a chromosomal scaffold.** (a) A swollen mitotic chromosome, produced in a buffer of low ionic strength, as seen in the electron microscope. Notice the appearance of chromatin loops at the margins. (b) Extraction of the histones leaves a proteinaceous chromosomal scaffold surrounded by naked DNA. (c) The DNA appears to be organized in loops attached at their base to the scaffold in the upper left corner; scale bar = 1 μm. The three images are at different magnifications. [(a, b) Don W. Fawcett/Science Source. (c) U. K. Laemmli et al., "Metaphase chromosome structure: The role of nonhistone proteins," *Cold Spring Harb. Symp. Quant. Biol.* 42:351, 1978. © Cold Spring Harbor Laboratory Press.]

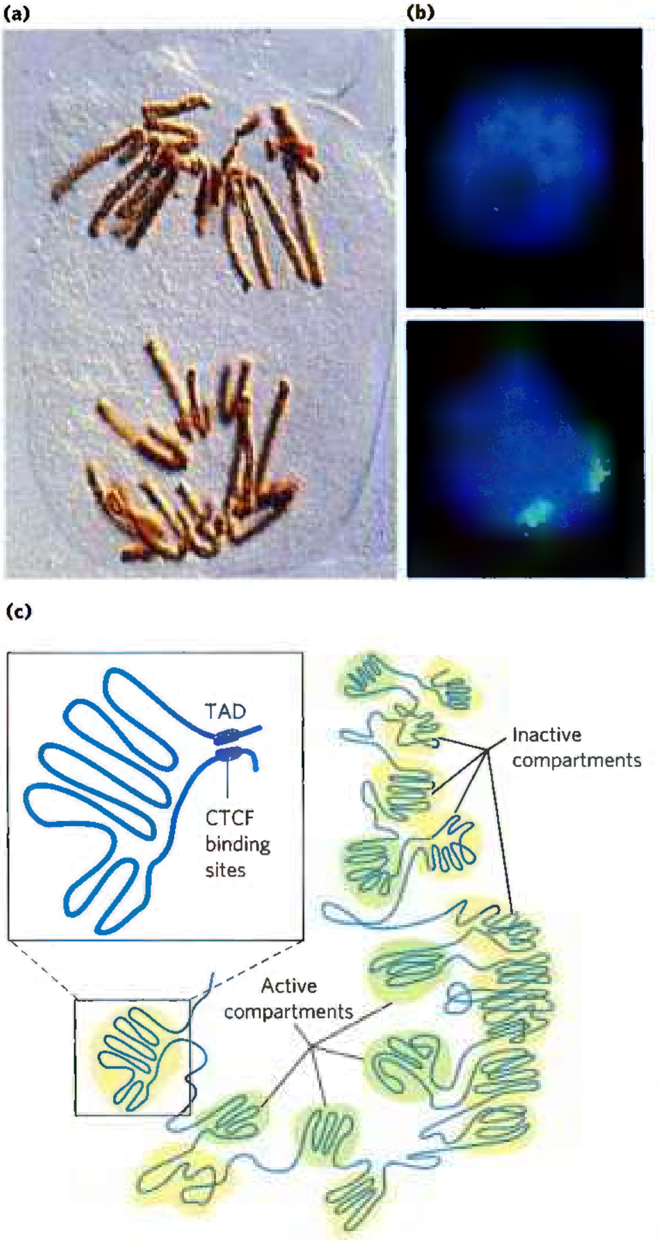

FIGURE 24-28 Chromosomal organization in the eukaryotic nucleus. (a) Condensed chromosomes at the mitotic anaphase in cells of the bluebell (*Endymion* sp.). (b) Interphase nuclei of human breast epithelial cells. The nucleus on the bottom has been treated so that its two copies of chromosome 11 fluoresce green. (c) Chromosomes are organized into active compartments, in which actively transcribed genes are clustered, and inactive compartments, made of heterochromatin within which genes are silenced. In both cases, the compartments feature large DNA loops called topologically associating domains (TADs), many constrained at their bases by DNA-binding proteins such as CTCF. Certain lncRNAs (Fig. 24-29) also play a role in defining loops within chromatin. Constraining a loop at its base not only provides a boundary for the loop, but also allows supercoiling within the loop to be controlled. [(a) Pr. G. Giménez-Martín/Science Source. (b) Karen Meaburn and Tom Misteli/National Cancer Institute.]

top), but they do not meander randomly in nuclear space (Fig. 24-28b, bottom). Each chromosome appears to be organized with two sets of compartments, one set that is transcriptionally active and the other that is transcriptionally inactive. The level of chromatin condensation is reduced in the transcriptionally active compartments. The highly condensed DNA in transcriptionally inactive regions or in regions lacking genes is also called **heterochromatin**. Within each compartment, large segments of DNA are organized in loops called **topologically associating domains**, or **TADs**. The TADs, which typically average about 800,000 bp, are often bordered by DNA sites recognized by the CCCTC-binding factor (CTCF). The binding by CTCF brings together sites that are otherwise quite distant in the linear DNA sequence, tethering the base of the loop (Fig. 24-28c).

P4 Another important component defining the structure of chromosomes is RNA, particularly a class of RNAs called **long noncoding RNAs (lncRNAs)**. RNA has the potential to take up a variety of structures (see Chapter 8), and can interact with DNA, proteins, or other RNA molecules. The lncRNAs, as the name implies, are functional RNAs, generally over 200 nucleotides long, that do not necessarily encode proteins. Many lncRNAs are now known and more are being discovered rapidly. Many of them provide a scaffold for proteins that both bind to the RNA and affect chromosome structure and function. Some proteins that bind to lncRNAs also have binding sites on DNA, and the RNAs provide a link that tethers distant parts of the chromosome together (Fig. 24-29). Other proteins that bind to lncRNAs help to position nucleosomes, modify histones,

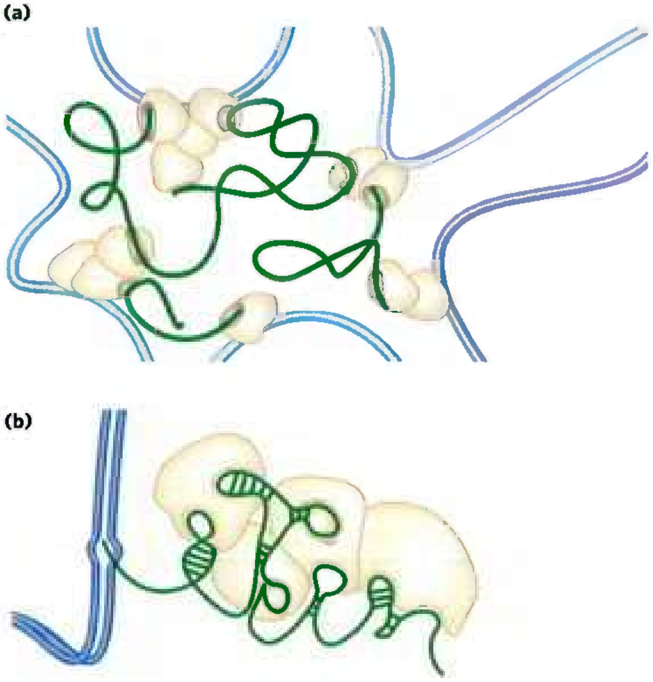

FIGURE 24-29 Effects of lncRNAs on chromosome architecture and gene expression. (a) Various lncRNAs can interact with DNA-binding proteins and in some cases with DNA to tether otherwise distant segments of DNA. (b) A transcribed lncRNA can interact with multiple proteins that have gene regulatory roles, suppressing or activating transcription of nearby genes. [Information from J. M. Engreitz et al., *Nat. Rev. Mol. Cell Biol.* 17:756, 2016, Fig. 5.]

BOX 24-1 METHODS

Epigenetics, Nucleosome Structure, and Histone Variants

Information that is passed from one generation to the next — to daughter cells at cell division or from parent to offspring — but is not encoded in DNA sequences is referred to as **epigenetic** information. Much of it is in the form of covalent modification of histones and/or the placement of histone variants in chromosomes. Understanding that placement in the context of a chromosome encompassing millions of base pairs is the focus of some powerful technologies.

Chromatin regions where active gene expression (transcription) is occurring tend to be partially decondensed and are called **euchromatin**. In these regions, histones H3 and H2A are often replaced by the histone variants H3.3 and H2AZ, respectively (Fig. 1). The complexes that deposit nucleosome cores containing histone variants on the DNA are similar to those that deposit nucleosome cores with the more common histones. Nucleosome cores containing histone H3.3 are deposited by a complex in which CAF1 (chromatin *a*ssembly *f*actor 1) is replaced by the protein HIRA (a name derived from a class of proteins called HIR, for *hi*stone *r*epressor). Both CAF1 and HIRA can be considered histone chaperones, helping to ensure the proper assembly and placement of nucleosomes. Histone H3.3 differs in sequence from H3 by only four amino acid residues, but these residues all play key roles in histone deposition.

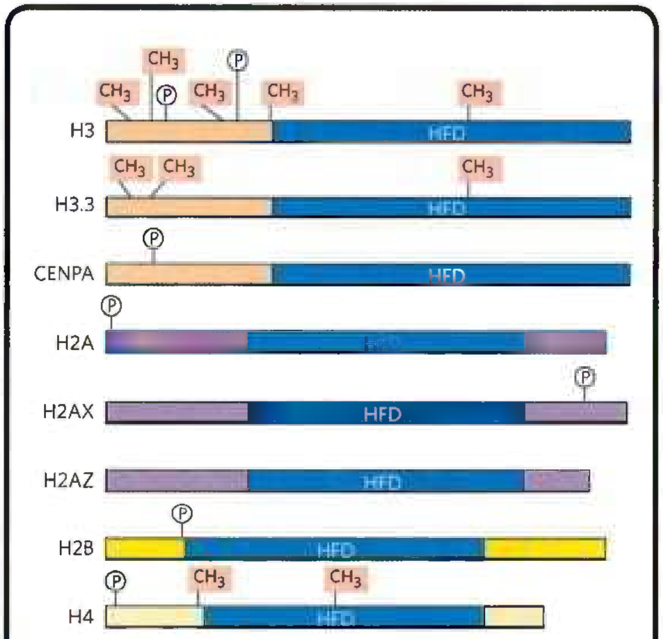

FIGURE 1 Shown here are the standard histones H3, H2A, and H2B and a few of the known variants. Sites of Lys/Arg residue methylation and Ser phosphorylation are indicated. HFD denotes the histone-fold domain, a structural domain common to all standard histones. Regions denoted in other colors define sequence and structural homologies. [Information from K. Sarma and D. Reinberg, *Nat. Rev. Mol. Cell Biol.* 6:139, 2005.]

Like histone H3.3, H2AZ is associated with a distinct nucleosome deposition complex, and it is generally associated with chromatin regions involved in active transcription. Incorporation of H2AZ stabilizes the nucleosome octamer, but it impedes some cooperative interactions between nucleosomes that are needed to compact the chromosome. This leads to a more open chromosome structure that enables the expression of genes in the region where H2AZ is located. The gene encoding H2AZ is essential in mammals. In fruit flies, loss of H2AZ prevents development beyond the larval stage.

Another H2A variant is H2AX, which is associated with DNA repair and genetic recombination. In mice, the absence of H2AX results in genome instability and male infertility. Modest amounts of H2AX seem to be scattered throughout the genome. When a double-strand break occurs, nearby molecules of H2AX become phosphorylated at Ser^{139} in the carboxyl-terminal region. If this phosphorylation is blocked experimentally, formation of the protein complexes necessary for DNA repair is inhibited.

The H3 histone variant known as CENPA (*cen*tromere *p*rotein *A*) is associated with the repeated DNA sequences in centromeres. The chromatin in the centromere region contains the histone chaperones CAF1 and HIRA, and both proteins could be involved in the deposition of nucleosome cores containing CENPA. Elimination of the gene for CENPA is lethal in mice.

The function and positioning of the histone variants can be studied by an application of technologies used in genomics. One useful technology is **chromatin immunoprecipitation,** or **chromatin IP (ChIP)**. Nucleosomes containing a particular histone variant are precipitated by an antibody that binds specifically to this variant. These nucleosome cores can be studied in isolation from their DNA, but more commonly the DNA associated with them is included in the study to determine where the nucleosome cores of interest bind. The DNA can be sequenced, yielding a map of genomic sequences to which those particular nucleosome cores bind. This technique is called a **ChIP-Seq** experiment (Fig. 2).

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methylate DNA at various locations to alter gene transcription, and generally affect chromosome structure in many different ways. Some well-studied examples include particular lncRNAs that play a major role in X chromosome inactivation in mammals (**Box 24-3**).

The entire structure of each chromosome is constrained within a subnuclear domain called a **chromosome territory** (**Fig. 24-30**). There is little or no intermingling of chromosomal DNA in different territories. The exact location of chromosome territories

The histone variants, along with the many covalent modifications that histones undergo, help define and isolate the functions of chromatin. They mark the chromatin, facilitating or suppressing specific functions such as chromosome segregation, transcription, and DNA repair. The histone modifications do not disappear at cell division or during meiosis, and thus they become part of the information transmitted from one generation to the next in all eukaryotic organisms.

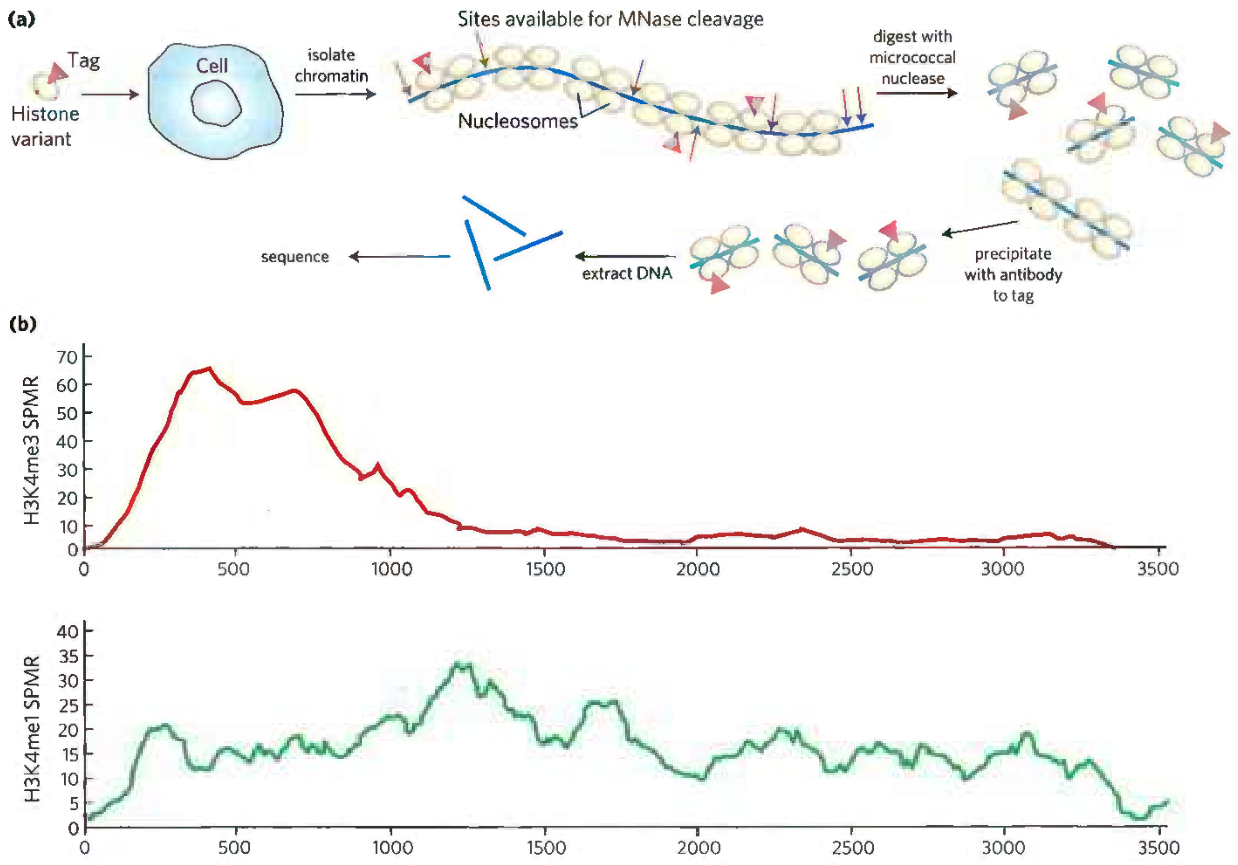


FIGURE 2 A ChIP-Seq experiment is designed to reveal the genomic DNA sequences to which a particular histone variant binds. (a) A histone variant with an epitope tag (a protein or chemical structure recognized by an antibody; see Chapters 5 and 9) is introduced into a particular cell type, where it is incorporated into nucleosomes. (In some cases, an epitope tag is unnecessary because antibodies are available that bind directly to the histone modification of interest.) Chromatin is isolated from the cells and digested briefly with micrococcal nuclease (MNase). The DNA bound in nucleosomes is protected from digestion, but the linker DNA is cleaved, releasing segments of DNA bound to one or two nucleosomes. An antibody that binds to the epitope tag is added, and the nucleosomes containing the epitope-tagged histone variant are selectively precipitated. The DNA in these nucleosomes is extracted from the precipitate and sequenced in depth to reveal the locations where the nucleosomes are bound. (b) In this example, the binding of different modified versions of histone H3 is characterized along the gene *YLR249W* from the yeast *S. cerevisiae*. Numbers along the bottom correspond to numbered nucleotide positions in this chromosomal segment. The top and bottom panels show the distributions of H3K4me3 (H3 with Lys^4 methylated three times) and H3K4me1, respectively. SPMR is sequence tags per million reads. [Information from L. M. Soares et al., *Mol. Cell* 68:773, 2017, Fig. 1D.]

varies from cell to cell in an organism, but some spatial patterns are evident. Some chromosomes have a higher density of genes than others (for example, human chromosomes 1, 16, 17, 19, and 22), and these tend to have territories in the center of the nucleus. Chromosomes with more heterochromatin tend to be located on the nuclear periphery. Spaces between chromosomes are often sites where transcriptional machinery and transcriptionally active genes on adjacent chromosomes are concentrated.

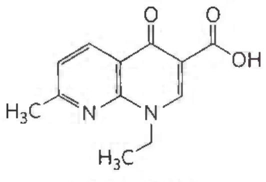
BOX 24-2 MEDICINE

Curing Disease by Inhibiting Topoisomerases

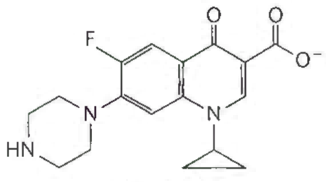
P3 The topological state of cellular DNA is intimately connected with its function. Without topoisomerases, cells cannot replicate or package their DNA, or express their genes—and they die. Inhibitors of topoisomerases have therefore become important pharmaceutical agents, targeted at infectious agents and malignant cells.

Two classes of bacterial topoisomerase inhibitors have been developed as antibiotics. The coumarins, including novobiocin and coumermycin A1, are natural products derived from *Streptomyces* species. They inhibit the ATP binding of the bacterial type II topoisomerases, DNA gyrase and topoisomerase IV. These antibiotics are not often used to treat infections in humans, but research continues to identify clinically effective variants.

The quinolone antibiotics, also inhibitors of bacterial DNA gyrase and topoisomerase IV, first appeared in 1962 with the introduction of nalidixic acid. This compound had limited effectiveness and is no longer used clinically in the United States, but the continued development of this class of drugs eventually led to the introduction of the fluoroquinolones, exemplified by ciprofloxacin (Cipro). The quinolones act by blocking the last step of the topoisomerase reaction, the resealing of the DNA strand breaks. Ciprofloxacin is a broad-spectrum antibiotic. It is one of the few antibiotics reliably effective in treating anthrax infections and is considered a valuable agent in protection against possible bioterrorism. Quinolones are selective for the bacterial topoisomerases, inhibiting the eukaryotic enzymes only at concentrations several orders of magnitude greater than the therapeutic doses.



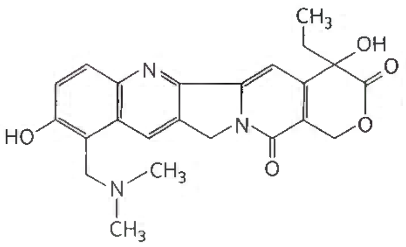
Nalidixic acid



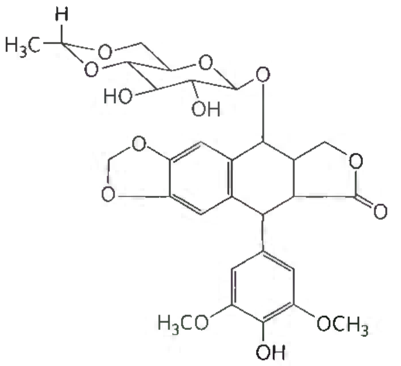
Ciprofloxacin

Some of the most important chemotherapeutic agents used in cancer treatment are inhibitors of human topoisomerases. Topoisomerases are generally present at elevated levels in tumor cells, and agents targeted to these enzymes are much more toxic to the tumors than to most other tissue types. Inhibitors of both type I and type II topoisomerases have been developed as anticancer drugs.

Camptothecin, isolated from a Chinese ornamental tree and first tested clinically in the 1970s, is an inhibitor of eukaryotic type I topoisomerases. Clinical trials indicated limited effectiveness, despite its early promise in preclinical work on mice. However, two effective derivatives, irinotecan (Campto) and topotecan (Hycamtin)—used to treat colorectal cancer and ovarian cancer, respectively—were developed in the 1990s. All of these drugs act by trapping the topoisomerase-DNA complex in which the DNA is cleaved, inhibiting religation.



Topotecan



Etoposide

The human type II topoisomerases are targeted by a variety of antitumor drugs, including doxorubicin (Adriamycin), etoposide (Etopophos), and ellipticine. Doxorubicin, effective against several kinds of human tumors, is an anthracycline in clinical use. Most of these drugs stabilize the covalent topoisomerase-DNA (cleaved) complex.

All of these anticancer agents generally increase the levels of DNA damage in the targeted, rapidly growing tumor cells. However, noncancerous tissues can also be affected, leading to a more general toxicity and unpleasant side effects that must be managed during therapy. As cancer therapies become more effective and survival statistics for cancer patients improve, the independent appearance of new tumors is becoming a greater problem. In the continuing search for new cancer therapies, topoisomerases are likely to remain prominent targets for research.

BOX 24-3

X Chromosome Inactivation by an lncRNA: Preventing Too Much of a Good (or Bad) Thing

In higher eukaryotes, the Y chromosome contains only a few genes. The X chromosome, in contrast, often contains more than a thousand genes, many essential for cell function and organismal development. Males have just one X chromosome, whereas females have two. Females thus could end up with a double dose of gene products, leading to a variety of potentially toxic outcomes. Mechanisms of gene dosage compensation vary among different groups of eukaryotes. In most mammals, dosage compensation is accomplished by X chromosome inactivation, randomly affecting just one of the two X chromosomes in a given cell. The effects of this can be seen in calico cats, all of which are female. Fur pigmentation genes are found on the X chromosome. When a cat inherits chromosomes with different pigmentation alleles on its two X chromosomes, its coloration patterns reflect the random inactivation of one or the other X chromosome in different sets of cells.

A calico cat. [krblokhin/Getty Images]

An lncRNA called Xist (X-inactive specific transcript) is uniquely expressed from its gene on the inactive X chromosome. Xist is approximately 17,000 nucleotides long, encodes no proteins, and is expressed only in cells with two X chromosomes (not in males). Xist is an essential component of the X inactivation process. This lncRNA interacts with many proteins and transcription factors (Fig. 1). As it migrates in the region immediately proximal to the gene from which it is transcribed, Xist gradually encompasses more and more DNA as that X chromosome undergoes a major condensation, giving rise to the inactive form called a **Barr body**.

The active X chromosome also transcribes the gene that produces Xist, but it does so in the opposite direction to produce a longer lncRNA called Tsix (Xist spelled backward). Tsix is synthesized using a different RNA polymerase binding site (promoter; Chapter 26) and is 40,000 nucleotides long. A large part of Tsix is perfectly complementary to Xist, and it antagonizes the function of any Xist that may appear near the active X chromosome.

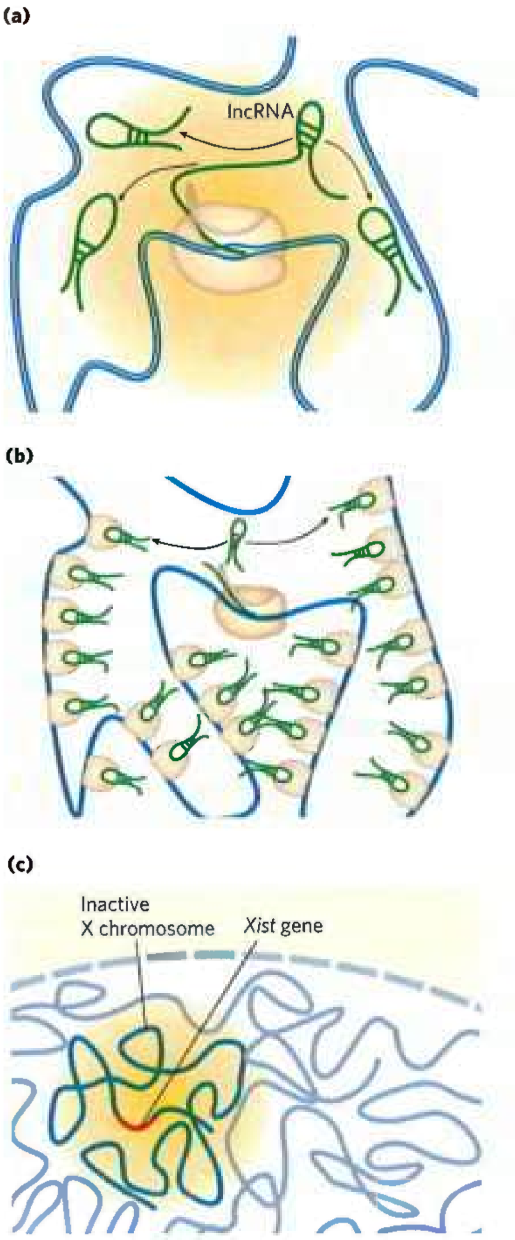


FIGURE 1 (a) As Xist RNA is transcribed, it migrates to nearby regions within an X chromosome, (b) binding to proteins including SAFA (scaffold attachment factor A). Xist binding spreads through the chromosome, leading to condensation and other architectural changes to form a Barr body. Other than the *Xist* gene itself, gene expression is suppressed throughout the chromosome. (c) The effects are quite local; Xist does not spread within the nucleus beyond the chromosome territory occupied by the inactivated X chromosome. [Information from J. M. Engreitz et al., *Nat. Rev. Mol. Cell Biol.* 17:756, 2016, Fig. 5.]

Random X inactivation occurs in the cells of a female embryo long before birth. The processes that initiate the inactivation of one, but not both, of the X chromosomes in a particular cell are still being elucidated.

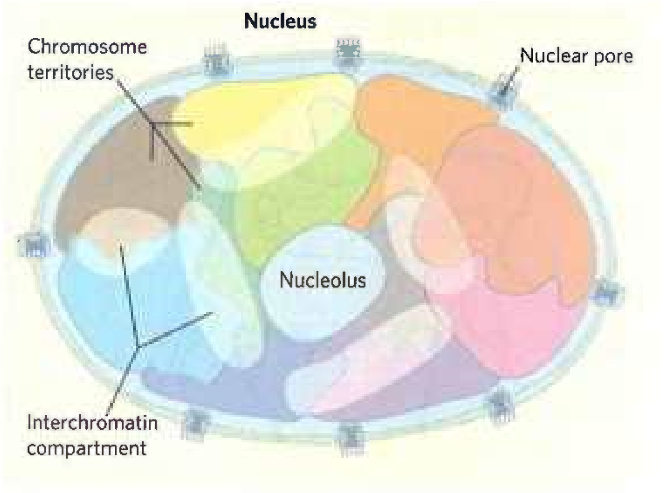


FIGURE 24-30 Chromosome territories. Cartoon showing chromosome territories in a eukaryotic nucleus. The interchromatin compartments are enriched in transcriptional machinery and have abundant actively transcribed genes. The nucleolus is a suborganelle within the nucleus where ribosomes are synthesized and assembled (Chapter 27).

Condensed Chromosome Structures Are Maintained by SMC Proteins

P4 **SMC proteins** (*s*tructural *m*aintenance of *c*hromosomes), the third major class of chromatin protein in addition to the histones and topoisomerases, are responsible for maintaining the structure and integrity of chromosomes following replication. The primary structure of SMC proteins consists of five distinct domains (**Fig. 24-31a**). The amino- and carboxyl-terminal globular domains, N and C, each of which contains part of an ATP-hydrolytic site, are connected by two regions of α-helical coiled-coil motifs (see Fig. 4-10) joined by a hinge domain. The proteins are generally dimeric, forming a V-shaped complex that is thought to be tied together through the protein's hinge domains. One N domain and one C domain come together like tweezers to form a complete ATP-hydrolytic site at each free end of the V (Fig. 24-31b).

Proteins in the SMC family are found in all types of organisms, from bacteria to humans. Eukaryotes have two major types, cohesins and condensins, both of which are bound by regulatory and accessory proteins (Fig. 24-31c, d). **Cohesins** play a substantial role in linking together sister chromatids immediately after replication and keeping them together as the chromosomes condense to metaphase. This linkage is essential if chromosomes are to segregate properly at cell division. Cohesins, along with a third protein, kleisin, are thought to form a ring around the replicated chromosomes that ties them together until separation is required. The ring may expand and contract in response to ATP hydrolysis. **Condensins** are essential to the condensation of chromosomes as cells enter mitosis. In the laboratory, condensins bind to DNA in a manner that creates positive supercoils; that is, condensin binding causes the DNA to become overwound, in contrast to the underwinding

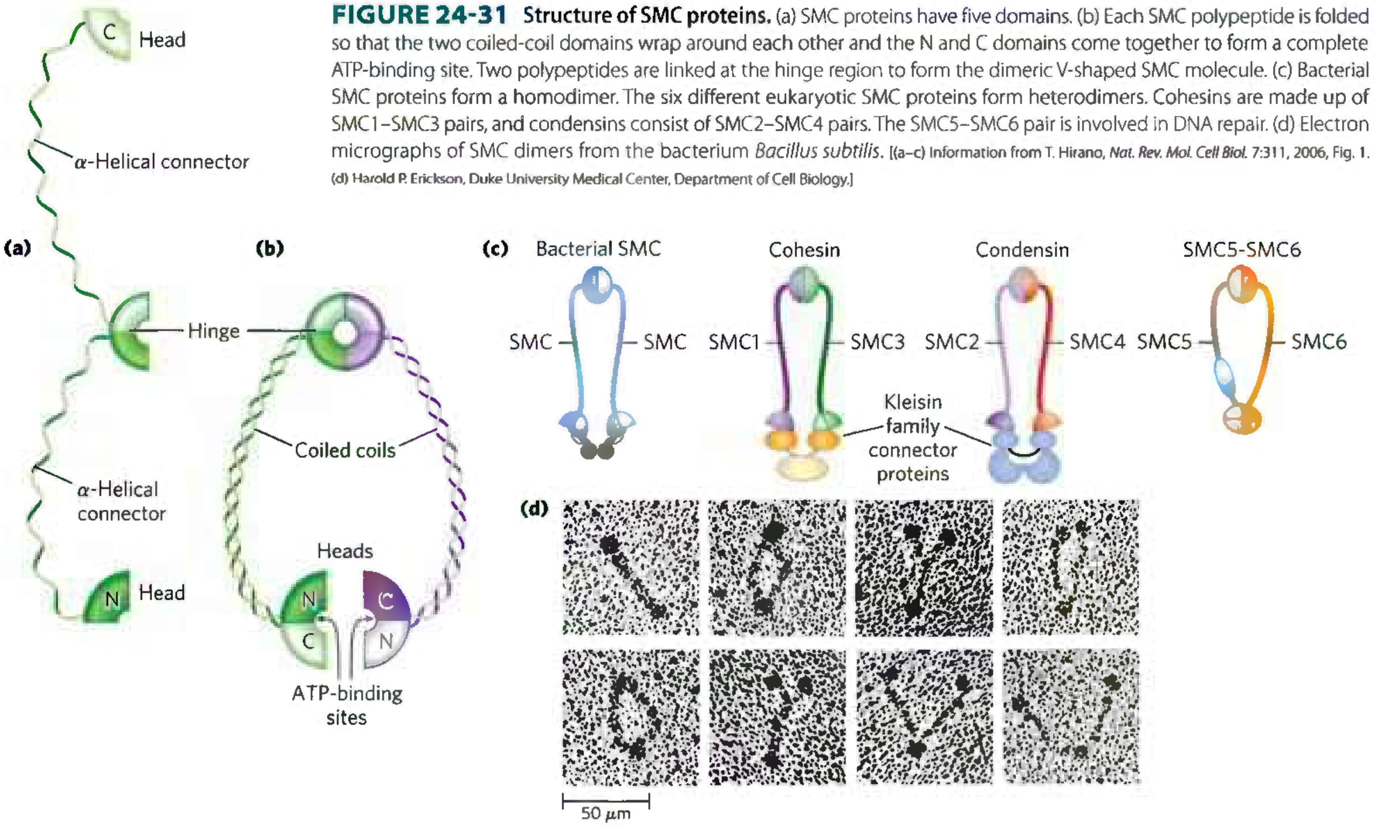


FIGURE 24-31 Structure of SMC proteins. (a) SMC proteins have five domains. (b) Each SMC polypeptide is folded so that the two coiled-coil domains wrap around each other and the N and C domains come together to form a complete ATP-binding site. Two polypeptides are linked at the hinge region to form the dimeric V-shaped SMC molecule. (c) Bacterial SMC proteins form a homodimer. The six different eukaryotic SMC proteins form heterodimers. Cohesins are made up of SMC1–SMC3 pairs, and condensins consist of SMC2–SMC4 pairs. The SMC5–SMC6 pair is involved in DNA repair. (d) Electron micrographs of SMC dimers from the bacterium *Bacillus subtilis*. [(a–c) Information from T. Hirano, *Nat. Rev. Mol. Cell Biol.* 7:311, 2006, Fig. 1. (d) Harold P. Erickson, Duke University Medical Center, Department of Cell Biology.]

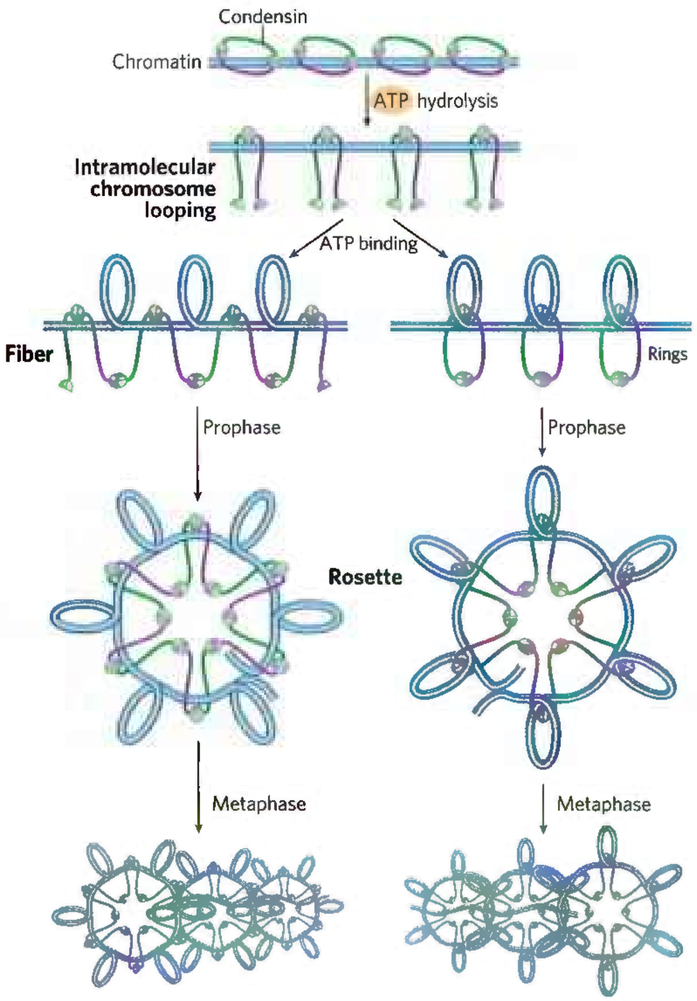


FIGURE 24-32 Two current models of the possible role of condensins in chromatin condensation. Initially, the DNA is bound at the hinge region of the SMC protein, in the interior of what can become an intramolecular SMC ring. ATP binding leads to head-to-head association, forming supercoiled loops in the bound DNA. Subsequent rearrangement of the head-to-head interactions to form rosettes condenses the DNA. Condensins may organize the looping of the chromosome segments in several ways. [Information from T. Hirano, *Nat. Rev. Mol. Cell Biol.* 7:311, 2006, Fig. 6.]

induced by the binding of nucleosomes. A model for the role of condensins in chromatin compaction is presented in **Figure 24-32**. In brief, as DNA is compacted to form tighter and tighter loops, the condensins stabilize the loops by binding at the base of each one. Cohesins and condensins are essential in orchestrating the many changes in chromosome structure during the eukaryotic cell cycle (**Fig. 24-33**).

Bacterial DNA Is Also Highly Organized

We now turn briefly to the structure of bacterial chromosomes. **P2** Bacterial DNA is compacted in a structure called the **nucleoid**, which can occupy a significant fraction of the cell volume (**Fig. 24-34**). The DNA seems to be attached at one or more points to the inner surface of the plasma membrane. Much less is known about the structure of the nucleoid than of eukaryotic chromatin, but a complex organization is slowly being revealed. **P3** In *E. coli*, a scaffoldlike structure seems to organize the *circular* chromosome into a series of about 500 looped domains, each encompassing, on average, 10,000 bp (**Fig. 24-35**), as described above for chromatin. The domains are topologically constrained; for example, if the DNA is cleaved in one domain, only the DNA within that domain will be relaxed. The domains do not have fixed end points. Instead, the boundaries are most likely in constant motion along the DNA, coordinated with DNA replication.

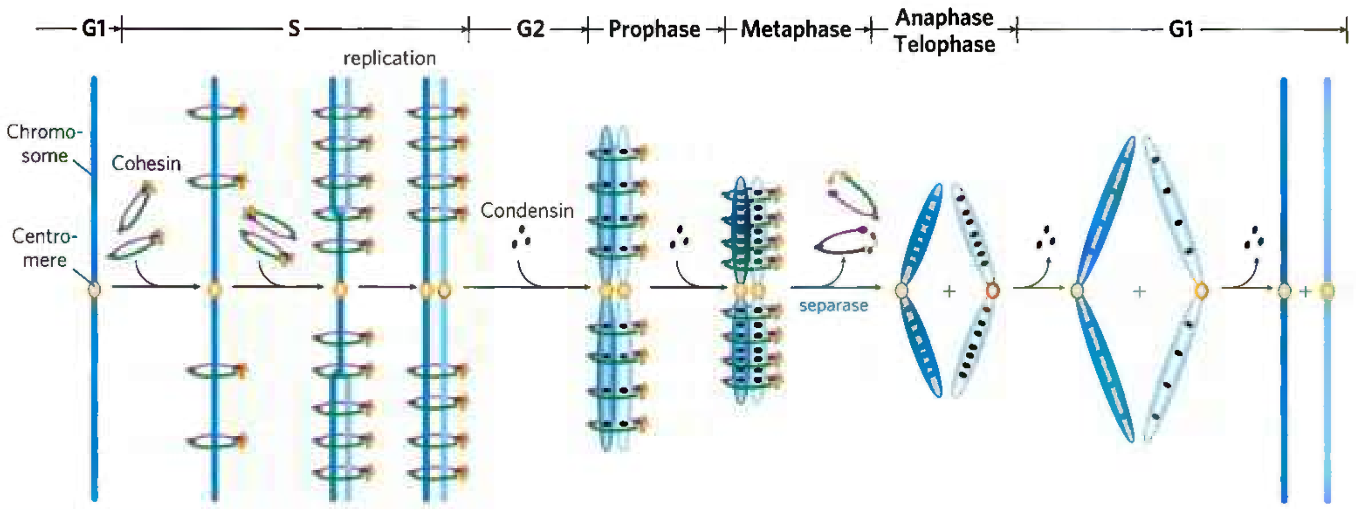


FIGURE 24-33 The roles of cohesins and condensins in the eukaryotic cell cycle. Cohesins are loaded onto the chromosomes during G1 (see Fig. 24-22), tying the sister chromatids together during replication. At the onset of mitosis, condensins bind and maintain the chromatids in a condensed state. During anaphase, the enzyme separase removes the cohesin links. Once the chromatids separate, condensins begin to unload and the daughter chromosomes return to the uncondensed state. [Information from D. P. Bazett-Jones et al., *Mol. Cell* 9:1183, 2002, Fig. 5.]

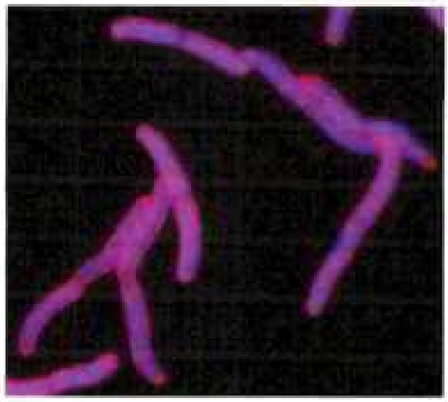


FIGURE 24-34 ***E. coli* nucleoids.** The DNA of these cells is stained with a dye that fluoresces blue when exposed to UV light. The blue areas define the nucleoids. Notice that some cells have replicated their DNA but have not yet undergone cell division and hence have multiple nucleoids. [Lars Renner.]

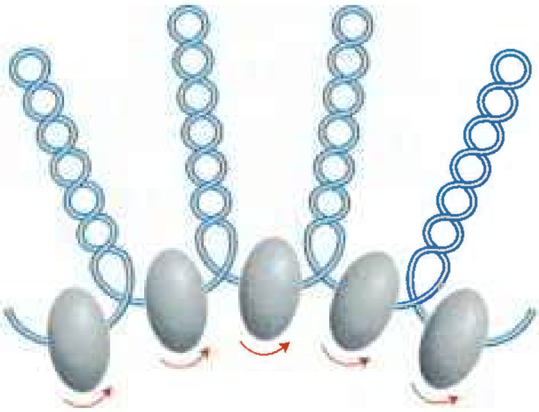


FIGURE 24-35 **Looped domains of the *E. coli* chromosome.** Each domain is about 10,000 bp long. The domains are not static but move along the DNA as replication proceeds. Barriers at the boundaries of the domains, of unknown composition, prevent the relaxation of DNA beyond the boundaries of the domain where a strand break occurs. The putative boundary complexes are shown as gray-shaded ovoids. The arrows denote movement of DNA through the boundary complexes.

Bacterial DNA does not seem to have any structure comparable to the local organization provided by nucleosomes in eukaryotes. P4 Histonelike proteins are abundant in *E. coli*—the best-characterized example is a two-subunit protein called HU (M_r 19,000)—but these proteins bind and dissociate within minutes, and no regular, stable DNA-histone structure has been found. The dynamic structural changes in the bacterial chromosome may reflect a requirement for more ready access to its genetic information. The bacterial cell division cycle can be as short as 15 min, whereas a typical eukaryotic cell may not divide for hours or even months. In addition, a much greater fraction of bacterial DNA is used to encode RNA and/or protein products. Higher rates of cellular metabolism in bacteria mean that a much higher proportion of the DNA is being transcribed or replicated at a given time than in most eukaryotic cells.

SUMMARY 24.3 *The Structure of Chromosomes*

- A eukaryotic chromosome is made of DNA, protein, and RNA, forming a structure called chromatin.
- Histones are small, basic DNA-binding proteins. Complexes of histones form nucleosomes, the fundamental structural unit of chromatin.
- The nucleosome consists of histones and a 200 bp segment of DNA. A core protein particle containing eight histone molecules (two copies each of histones H2A, H2B, H3, and H4) is encircled by a segment of DNA (about 146 bp) in the form of a left-handed solenoidal supercoil.
- Higher-order folding of chromosomes involves attachment to a chromosomal scaffold. Transcriptionally active and inactive regions of chromosomes are separated into compartments, each featuring large loops of DNA, each loop constrained at its base by proteins and lncRNAs. Individual chromosomes are constrained within nuclear subdomains called territories. Histone H1, topoisomerase II, and SMC proteins play organizational roles in chromosomes.
- The SMC proteins, principally cohesins and condensins, have important roles in keeping the chromosomes organized during each stage of the cell cycle.
- The bacterial chromosome is extensively compacted into the nucleoid, but the chromosome seems to be much more dynamic and irregular in structure than eukaryotic chromatin, reflecting the shorter cell cycle and very active metabolism of a bacterial cell.

KEY TERMS

Terms in bold are defined in the glossary.

chromosome	885
phenotype	886
mutation	886
gene	886
regulatory sequence	886
plasmid	887
intron	889
exon	889
simple-sequence DNA	890
satellite DNA	890
centromere	890
telomere	890
supercoil	891
relaxed DNA	891
topology	891
linking number	893
specific linking difference	894
superhelical density (σ)	894
topoisomers	894
topoisomerases	895
catenane	896
plectonemic	897
solenoidal	898
chromatin	899
histones	899
nucleosome	899
histone exchange factors	902
heterochromatin	903
topologically associating domains (TADs)	903
long noncoding RNA (lncRNA)	903
epigenetic	904
euchromatin	904
chromosome territory	904
Barr body	907
SMC proteins	908
cohesins	908
condensins	908
nucleoid	909

PROBLEMS

1. **Packaging of DNA in a Virus** Bacteriophage T2 has a DNA of molecular weight 120×10^6 contained in a head about 210 nm long. Calculate the length of the DNA and compare it with the length of the T2 head. Assume the molecular weight of a nucleotide base pair is 650 and that the DNA is in the B form and relaxed.

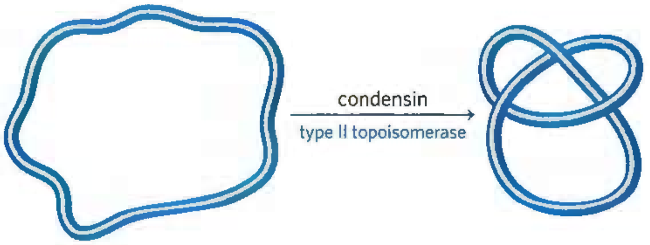
2. **The DNA of Phage M13** The base composition of the DNA within a bacteriophage M13 viral particle is A, 23%; T, 36%; G, 21%; C, 20%. What does this tell you about this DNA molecule?

3. **The *Mycoplasma* Genome** The complete genome of the simplest bacterium known, *Mycoplasma genitalium,* is a circular DNA molecule with 580,070 bp. Calculate the molecular weight and contour length (when relaxed) of this molecule. What is Lk_0 for the *Mycoplasma* chromosome? If $\sigma = -0.06$, what is *Lk*?

4. **Size of Eukaryotic Genes** An enzyme isolated from rat liver has 192 amino acid residues and is encoded by a gene with 1,440 bp. Explain the relationship between the number of amino acid residues in the enzyme and the number of nucleotide pairs in its gene.

5. **Linking Number** A closed-circular DNA molecule in its relaxed form has an *Lk* of 500. Approximately how many base pairs are in this DNA? How is *Lk* altered (increases, decreases, doesn't change, becomes undefined) when **(a)** a protein complex binds to form a nucleosome, **(b)** one DNA strand is broken, **(c)** DNA gyrase and ATP are added to the DNA solution, or **(d)** the double helix is denatured by heat?

6. **DNA Topology** In the presence of a eukaryotic condensin and a bacterial type II topoisomerase, the *Lk* of a relaxed closed-circular DNA molecule does not change. However, the DNA becomes highly knotted.

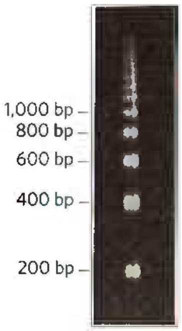


Formation of the knots requires breakage of the DNA, passage of a segment of DNA through the break, and religation by the topoisomerase. Given that every reaction of the topoisomerase would be expected to result in a change in linking number, how can *Lk* remain the same?

7. **Superhelical Density** Bacteriophage λ infects *E. coli* by integrating its DNA into the bacterial chromosome. The success of this recombination depends on the topology of the *E. coli* DNA. When the superhelical density (σ) of the *E. coli* DNA is greater than -0.045, the probability of integration is less than 20%; when σ is less than -0.06, the probability is $>70\%$. Plasmid DNA isolated from an *E. coli* culture is found to have a length of 13,800 bp and an *Lk* of 1,222. Calculate σ for this DNA and predict the likelihood that bacteriophage λ will be able to infect this culture.

8. **Altering Linking Number (a)** What is the *Lk* of a 5,000 bp circular duplex DNA molecule with a nick in one strand? **(b)** What is the *Lk* of the molecule in (a) when the nick is sealed (relaxed)? **(c)** How would the *Lk* of the molecule in (b) be affected by the action of a single molecule of *E. coli* topoisomerase I? **(d)** What is the *Lk* of the molecule in (b) after eight enzymatic turnovers by a single molecule of DNA gyrase in the presence of ATP? **(e)** What is the *Lk* of the molecule in (d) after four enzymatic turnovers by a single molecule of bacterial type I topoisomerase? **(f)** What is the *Lk* of the molecule in (d) after binding of one nucleosome core?

9. **Chromatin** The agarose gel shown, in which the thick bands represent DNA, helped researchers define nucleosome structure. They generated this result by briefly treating chromatin with an enzyme that degrades DNA, then removing all protein and subjecting the purified DNA to electrophoresis. Numbers at the side of the gel denote the position to which a linear DNA of the indicated size would migrate. What does this gel demonstrate about chromatin structure? Why are the DNA bands thick and spread out rather than sharply defined?



[Courtesy Dr. Roger D. Kornberg, Stanford University School of Medicine.]

10. **DNA Structure** Explain how the underwinding of a B-DNA helix might facilitate or stabilize the formation of Z-DNA (see Fig. 8-17).

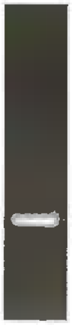
11. **Maintaining DNA Structure (a)** Describe two structural features required for a DNA molecule to maintain a negatively supercoiled state. **(b)** List three structural changes that become more favorable when a DNA molecule is negatively supercoiled. **(c)** What *E. coli* enzyme, with the aid of ATP, can generate negative superhelicity in DNA? **(d)** Describe the physical mechanism by which this enzyme acts.

12. **Yeast Artificial Chromosomes (YACs)** Researchers use YACs to clone large pieces of DNA in yeast cells. What three types of DNA sequence do researchers require to ensure proper replication and propagation of a YAC in a yeast cell?

13. **Nucleoid Structure in Bacteria** In bacteria, DNA topology affects the transcription of a subset of genes, with expression increasing or (more often) decreasing when the DNA is relaxed. Following cleavage of a bacterial chromosome at a specific site by a restriction enzyme (one that cuts at a long, and thus rare, sequence), only nearby genes (within 10,000 bp) exhibit either an increase or a decrease in expression. The transcription of genes elsewhere in the chromosome is unaffected. Explain. (Hint: See Fig. 24-35.)

14. **DNA Topology** When DNA is subjected to electrophoresis in an agarose gel, shorter molecules migrate faster than

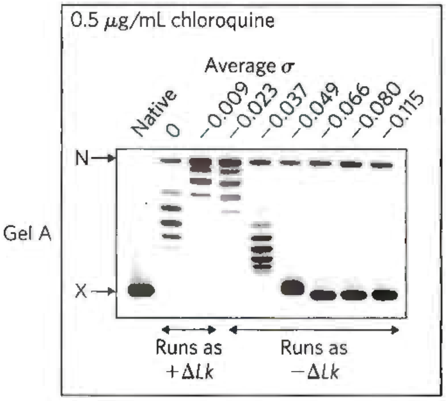
longer ones. Closed-circular DNAs of the same size but with different linking numbers also can be separated on an agarose gel: topoisomers that are more supercoiled, and thus more condensed, migrate faster through the gel. In the gel shown, purified plasmid DNA has migrated from top to bottom. There are two bands, with the faster band much more prominent.

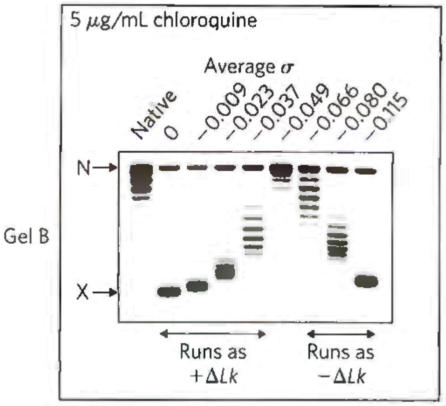


[Michael Cox Laboratory.]

(a) What are the DNA species in the two bands? **(b)** If topoisomerase I were added to a solution of this DNA, what would happen to the upper and lower bands after electrophoresis? **(c)** If DNA ligase were added to the DNA, would the appearance of the bands change? Explain your answer. **(d)** If DNA gyrase plus ATP were added to the DNA after the addition of DNA ligase, how would the band pattern change?

15. DNA Topoisomers When DNA is subjected to electrophoresis in an agarose gel, shorter molecules migrate faster than longer ones. Closed-circular DNAs of the same size but different linking number also can be separated on an agarose gel: topoisomers that are more supercoiled, and thus more condensed, migrate faster through the gel—from top to bottom in the two gels shown. An investigator added a dye, chloroquine, to these gels. Chloroquine intercalates between base pairs and stabilizes a more underwound DNA structure. When the dye binds to a relaxed closed-circular DNA, the DNA is underwound where the dye binds, and unbound regions take on positive supercoils to compensate. In the experiment shown here, an investigator used topoisomerases to make preparations of the same DNA circle with different superhelical densities (σ). Completely relaxed DNA migrated to the position labeled N (for *n*icked), and highly supercoiled DNA (above the limit where individual topoisomers can be distinguished) migrated to the position labeled X.





[R. P. Bowater (2005), "Supercoiled DNA: structure," in *Encyclopedia of Life Sciences*, John Wiley & Sons/Wiley InterScience, www.els.net.]

(a) In gel A, why does the $\sigma = 0$ lane (i.e., DNA prepared so that $\sigma = 0$, on average) have multiple bands?
(b) In gel B, is the DNA from the $\sigma = 0$ preparation positively or negatively supercoiled in the presence of the intercalating dye?
(c) In both gels, the $\sigma = -0.115$ lane has two bands, one a highly supercoiled DNA and one relaxed. Propose a reason for the presence of relaxed DNA in these lanes (and others).
(d) The native DNA (leftmost lane in each gel) is the same DNA circle isolated from bacterial cells and untreated. What is the approximate superhelical density of this native DNA?

16. Nucleosomes The human genome comprises just over 3.1 billion base pairs. Assuming it contains nucleosomes that are spaced as described in this chapter, how many molecules of histone H2A are present in one somatic human cell? (Ignore reductions in H2A due to its replacement in some regions by H2A variants.) How would the number change after DNA replication but before cell division?

17. Bacterial DNA Topoisomerase IV The gene encoding topoisomerase IV in *E. coli* is essential, even though another type II topoisomerase (topoisomerase II or gyrase) is present. Suggest a reason for the requirement for topoisomerase IV.

18. Chromosome Topology Eukaryotic chromosomes are linear DNA molecules, yet the DNA of a chromosome retains a high level of underwinding (supercoiling) throughout its length. How does the organization of chromosomal DNA into loops called TADs contribute to the maintenance of supercoiling?

DATA ANALYSIS PROBLEM

19. Defining the Functional Elements of Yeast Chromosomes Figure 24-7 shows the major structural elements of a chromosome of budding yeast (*S. cerevisiae*). Heiter, Mann, Snyder, and Davis (1985) determined the properties of some of these elements. They based their study on the finding that in yeast cells, plasmids (which have genes and an origin of replication) act differently from chromosomes (which have these elements plus centromeres and telomeres) during mitosis. The plasmids are not manipulated by the mitotic apparatus and segregate randomly between daughter cells. Without a selectable marker to force the host cells to retain them (see Fig. 9-4), these plasmids are rapidly lost. In contrast, chromosomes, even without a selectable marker, are manipulated by the mitotic apparatus and are lost at a very low frequency (about 10^{-5} per cell division).

Heiter and colleagues set out to determine the important components of yeast chromosomes by constructing plasmids with various parts of chromosomes and observing whether these "synthetic chromosomes" segregated properly during mitosis. To measure the frequencies of different types of failed chromosome segregation, the researchers needed a rapid assay to determine the number of copies of synthetic chromosomes present in different cells. The assay took advantage of the fact that wild-type yeast colonies are white whereas certain adenine-requiring (ade$^-$) mutants yield red colonies on nutrient media; *ade2*$^-$ cells lack functional AIR carboxylase (the enzyme of step 6a in Fig. 22-35) and accumulate AIR (5-aminoimidazole ribonucleotide) in their cytoplasm, and the excess AIR is converted to a conspicuous red pigment. The other part of the assay involved the gene *SUP11*, which encodes an ochre suppressor (a type of nonsense suppressor in which a termination codon specifies an amino acid) that suppresses the phenotype of some *ade2*$^-$ mutants.

Heiter and coworkers started with a diploid strain of yeast homozygous for *ade2*$^-$; these cells are red. When the mutant cells contain one copy of *SUP11*, the metabolic defect is partly suppressed and the cells are pink. When the cells contain two or more copies of *SUP11*, the defect is completely suppressed and the cells are white.

The researchers inserted one copy of *SUP11* into synthetic chromosomes containing various elements thought to be important in chromosome function, and then observed how well these chromosomes were passed from one generation to the next. These pink cells were plated on nonselective media, and the behavior of the synthetic chromosomes was observed. Heiter and coworkers looked for colonies in which the synthetic chromosomes segregated improperly at the first division after plating, giving rise to a colony that was half one genotype and half the other. Because yeast cells are nonmotile, this would be a sectored colony, with one half one color and the other half another color.

(a) One way for the mitotic process to fail is *nondisjunction*: the chromosome replicates but the sister chromatids fail to separate, so both copies of the chromosome end up in the same daughter cell. Explain how nondisjunction of the synthetic chromosome would give rise to a colony that is half red and half white.

(b) Another way for the mitotic process to fail is *chromosome loss*: the chromosome does not enter the daughter nucleus or is not replicated. Explain how loss of the synthetic chromosome would give rise to a colony that is half red and half pink.

By counting the frequency of the different colony types, Heiter and colleagues could estimate the frequency of these aberrant mitotic events with different types of synthetic chromosome. First, they explored the requirement for centromeric sequences by constructing synthetic chromosomes with DNA fragments of different sizes containing a known centromere. Their results are shown here.

Synthetic chromosome	Size of centromere-containing fragment (kbp)	Chromosome loss (%)	Nondisjunction (%)
1	None	—	>50
2	0.63	1.6	1.1
3	1.6	1.9	0.4
4	3.0	1.7	0.35
5	6.0	1.6	0.35

(c) Based on these data, what can you conclude about the size of the centromere required for normal mitotic segregation? Explain your reasoning.

(d) All the synthetic chromosomes created in these experiments were circular and lacked telomeres. Explain how they could be replicated more-or-less properly.

Heiter and colleagues next constructed a series of linear synthetic chromosomes that included the functional centromeric sequence and telomeres, and they measured the total mitotic error frequency (% loss + % nondisjunction) as a function of size.

Synthetic chromosome	Size (kbp)	Total error frequency (%)
6	15	11.0
7	55	1.5
8	95	0.44
9	137	0.14

(e) Based on these data, what can you conclude about the chromosome size required for normal mitotic segregation? Explain your reasoning.

(f) Normal yeast chromosomes are linear, range from 250 kbp to 2,000 kbp in length, and, as noted above, have a mitotic error frequency of about 10^{-5} per cell division. Extrapolating the results from (e), do the centromeric and telomeric sequences used in these experiments explain the mitotic stability of normal yeast chromosomes, or must other elements be involved? Explain your reasoning. (Hint: A plot of log of error frequency vs. length will be helpful.)

Reference

Heiter, P., C. Mann, M. Snyder, and R.W. Davis. 1985. Mitotic stability of yeast chromosomes: A colony color assay that measures nondisjunction and chromosome loss. *Cell* 40:381–392.

Chapter 25

DNA METABOLISM

25.1 DNA Replication *915*
25.2 DNA Repair *930*
25.3 DNA Recombination *940*

As the repository of genetic information, DNA occupies a unique and central place among biological macromolecules. The nucleotide sequences of DNA encode the primary structures of all cellular RNAs and proteins and, through enzymes, indirectly affect the synthesis of all other cellular constituents. This passage of information from DNA to RNA and protein guides the size, shape, and functioning of every living thing.

DNA is a marvelous device for the stable storage of genetic information. The phrase "stable storage," however, conveys a static and misleading picture. It fails to capture the complexity of processes by which genetic information is preserved in an uncorrupted state and then transmitted from one generation of cells to the next. DNA metabolism comprises both the process that gives rise to faithful copies of DNA molecules (replication) and the processes that affect the inherent structure of the information (repair and recombination). Together, these activities are both the focus of this chapter and the underpinning for several guiding principles:

P1 **Along with catalysis, biological information is one of the two key prerequisites for life.** The faithful maintenance and transmission of genetic information from one generation to another ensures continuity within each species.

P2 **Information is expensive.** The chemistry of joining one nucleotide to the next in DNA replication is elegant and simple, almost deceptively so. However, the enzymatic and thermodynamic commitment to linking one nucleotide to another in DNA far exceeds what would normally be required to successfully form a phosphodiester bond. It is not enough to synthesize a phosphodiester bond; that bond must accurately link two *particular* nucleotides.

P3 **The fidelity of genome maintenance and transmission is not perfect.** DNA damage happens, often by spontaneous processes. DNA replication and repair deal with the vast majority of DNA lesions, providing a high degree of genetic fidelity and stability. The few DNA damage events that slip through uncorrected provide fuel for evolution.

P4 **Although considered separately, the processes of replication, repair, and recombination of DNA are not distinct.** These processes are highly integrated in cells, and they are required for proper genome maintenance.

We give the *enzymes* of DNA metabolism special emphasis in this chapter. These enzymes are not only intrinsically important biologically; they are also important in medicine and biochemical technologies. Many of the seminal discoveries in DNA metabolism have been made with *Escherichia coli*, so its well-understood enzymes are generally used to illustrate the ground rules.

KEY CONVENTION Before taking a closer look at replication, we make a short digression into the use of abbreviations in naming genes and proteins — you will encounter many of these in this and later chapters. Bacterial genes generally are named using three italicized, lowercase letters that often reflect a gene's apparent function. For example, the *dna*, *uvr*, and *rec* genes affect *DNA* replication, resistance to the damaging effects of *UV* radiation, and *rec*ombination, respectively. Where several genes affect the same process, the letters *A*, *B*, *C*, and so forth, are added — as in *dnaA*, *dnaB*, *dnaQ*, for example — usually reflecting their order of discovery rather than the order of their gene products in a reaction sequence. Similar conventions exist for naming eukaryotic genes, although the exact form of the abbreviations may vary with the species, and no single convention applies to all eukaryotic systems. For example, in the budding yeast *Saccharomyces cerevisiae*, gene names are generally three uppercase letters followed by a number, all italicized (e.g., the gene *COX1* encodes a subunit of cytochrome oxidase). Gene names that predate current conventions may differ in format.

The use of abbreviations in naming proteins is less straightforward. During genetic investigations, the protein product of each gene is usually isolated and characterized. Many bacterial genes were identified and named before the roles of their protein products were understood in detail. Sometimes the gene product was found to be a previously isolated protein, and some renaming occurred. Often, however, the product turned out to be an as yet unknown protein, with an activity not easily described by a simple enzyme name.

Bacterial proteins often retain the name of their genes. When referring to the protein product of an *E. coli* gene, we use roman type and capitalize the first letter: for example, the *dnaA* and *recA* gene products are the DnaA and RecA proteins, respectively. Conventions for eukaryotic proteins are again complex. For yeast, some proteins have long common names (such as cytochrome oxidase). Others have the same name as the gene, in which case the protein name usually has one uppercase and two lowercase letters, followed by a number and the letter "p," all in roman type (such as Rad51p). The "p" is to emphasize that this is a protein and to prevent confusion with naming conventions for other organisms. ■

25.1 DNA Replication

P1 Long before the structure of DNA was known, scientists wondered at the ability of organisms to create faithful copies of themselves and, later, at the ability of cells to produce many identical copies of large, complex macromolecules. Speculation about these problems centered around the concept of a **template**, a structure that would allow molecules to be lined up in a specific order and joined to create a macromolecule with a unique sequence and function. The 1940s brought the revelation that DNA was the genetic molecule, but not until James Watson and Francis Crick deduced its structure did the way in which DNA could act as a template for the replication and transmission of genetic information become clear: *one strand is the complement of the other.* The strict base-pairing rules mean that each strand provides the template for a new strand with a predictable and complementary sequence (see Figs. 8-14, 8-15). The fundamental properties of the DNA replication process and the mechanisms used by the enzymes that catalyze it have proved to be essentially identical in all species.

DNA Replication Follows a Set of Fundamental Rules

Early research on bacterial DNA replication and its enzymes helped to establish several basic properties that have proven applicable to DNA synthesis in every organism.

DNA Replication Is Semiconservative **P1** Each DNA strand serves as a template for the synthesis of a new strand, producing two new DNA molecules, each with one new strand and one old strand. This is **semiconservative replication**. The semiconservative nature of replication was established by Matthew Meselson and Frank Stahl in 1957.

Replication Begins at an Origin and Usually Proceeds Bidirectionally Following the confirmation of a semiconservative mechanism of replication, a host of questions arose. Are the parent DNA strands completely unwound before each is replicated? Does replication begin at random places or at a unique point? After initiation at any point in the DNA, does replication proceed in one direction or both?

Photographic images of tritium (^{3}H)–labeled bacterial DNA made by John Cairns revealed that the intact chromosome of *E. coli* is a single huge circle, 1.7 mm long. Radioactive DNA isolated from cells during replication showed an extra loop (**Fig. 25-1**). Cairns concluded that the loop resulted from the formation of two radioactive daughter strands, each complementary to a parent

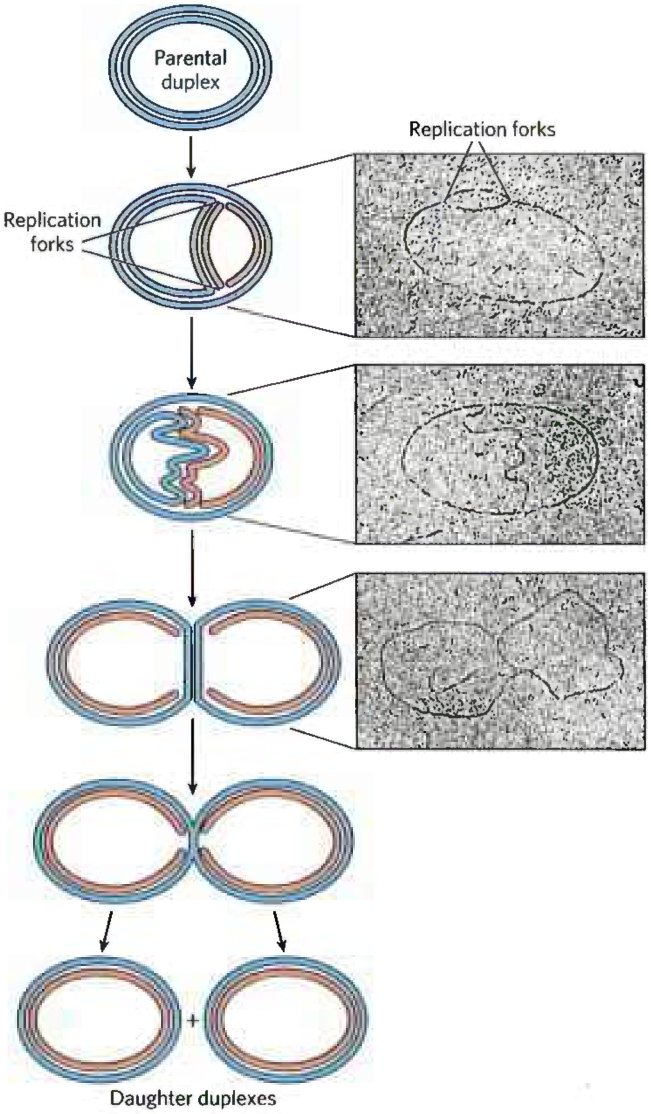


FIGURE 25-1 Visualization of DNA replication. Stages in the replication of circular DNA molecules have been visualized by electron microscopy. Replication of a circular chromosome produces a structure resembling the Greek letter theta, θ, as both strands are replicated simultaneously (new strands shown in light red). The electron micrographs show images of plasmid DNA being replicated from a single replication origin. [Electron micrographs: Cairns, J. (1963), The Chromosome of *Escherichia coli*, *Cold Spring Harbor Symp Quant Biol*, 28, 43–46. © Cold Spring Harbor Laboratory Press.]

strand. One or both ends of the loop are dynamic points, termed **replication forks**, where parent DNA is being unwound and the separated strands quickly replicated. Cairns's results demonstrated that both DNA strands are replicated simultaneously, and variations on his experiment showed that replication of bacterial chromosomes is bidirectional: both ends of the loop have active replication forks.

Determination of whether the replication loops originate at a unique point in the DNA required landmarks along the DNA molecule. These landmarks were provided by a technique called **denaturation mapping**, developed by Ross Inman and colleagues. Using the 48,502 bp chromosome of bacteriophage λ, Inman showed that DNA could be selectively denatured at sequences unusually rich in A=T base pairs, generating a reproducible pattern of single-strand bubbles (see Fig. 8-28). Using the denatured regions as points of reference, investigators have subsequently been able to measure the position and progress of the replication forks. Inman and colleagues found that the replication loops always initiated at a unique point, which was termed an **origin**. They also confirmed the earlier observation that replication is usually bidirectional. For circular DNA molecules, the two replication forks meet at a point on the side of the circle opposite to the origin. Specific origins of replication have since been identified and characterized in bacteria and eukaryotes.

DNA Synthesis Proceeds in a 5′→3′ Direction and Is Semidiscontinuous A new strand of DNA is always synthesized in the 5′→3′ direction, with the free 3′-OH as the point at which the DNA is elongated. (Recall from Chapter 8 that the 5′ end lacks a nucleotide attached to the 5′ position, and the 3′ end lacks a nucleotide attached to the 3′ position.) Because the two DNA strands are antiparallel, the strand serving as the template is read from its 3′ end toward its 5′ end.

If synthesis always proceeds in the 5′→3′ direction, how can both strands be synthesized simultaneously? If both strands were synthesized *continuously* while the replication fork moved, one strand would have to undergo 3′→5′ synthesis. This problem was resolved by Reiji Okazaki and colleagues in the 1960s. Okazaki found that one of the new DNA strands is synthesized in short pieces, now called **Okazaki fragments**. Thus, one strand is synthesized continuously and the other discontinuously (Fig. 25-2). The continuous strand, or **leading strand**, is the one for which 5′→3′ synthesis proceeds in the *same* direction that the replication fork moves. The discontinuous strand, or **lagging strand**, is the one in which 5′→3′ synthesis proceeds in the direction *opposite* to the direction of fork movement. Okazaki fragments are typically 150 to 200 nucleotides long in eukaryotes, and 1,000 to 2,000 nucleotides long in bacteria. As we shall see, leading and lagging strand syntheses are tightly coordinated.

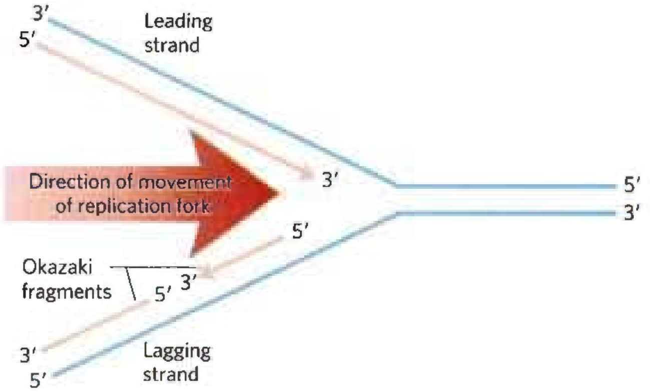
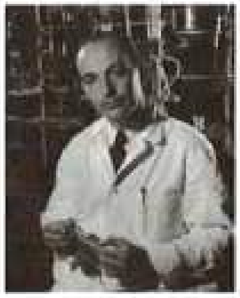


FIGURE 25-2 Defining DNA strands at the replication fork. A new DNA strand (light red) is always synthesized in the 5′→3′ direction. The template is read in the opposite direction, 3′→5′. The leading strand is continuously synthesized in the direction taken by the replication fork. The other strand, the lagging strand, is synthesized discontinuously in short pieces (Okazaki fragments) in a direction opposite to that in which the replication fork moves. The Okazaki fragments are spliced together by DNA ligase. In bacteria, Okazaki fragments are ~1,000 to 2,000 nucleotides long. In eukaryotic cells, they are 150 to 200 nucleotides long.

DNA Is Degraded by Nucleases

To explain the enzymology of DNA replication, we first introduce the enzymes that degrade DNA rather than synthesize it. These enzymes are known as **nucleases**, or **DNases** if they are specific for DNA rather than RNA. Every cell contains several different nucleases, belonging to two broad classes: exonucleases and endonucleases. **Exonucleases** degrade nucleic acids from one end of the molecule. Many operate in only the 5′→3′ direction or the 3′→5′ direction, removing nucleotides only from the 5′ end or the 3′ end, respectively, of one strand of a double-stranded nucleic acid or of a single-stranded DNA. **Endonucleases** can begin to degrade at specific internal sites in a nucleic acid strand or molecule, reducing it to smaller and smaller fragments. A few exonucleases and endonucleases degrade only single-stranded DNA. A few important classes of endonucleases cleave only at specific nucleotide sequences (such as the restriction endonucleases that are so important in biotechnology; see Chapter 9, Fig. 9-2). You will encounter many types of nucleases in this and subsequent chapters.

DNA Is Synthesized by DNA Polymerases



Arthur Kornberg, 1918–2007
[World History Archive/Alamy.]

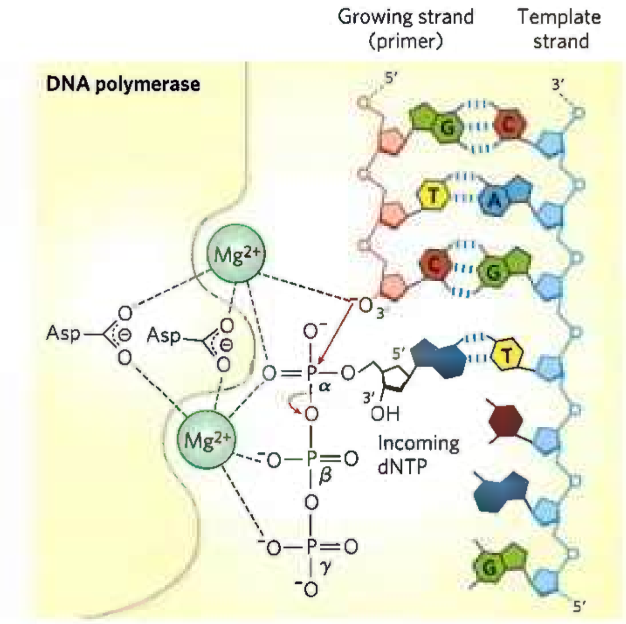
The search for an enzyme that could synthesize DNA began in 1955. Work by Arthur Kornberg and colleagues led to the purification and characterization of a **DNA polymerase** from *E. coli* cells, a single-polypeptide enzyme now called **DNA polymerase I** (M_r 103,000; encoded by the *polA* gene). Much later, investigators found that *E. coli* contains at least four additional distinct DNA polymerases, described below.

Detailed studies of DNA polymerase I revealed features of the DNA synthetic process that are now known to be common to all DNA polymerases. The fundamental reaction is a phosphoryl group transfer. The nucleophile is the 3′-hydroxyl group of the nucleotide at the 3′ end of the growing strand. Nucleophilic attack occurs at the α phosphorus of the incoming deoxynucleoside 5′-triphosphate (**Fig. 25-3**). Inorganic pyrophosphate is released in the reaction. The general reaction is

$$\underset{\text{DNA}}{(\text{dNMP})_n} + \text{dNTP} \longrightarrow \underset{\substack{\text{Lengthened}\\\text{DNA}}}{(\text{dNMP})_{n+1}} + \text{PP}_\text{i} \qquad (25\text{-}1)$$

where dNMP and dNTP are a deoxynucleoside 5′-monophosphate and 5′-triphosphate, respectively. Catalysis by virtually all DNA polymerases prominently involves two Mg^{2+} ions at the active site. One of these helps to deprotonate the 3′-hydroxyl group, rendering it a more effective nucleophile. The other binds to the incoming dNTP and facilitates departure of the pyrophosphate.

P2 The reaction seems to proceed with only a minimal change in free energy, given that one phosphodiester bond is formed at the expense of a somewhat less stable phosphate anhydride. However, noncovalent base-stacking and base-pairing interactions provide additional stabilization to the lengthened DNA product relative to the free nucleotide. Also, the formation of products is facilitated in the cell by the 19 kJ/mol generated in the subsequent hydrolysis of the pyrophosphate product by the enzyme pyrophosphatase (p. 485).



MECHANISM FIGURE 25-3 The DNA polymerase reaction. The catalytic mechanism for addition of a new nucleotide by DNA polymerase involves two Mg^{2+} ions, coordinated to the phosphate groups of the incoming nucleotide triphosphate, the 3′-hydroxyl group that will act as a nucleophile, and three Asp residues, two of which are highly conserved in all DNA polymerases. The Mg^{2+} ion depicted at the top facilitates attack of the 3′-hydroxyl group of the primer on the α phosphate of the nucleotide triphosphate; the other Mg^{2+} ion facilitates displacement of the pyrophosphate. Both ions stabilize the structure of the pentacovalent transition state. RNA polymerases use a similar mechanism.

Early work on DNA polymerase I led to the definition of two central requirements for DNA polymerization (**Fig. 25-4**). First, all DNA polymerases require a template. The polymerization reaction is guided by a template DNA strand according to the base-pairing rules predicted by Watson and Crick: where a guanine is present in the template, a cytosine deoxynucleotide is added to the new strand, and so on. P1 This was a particularly important discovery, not only because it provided a chemical basis for accurate semiconservative DNA replication, but also because it represented the first example of the use of a template to guide a biosynthetic reaction.

Second, the polymerases require a **primer**. A primer is a strand segment (complementary to the template) with a free 3′-hydroxyl group to which a nucleotide can be added; the free 3′ end of the primer is called the **primer terminus** (Fig. 25-4a). In other words, part of the new strand must already be in place: all DNA polymerases can add nucleotides only to a preexisting strand. Many primers are oligonucleotides of RNA rather than DNA, and specialized enzymes synthesize primers when and where they are required.

A DNA polymerase active site has two parts (Fig. 25-4a). The incoming nucleotide is initially positioned in the **insertion site**. Once the phosphodiester bond is formed, the polymerase slides forward on the DNA and the new base pair is positioned in the **postinsertion site**. These sites are located in a pocket that resembles the palm of a hand (Fig. 25-4b, c).

After adding a nucleotide to a growing DNA strand, a DNA polymerase either dissociates or moves along the template and adds another nucleotide. Dissociation and reassociation of the polymerase can limit the overall polymerization rate—the process is generally faster when a polymerase adds more nucleotides without dissociating from the template. The average number of nucleotides added before a polymerase dissociates defines its **processivity**. DNA polymerases vary greatly in processivity; some add just a few nucleotides before dissociating, whereas others add many thousands.

Replication Is Very Accurate

Replication proceeds with an extraordinary degree of fidelity. In *E. coli*, a mistake is made only once for every 10^9 to 10^{10} nucleotides added. For the *E. coli* chromosome of $\sim 4.6 \times 10^6$ bp, this means that an error occurs only once per 1,000 to 10,000 replications. During polymerization, discrimination between correct and incorrect nucleotides relies not just on the hydrogen bonds that specify the correct pairing between complementary bases but also on the common geometry of the standard A=T and G≡C base pairs (**Fig. 25-5**). The active site of DNA polymerase I

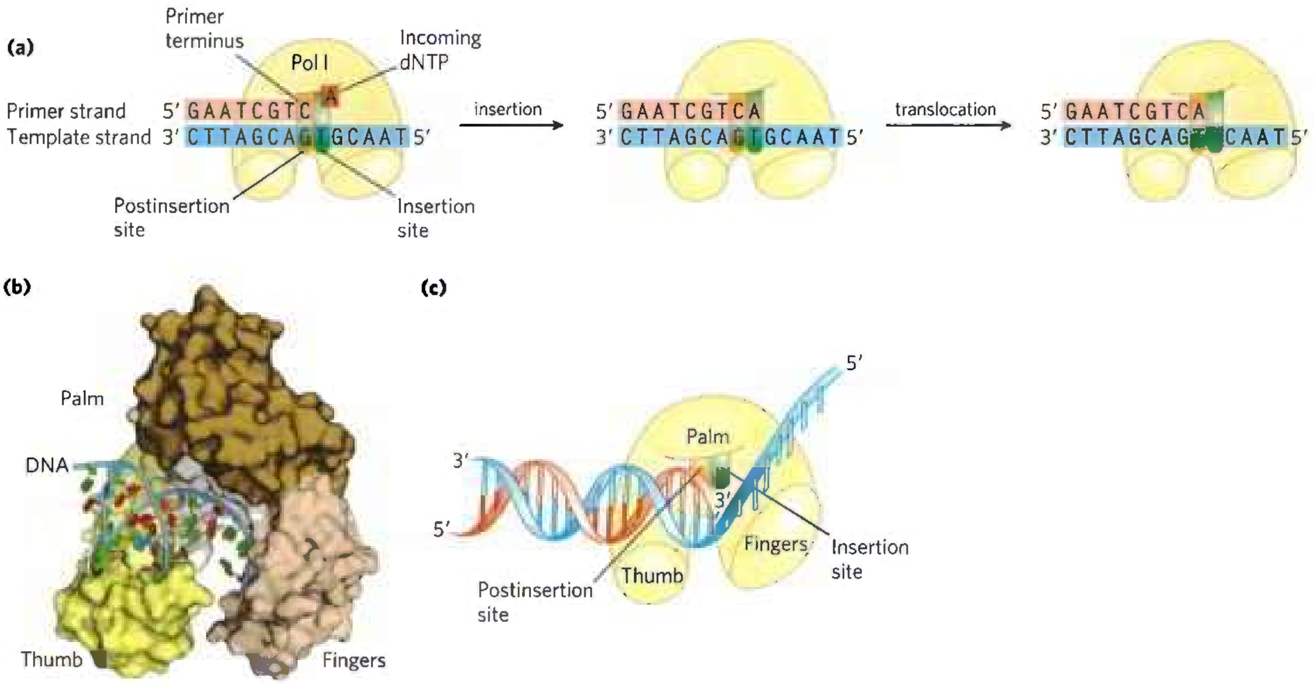
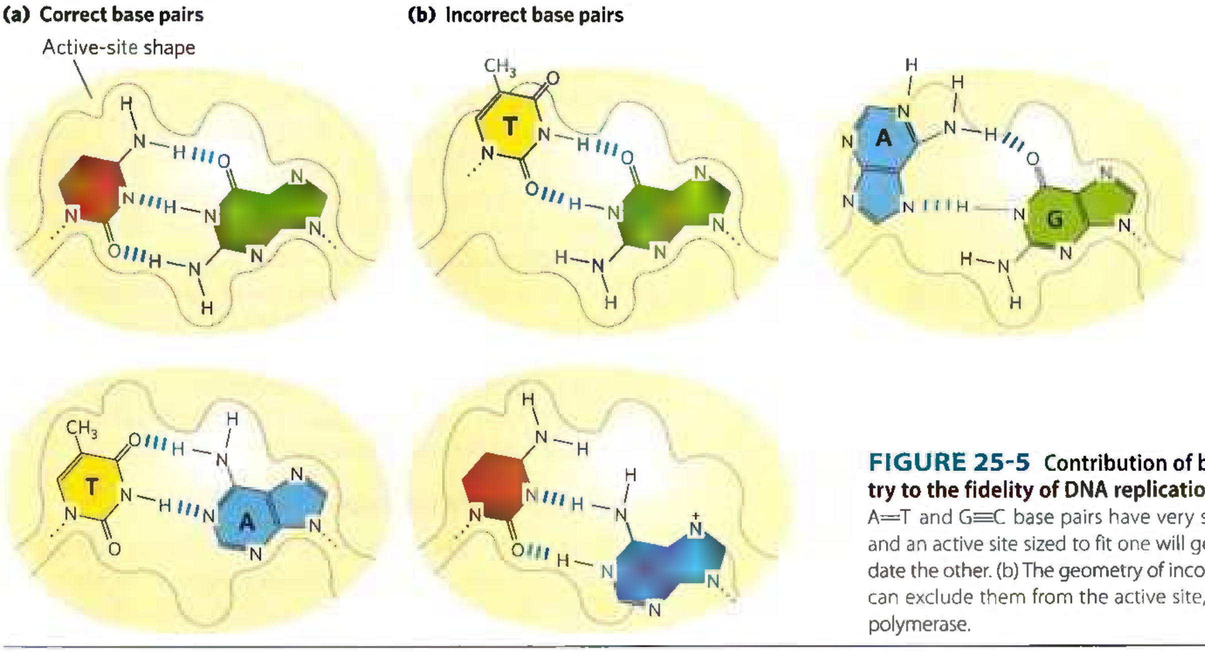

(a)
Primer terminus
Pol I
Incoming dNTP
Primer strand 5′ GAATCGTC
A
Template strand 3′ CTTAGCAGTGCAAT 5′
Postinsertion site
Insertion site
insertion
5′ GAATCGTCA
3′ CTTAGCAGTGCAAT 5′
translocation
5′ GAATCGTCA
3′ CTTAGCAGTGCAAT 5′
(b)
Palm
DNA
Thumb
Fingers
(c)
5′
3′
5′
Palm
3′
Fingers
Insertion site
Thumb
Postinsertion site

FIGURE 25-4 Elongation of a DNA chain. (a) DNA polymerase I activity requires a single unpaired strand to act as template and a primer strand to provide the free hydroxyl group at the 3′ end to which the new nucleotide unit is added. Each incoming complementary nucleotide is bound selectively, in part by base-pairing to the appropriate nucleotide in the template strand. The reaction product has a new free 3′ hydroxyl, allowing the addition of another nucleotide. The newly formed base pair translocates to make the active site available to the next pair to be formed. (b) The core of most DNA polymerases is shaped like a human hand that wraps around the active site. The structure shown here is DNA polymerase I of *Thermus aquaticus*, bound to DNA. (c) A cartoon interpretation shows the insertion site, where the nucleotide addition occurs, and the postinsertion site, to which the newly formed base pair translocates. [(b) Data from PDB ID 4KTQ, Y. Li et al., *EMBO J.* 17:7514, 1998.]


(a) Correct base pairs

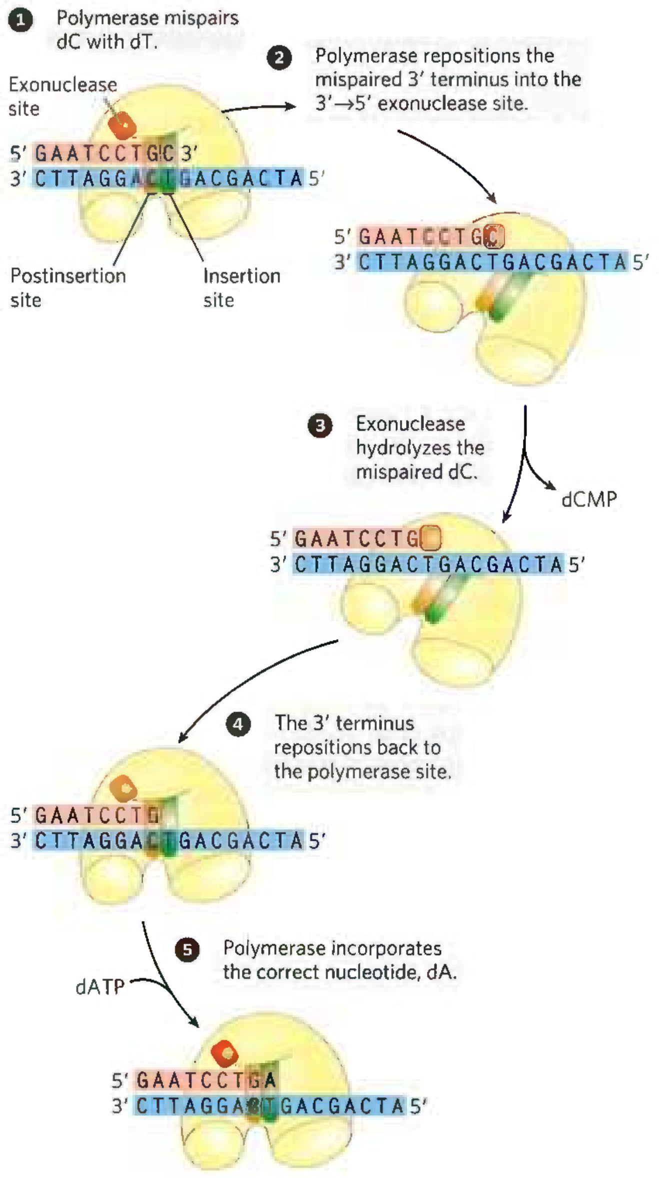
(b) Incorrect base pairs
Active-site shape
H
N—H
O
N
N
N
N—H
O—H—N
N
H
CH3
O
T
N—H
O
N
O—H—N
N
N
H—N
H
H
H
N
A
N—H
O
N
N
N—H—N
G
N
N
H—N
H
CH3
O—H—N
H
T
N—H—N
A
N
N
O
N
H
N—H
H
N—H—N
N+
O—H—N
N
N

FIGURE 25-5 Contribution of base-pair geometry to the fidelity of DNA replication. (a) The standard A═T and G≡C base pairs have very similar geometries, and an active site sized to fit one will generally accommodate the other. (b) The geometry of incorrectly paired bases can exclude them from the active site, as occurs on DNA polymerase.

accommodates only base pairs with this geometry. An incorrect nucleotide may be able to hydrogen-bond with a base in the template, but it generally will not fit into the active site. Incorrect bases can be rejected before the phosphodiester bond is formed.

The accuracy of the polymerization reaction itself, however, is insufficient to account for the high degree of fidelity in replication. Careful measurements in vitro have shown that DNA polymerases insert one incorrect nucleotide for every 10^4 to 10^5 correct ones. These mistakes sometimes occur because a base is briefly in an unusual tautomeric form (see Fig. 8-9), allowing it to hydrogen-bond with an incorrect partner. In vivo, the error rate is reduced by additional enzymatic mechanisms.

One mechanism intrinsic to many DNA polymerases is a separate 3′→5′ exonuclease activity that double-checks each nucleotide after it is added. This nuclease activity permits the enzyme to remove a newly added nucleotide and is highly specific for mismatched base pairs (**Fig. 25-6**). If the polymerase has added the wrong nucleotide, translocation of the enzyme to the position where the next nucleotide is to be added is inhibited. This kinetic pause provides the opportunity for a correction. The 3′→5′ exonuclease activity cleaves the most recently added phosphodiester bond and removes the mispaired nucleotide; the polymerase then adds another nucleotide to begin active synthesis again. This activity, known as **proofreading**, is not simply the reverse of the polymerization reaction (Eqn 25-1). P2 Instead, replacement of the incorrect nucleotide requires the expenditure of three high-energy bonds. The polymerizing and proofreading activities of a DNA polymerase can be measured separately. Proofreading improves the inherent accuracy of the polymerization reaction 10^2- to 10^3-fold. In the monomeric DNA polymerase I, the polymerizing and proofreading activities have separate active sites within the same polypeptide.



① Polymerase mispairs dC with dT.
Exonuclease site
5′ GAATCCTGC 3′
3′ CTTAGGACTGACGACTA 5′
Postinsertion site
Insertion site
② Polymerase repositions the mispaired 3′ terminus into the 3′→5′ exonuclease site.
5′ GAATCCTGC
3′ CTTAGGACTGACGACTA 5′
③ Exonuclease hydrolyzes the mispaired dC.
dCMP
5′ GAATCCTG
3′ CTTAGGACTGACGACTA 5′
④ The 3′ terminus repositions back to the polymerase site.
5′ GAATCCTG
3′ CTTAGGACTGACGACTA 5′
⑤ Polymerase incorporates the correct nucleotide, dA.
dATP
5′ GAATCCTGA
3′ CTTAGGACTGACGACTA 5′

FIGURE 25-6 An example of error correction by the 3′→5′ exonuclease activity of DNA polymerase I. Structural analysis has located the exonuclease activity behind the polymerase activity as the enzyme is oriented in its movement along the DNA. A mismatched base (here, a C–T mismatch) impedes translocation of DNA polymerase I (Pol I) to the next site.

P3 When base selection and proofreading are combined, DNA polymerase leaves behind one net error for every 10^6 to 10^8 bases added. Yet the measured accuracy of replication in *E. coli* is higher still. The additional accuracy is provided by a separate enzyme system that repairs the mismatched base pairs remaining after replication. We describe this mismatch repair, along with other DNA repair processes, in Section 25.2.

E. coli Has at Least Five DNA Polymerases

More than 90% of the DNA polymerase activity observed in *E. coli* extracts can be accounted for by DNA polymerase I. Soon after the isolation of this enzyme in 1955, however, evidence began to accumulate that it is not suited for replication of the large *E. coli* chromosome. First, the rate at which it adds nucleotides (600 nucleotides/min) is too slow (by a factor of 100 or more) to account for the rates at which the replication fork moves in the bacterial cell. Second, DNA polymerase I has a relatively low processivity. Third, genetic studies have demonstrated that many genes, and therefore many proteins, are involved in replication: DNA polymerase I clearly does not act alone. Fourth, and most important, in 1969 John Cairns isolated a bacterial strain with an altered gene for DNA polymerase I that produced an inactive enzyme. Although this strain was abnormally sensitive to agents that damaged DNA, it was nevertheless viable.

A search for other DNA polymerases led to the discovery of *E. coli* **DNA polymerase II** and **DNA polymerase III** in the early 1970s. DNA polymerase II is an enzyme involved in one type of DNA repair (Section 25.3). DNA polymerase III is the principal replication enzyme in *E. coli*. DNA polymerases IV and V, identified in 1999, are involved in an unusual form of DNA repair (Section 25.2). The properties of these five DNA polymerases are compared in **Table 25-1**.

DNA polymerase I, then, is not the primary enzyme of replication; instead, it performs a host of cleanup functions during replication, recombination, and repair. The polymerase's special functions are enhanced by its 5′→3′ exonuclease activity. This activity, distinct from the 3′→5′ proofreading exonuclease (Fig. 25-6), is

TABLE 25-1 Comparison of the Five DNA Polymerases of *E. coli*

	DNA polymerase				
	I	II[a]	III	IV[a]	V[a]
Structural gene[b]	*polA*	*polB*	*polC* (*dnaE*)	*dinB*	*umuC*
Subunits (number of different types)	1	7	9	1	3
M_r	103,000	88,000[c]	1,065,400	39,100	110,000
3′→5′ exonuclease (proofreading)	Yes	Yes	Yes	No	No
5′→3′ exonuclease	Yes	No	No	No	No
Polymerization rate (nucleotides/s)	10–20	40	250–1,000	2–3	1
Processivity (nucleotides added before polymerase dissociates)	3–200	1,500	≥500,000	1	6–8

[a] Translesion (mutagenic) DNA polymerases. For DNA polymerase IV, processivity is increased substantially by association with a β clamp. These polymerases are slowed when a DNA lesion is present in the DNA template strand.

[b] For enzymes with more than one subunit, the gene listed here encodes the subunit with polymerization activity. Note that *dnaE* is an earlier designation for the gene now referred to as *polC*.

[c] Polymerization subunit only. DNA polymerase II shares several subunits with DNA polymerase III, including the $\beta, \delta, \delta', \chi$, and ψ subunits (see Table 25-2).

located in a structural domain that can be separated from the rest of the enzyme by mild protease treatment. When the 5′→3′ exonuclease domain is removed, the remaining fragment (M_r 68,000), the **large fragment** or **Klenow fragment**, retains the polymerization and proofreading activities. The 5′→3′ exonuclease activity of intact DNA polymerase I can replace a segment of DNA (or RNA) paired to the template strand, in a process known as nick translation (**Fig. 25-7**). Most other DNA polymerases lack a 5′→3′ exonuclease activity.

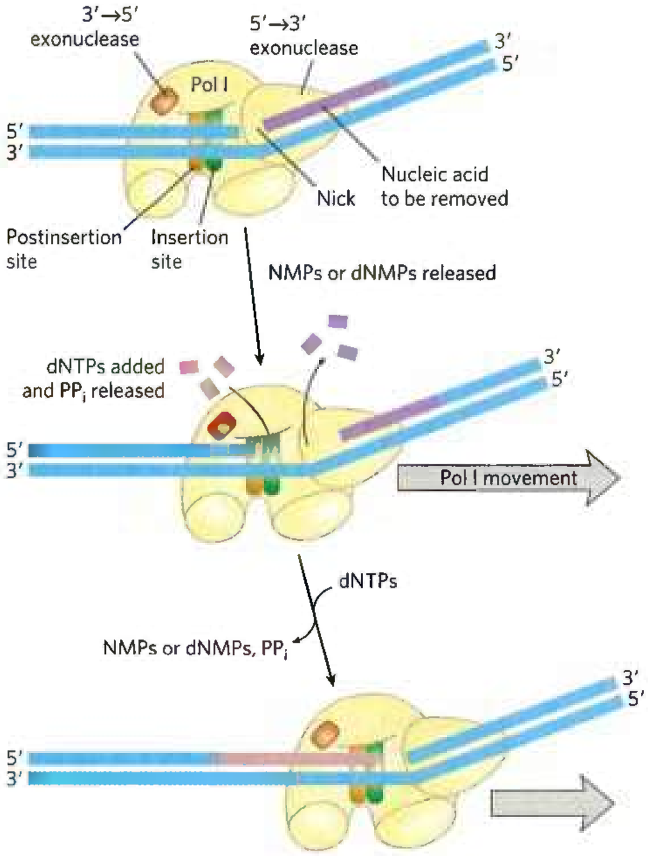


FIGURE 25-7 Nick translation. The bacterial DNA polymerase I has three domains, catalyzing its DNA polymerase, 5′→3′ exonuclease, and 3′→5′ exonuclease activities. The 5′→3′ exonuclease domain is in front of the enzyme as it moves along the DNA and is not shown in Figure 25-4. By degrading the DNA strand ahead of the enzyme and synthesizing a new strand behind, DNA polymerase I can promote nick translation, in which a break or nick in the DNA is effectively moved along with the enzyme. This process has a role in DNA repair and in the removal of RNA primers during replication (both described later in this chapter). The strand of nucleic acid to be removed (either DNA or RNA) is shown in purple, the replacement strand in red. DNA synthesis begins at a nick (a broken phosphodiester bond, leaving a free 3′ hydroxyl and a free 5′ phosphate). A nick remains where DNA polymerase I eventually dissociates, and the nick is later sealed by another enzyme.

DNA polymerase III is much more complex than DNA polymerase I, with nine different kinds of subunits (**Table 25-2**). Its polymerization and proofreading activities reside in its α and ε subunits, respectively. The θ subunit associates with α and ε to form a core polymerase, which can polymerize DNA but with limited processivity. Up to three core polymerases can be linked by a clamp-loading complex consisting of five subunits of three different types, $\tau_3\delta\delta'$. The core polymerases are linked through the τ (tau) subunits. Two additional subunits, χ (chi) and ψ (psi), are bound to the clamp-loading complex. The entire assembly of 16 protein subunits (eight different types) is called DNA polymerase III* (**Fig. 25-8a**).

DNA polymerase III* can polymerize DNA, but with a much lower processivity than one would expect for the organized replication of an entire chromosome. The necessary increase in processivity is provided by the addition of the β subunits. The β subunits associate in pairs to form donut-shaped structures that encircle the DNA and act like clamps (Fig. 25-8b). Each dimer associates with a core subassembly of polymerase III* (one dimeric clamp per active core subassembly) and slides along the DNA as replication proceeds. The β sliding clamp prevents the dissociation of DNA polymerase III from DNA, dramatically increasing processivity—to greater than 500,000

TABLE 25-2 Subunits of DNA Polymerase III of *E. coli*

Subunit	Number of subunits per holoenzyme	M_r of subunit	Gene	Function of subunit	
α	3	129,900	*polC* (*dnaE*)	Polymerization activity	Core polymerase
ε	3	27,500	*dnaQ* (*mutD*)	$3' \rightarrow 5'$ proofreading exonuclease	
θ	3	8,600	*holE*	Stabilization of ε subunit	
τ	3	71,100	*dnaX*	Stable template binding; core enzyme dimerization	Clamp-loading (γ) complex that loads β subunits on lagging strand at each Okazaki fragment[a]
δ	1	38,700	*holA*	Clamp opener	
δ'	1	36,900	*holB*	Clamp loader	
χ	1	16,600	*holC*	Interaction with SSB	
ψ	1	15,200	*holD*	Interaction with τ and χ	
β	6	40,600	*dnaN*	DNA clamp required for optimal processivity	

[a] The γ subunit is encoded by a portion of the gene for the τ subunit (*dnaX*), such that the amino-terminal 66% of the τ subunit has the same amino acid sequence as the γ subunit. The γ subunit is generated by a translational frameshifting mechanism (p. 1013) that leads to premature translational termination. The γ subunit shares the clamp-loading functions of τ but lacks the protein segments that interact with the core polymerase or with DnaB helicase. Clamp-loading complexes incorporating γ subunits may operate independently of the DNA polymerase III holoenzyme, promoting the unloading of β clamps discarded on the lagging strand as the replication fork progresses. They may also promote loading of β clamps for some DNA repair processes that require DNA synthesis away from the replication fork.

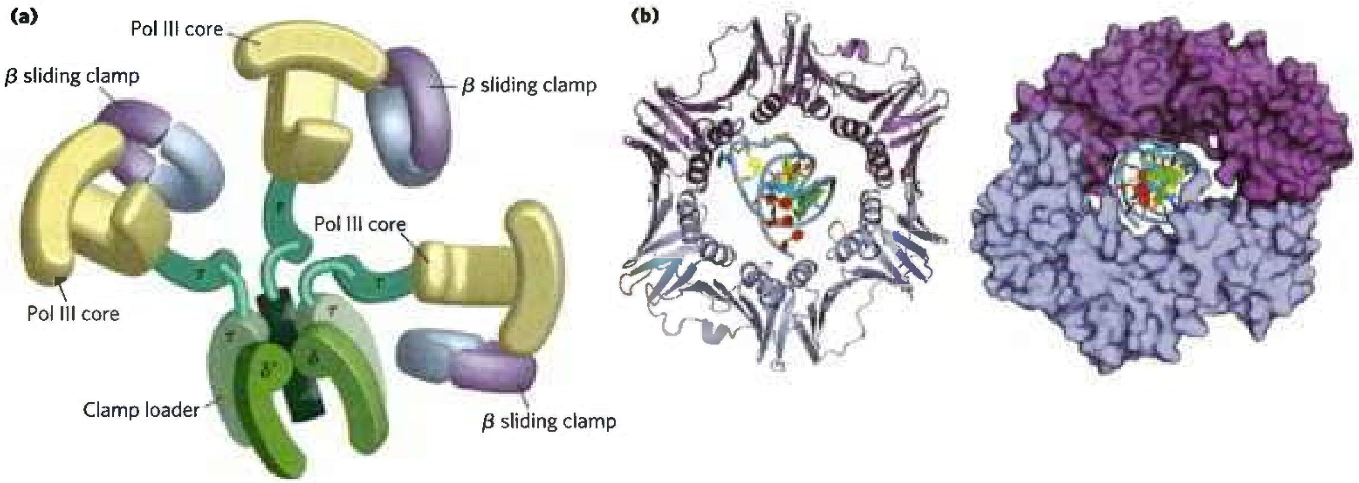


FIGURE 25-8 DNA polymerase III. (a) Architecture of bacterial DNA polymerase III (Pol III). Three core domains, composed of subunits α, ε, and θ, are linked by a five-subunit clamp-loading complex, with the composition $\tau_3\delta\delta'$. The core subunits and clamp-loading complex constitute DNA polymerase III*. The other two subunits of DNA polymerase III*, χ and ψ (not shown), also bind to the clamp-loading complex. Three β clamps interact with the three core subassemblies, each clamp a dimer of the β subunit. The complex interacts with the DnaB helicase (described later in the text) through the τ subunits. (b) Two β subunits of *E. coli* polymerase III form a circular clamp that surrounds the DNA. The clamp slides along the DNA molecule, increasing the processivity of the polymerase III holoenzyme to more than 500,000 nucleotides by preventing its dissociation from the DNA. The two β subunits are shown in two shades of purple as ribbon structures (left) and surface contour images (right), surrounding the DNA. [(a) Information from N. Yao and M. O'Donnell, *Mol. Biosyst.* 4:1075, 2008. (b) Data from PDB ID 2POL, X.-P. Kong et al., *Cell* 69:425, 1992.]

(Table 25-1). The addition of the β subunits converts DNA polymerase III* to DNA polymerase III holoenzyme.

DNA Replication Requires Many Enzymes and Protein Factors

Replication in *E. coli* requires not just a single DNA polymerase but 20 or more different enzymes and proteins, each performing a specific task. The entire complex has been termed the **DNA replicase system** or **replisome**. The enzymatic complexity of replication reflects the constraints imposed by the structure of DNA and by the requirements for accuracy. The main classes of replication enzymes are considered here in terms of the problems they overcome.

The DNA must be separated into two strands that each act as a template. P2 This is generally accomplished by **helicases**, enzymes that move along the DNA and separate the strands, using chemical energy from ATP. Strand separation creates topological stress in the helical DNA structure (see Fig. 24-11), which is relieved by the action of **topoisomerases**. The separated strands are stabilized by **DNA-binding proteins**. As noted earlier, before DNA polymerases can begin synthesizing DNA, primers must be present on the template—generally, short segments of RNA synthesized by enzymes known as **primases**. Ultimately, the RNA primers are removed and replaced by DNA; in *E. coli*, this is one of the many functions of DNA polymerase I. A specialized nuclease that degrades RNA in RNA-DNA hybrids, called RNase H1, also removes some RNA primers. After an RNA primer is removed and the gap is filled in with DNA, a nick remains in the DNA backbone in the form of a broken phosphodiester bond. These nicks are sealed by **DNA ligases**. All these processes require coordination and regulation best characterized in the *E. coli* system.

Replication of the *E. coli* Chromosome Proceeds in Stages

The synthesis of a DNA molecule can be divided into three stages: initiation, elongation, and termination, distinguished both by the reactions taking place and by the enzymes required. As you will find here and in the next two chapters, synthesis of the major information-containing biological polymers — DNAs, RNAs, and proteins — can be understood in terms of these same three stages, with the stages of each pathway having unique characteristics. The events described below reflect information derived primarily from in vitro experiments using purified *E. coli* proteins, although the principles are highly conserved in all replication systems.

Initiation The *E. coli* replication origin, *oriC*, consists of 245 bp and contains DNA sequence elements that are highly conserved among bacterial replication origins. The general arrangement of the conserved sequences is illustrated in Figure 25-9. Two types of sequences are of special interest: five repeats of a 9 bp sequence (R sites) that serve as binding sites for the key initiator protein, DnaA, and a region rich in A=T base pairs called the **DNA unwinding element (DUE)**. There are three additional DnaA-binding sites (I sites), and binding sites for the proteins IHF (integration host factor) and FIS (factor for inversion stimulation). These two proteins were discovered as necessary components of certain recombination reactions described later in this chapter, and their names reflect those roles. Another DNA-binding protein, HU (a histonelike bacterial protein originally dubbed factor U), also participates but does not have a specific binding site.

At least 10 different enzymes or proteins (summarized in Table 25-3) participate in the initiation phase of replication. They open the DNA helix at the origin and establish a prepriming complex for subsequent reactions. The crucial component in the initiation process is the DnaA protein, a member of the **AAA+ ATPase** protein family (*A*TPases *a*ssociated with diverse cellular *a*ctivities). Many AAA+ ATPases, including DnaA, form oligomers and hydrolyze ATP relatively slowly. This ATP hydrolysis acts as a switch that mediates interconversion of the protein between two states. In the case of DnaA, the ATP-bound form is active and the ADP-bound form is inactive.

Eight DnaA protein molecules, all in the ATP-bound state, assemble to form a helical complex encompassing the R and I sites in *oriC* (Fig. 25-10). DnaA has a higher affinity for R sites than I sites, and it binds R sites equally well in its ATP- or ADP-bound form. The I sites, which bind only the ATP-bound DnaA, allow discrimination between the active and inactive forms of DnaA. The tight right-handed wrapping of the DNA around this complex introduces a positive supercoil (see Chapter 24). The associated strain in the nearby DNA, combined with the binding of additional DnaA protein to the DUE region, leads to strand separation in the A=T-rich DUE. The complex formed at the replication origin also includes several DNA-binding proteins—HU, IHF, and FIS—that facilitate DNA bending.

The DnaC protein, another AAA+ ATPase, then loads the DnaB protein onto the separated DNA strands in the denatured region. A hexamer of DnaC, each subunit bound to ATP, forms a tight complex with the ring-shaped, hexameric DnaB helicase. This DnaC-DnaB interaction opens the DnaB ring, the process being aided by a further interaction between DnaB and DnaA. Two of the ring-shaped DnaB hexamers are loaded in the DUE, one onto each DNA strand. The ATP bound to DnaC is hydrolyzed, releasing the DnaC and leaving the DnaB bound to the DNA.

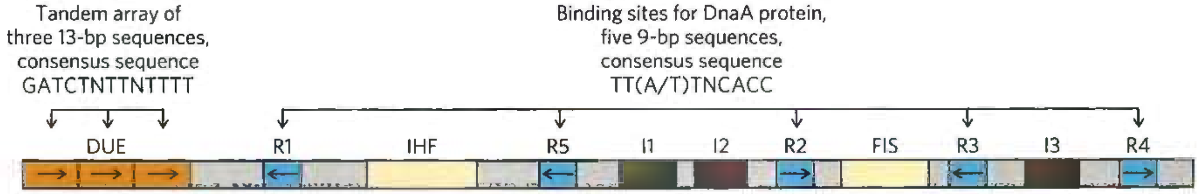


FIGURE 25-9 Arrangement of sequences in the *E. coli* replication origin, *oriC*. Conserved sequences for key repeated elements are shown. N represents any of the four nucleotides. The horizontal arrows indicate the orientations of the nucleotide sequences (left-to-right arrow denotes a sequence in the top strand; right-to-left, in the bottom strand). FIS and IHF are binding sites for proteins described in the text. R sites are bound by DnaA. I sites are additional DnaA-binding sites (with different sequences, labeled I1, I2, and I3), for DnaA only when the protein is complexed with ATP.

TABLE 25-3 Proteins Required to Initiate Replication at the *E. coli* Origin

Protein	M_r	Number of subunits	Function
DnaA protein	52,000	1	Recognizes *oriC* sequence; opens duplex at specific sites in origin
DnaB protein (helicase)	300,000	6^a	Unwinds DNA
DnaC protein	174,000	6^a	Required for DnaB binding at origin
HU	19,000	2	Histonelike protein; DNA-binding protein; stimulates initiation
FIS	22,500	2^a	DNA-binding protein; stimulates initiation
IHF	22,000	2	DNA-binding protein; stimulates initiation
Primase (DnaG protein)	60,000	1	Synthesizes RNA primers
Single-stranded DNA–binding protein (SSB)	75,600	4^a	Binds single-stranded DNA
DNA gyrase (DNA topoisomerase II)	400,000	4	Relieves torsional strain generated by DNA unwinding
Dam methylase	32,000	1	Methylates (5′)GATC sequences at *oriC*

[a] Subunits in these cases are identical.

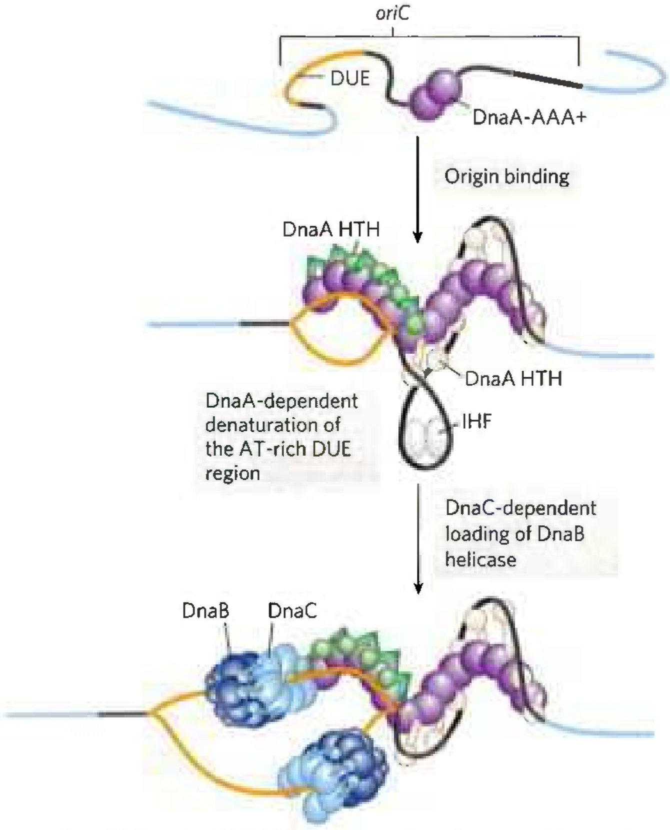

oriC
DUE
DnaA-AAA+
Origin binding
DnaA HTH
DnaA HTH
IHF
DnaA-dependent denaturation of the AT-rich DUE region
DnaC-dependent loading of DnaB helicase
DnaB
DnaC

FIGURE 25-10 Model for initiation of replication at the *E. coli* origin, *oriC*. DnaA protein molecules bind initially at the five specific R sites. Upon ATP binding, additional DnaA molecules bind at the I sites in the origin, forming a right-handed helical complex and drawing in more DnaA molecules that continue the helix into the DUE region (see Fig. 25-9). The DNA is wrapped around this complex. DnaA molecules bound at the R and I sites bind the DNA with an HTH doman (referring to a DNA binding motif called helix-turn-helix). The A═T-rich DUE region is denatured as a result of the strain imparted by the DnaA binding. Within the DUE, single-stranded DNA is bound by the ATPase domain of DnaA rather than by the HTH. Formation of the helical DnaA complex is facilitated by the proteins HU, IHF, and FIS. The detailed structural roles of these proteins are not known, but IHF may stabilize a transient DNA loop, as shown here. Hexamers of the DnaB protein bind to each strand, with the aid of DnaC protein. The DnaB helicase activity further unwinds the DNA in preparation for priming and DNA synthesis. [Information from J. P. Erzberger et al., *Nat. Struct. Mol. Biol.* 13:676, 2006.]

Loading of the DnaB helicase is the key step in replication initiation. As a replicative helicase, DnaB migrates along the single-stranded DNA in the 5′→3′ direction, unwinding the DNA as it travels. The DnaB helicases loaded onto the two DNA strands thus travel in opposite directions, creating two potential replication forks. All other proteins at the replication fork are linked directly or indirectly to DnaB. The DNA polymerase III holoenzyme is linked through its τ subunits; additional DnaB interactions are described below. As replication begins and the DNA strands are separated at the fork, many molecules of single-stranded DNA–binding protein (SSB) bind to and stabilize the separated strands, and DNA gyrase (DNA topoisomerase II) relieves the topological stress induced ahead of the fork by the unwinding reaction.

Initiation is the only phase of DNA replication that is known to be regulated, and it is regulated such that replication occurs only once in each cell cycle. The mechanism of regulation is not yet entirely understood, but genetic and biochemical studies have provided insights into several separate regulatory mechanisms.

Once DNA polymerase III has been loaded onto the DNA, along with the β subunits (signaling completion of the initiation phase), the protein Hda binds to the β subunits and interacts with DnaA to stimulate hydrolysis of its bound ATP. Hda is yet another AAA+ ATPase closely related to DnaA (its name is derived from *h*omologous to *DnaA*). This ATP hydrolysis leads to disassembly of the DnaA complex at the origin. Slow release of ADP by DnaA and rebinding of ATP cycles the protein between its inactive (with bound ADP) and active (with bound ATP) forms on a time scale of 20 to 40 minutes.

The timing of replication initiation is affected by DNA methylation and interactions with the bacterial plasma membrane. The *oriC* DNA is methylated by the Dam methylase (Table 25-3), which methylates the N^6 position of adenine within the palindromic sequence (5′)GATC. (Dam is not a biochemical expletive; it stands for *D*NA *a*denine *m*ethylation.) The *oriC* region of *E. coli* is highly enriched in GATC sequences—it has 11 in its 245 bp; the average frequency of GATC in the *E. coli* chromosome as a whole is 1 in 256 bp.

Immediately after replication, the DNA is hemimethylated: the parent strands have methylated *oriC* sequences but the newly synthesized strands do not. The hemimethylated *oriC* sequences are now sequestered by interaction with the plasma membrane (the mechanism is unknown) and by binding of the protein SeqA. After a time, *oriC* is released from the plasma membrane, SeqA dissociates, and the DNA must be fully methylated by Dam methylase before it can again bind DnaA and initiate a new round of replication.

Elongation The elongation phase of replication includes two distinct but related operations: leading strand synthesis and lagging strand synthesis. Several enzymes at the replication fork are important to the synthesis of both strands. Parent DNA is first unwound by DNA helicases, and the resulting topological stress is relieved by topoisomerases. Each separated strand is then stabilized by SSB. From this point, synthesis of leading and lagging strands is sharply different.

Leading strand synthesis, the more straightforward of the two, begins with the synthesis by primase (DnaG protein) of a short (10 to 60 nucleotide) RNA primer at the replication origin. DnaG interacts with DnaB helicase to carry out this reaction, and the primer is synthesized in the direction opposite to that in which the DnaB helicase is moving. In effect, the DnaB helicase moves along the strand that becomes the lagging strand in DNA synthesis; however, the first primer laid down in the first DnaG-DnaB interaction serves to prime leading strand DNA synthesis in the opposite direction. Deoxyribonucleotides are added to this primer by a DNA polymerase III complex linked to the DnaB helicase tethered to the opposite DNA strand. Leading strand synthesis then proceeds continuously, keeping pace with the unwinding of DNA at the replication fork.

Lagging strand synthesis, as we have noted, is accomplished in short Okazaki fragments (**Fig. 25-11a**). First, an RNA primer is synthesized by primase, and, as in leading strand synthesis, DNA polymerase III binds to the RNA primer and adds deoxyribonucleotides (Fig. 25-11b). On this level, the synthesis of each Okazaki fragment seems straightforward, but the details are quite complex. The complexity lies in the *coordination* of leading and lagging strand synthesis. Both strands are produced by a *single* asymmetric DNA polymerase III dimer; this is accomplished by looping the DNA of the lagging strand as shown in **Figure 25-12**, bringing together the two points of polymerization.

The synthesis of Okazaki fragments on the lagging strand entails some elegant enzymatic choreography.

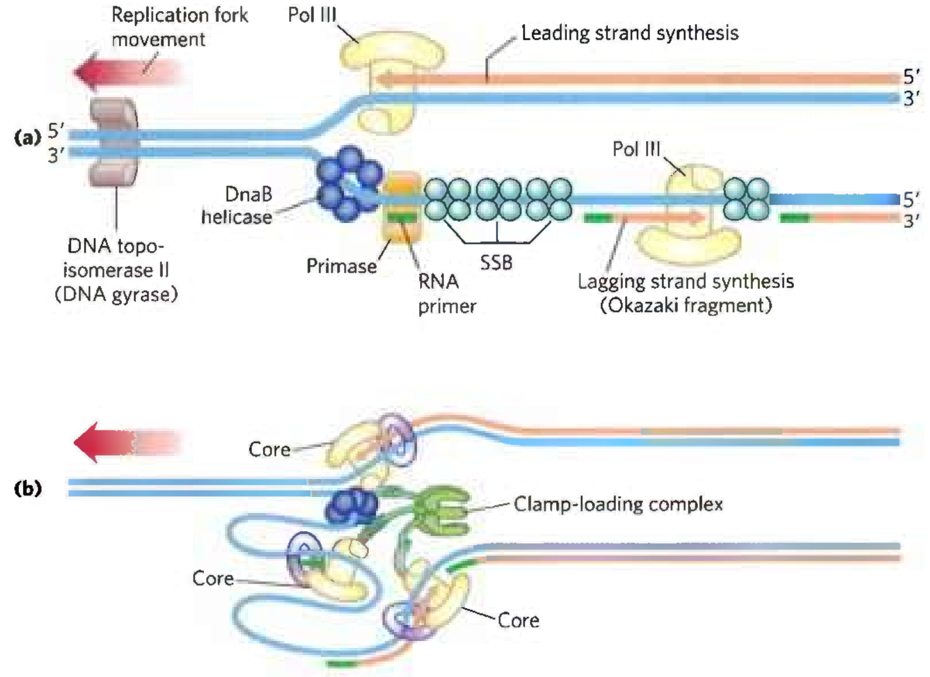
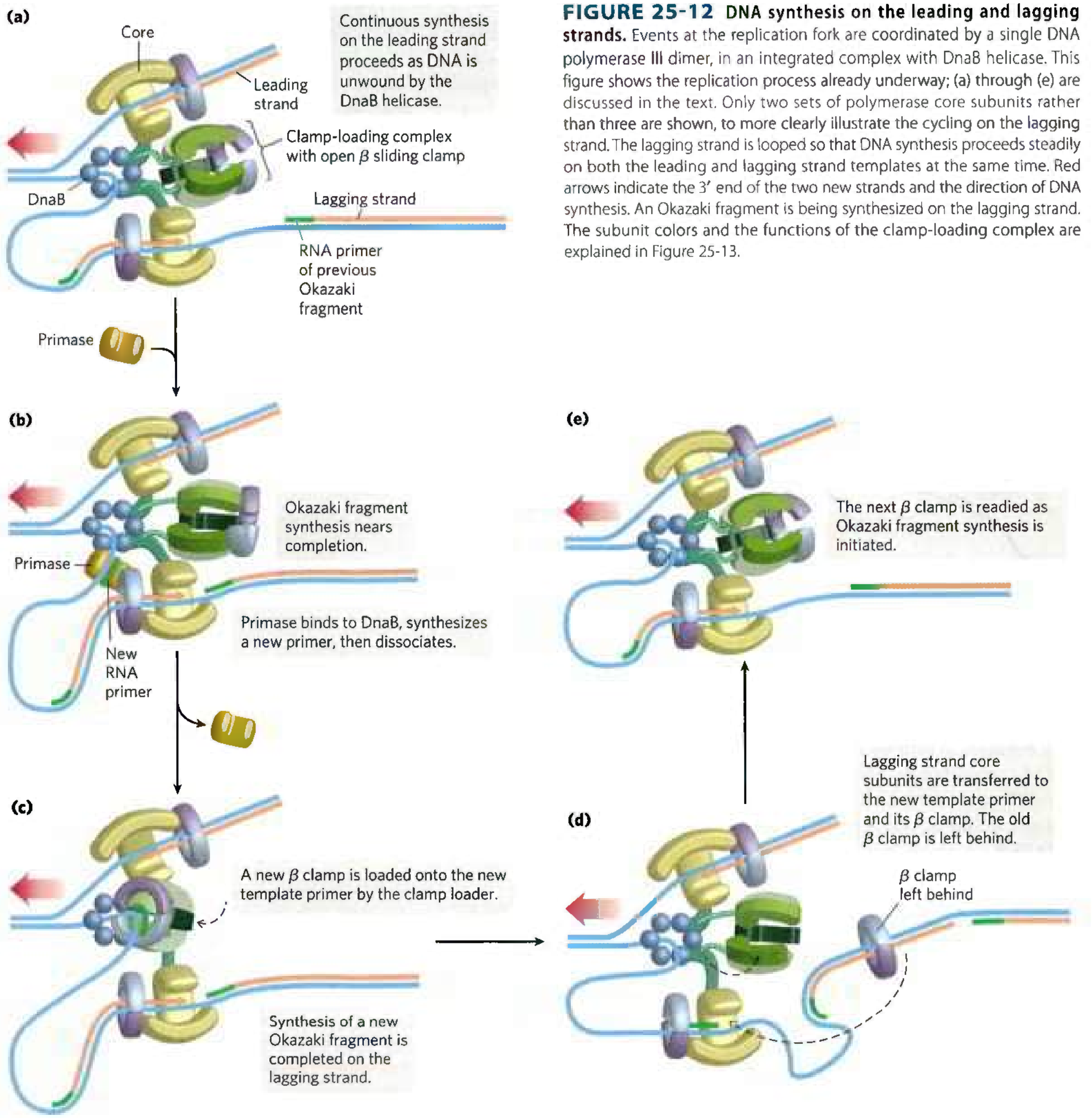


FIGURE 25-11 Synthesis of Okazaki fragments. (a) At intervals, primase synthesizes an RNA primer for a new Okazaki fragment. If we consider the two template strands as lying side by side, lagging strand synthesis formally proceeds in the opposite direction from fork movement. Each primer is extended by DNA polymerase III. DNA synthesis continues until the fragment extends as far as the primer of the previously added Okazaki fragment. A new primer is synthesized near the replication fork to begin the process again. (b) In the replisome complex, DNA synthesis on the leading and lagging strands is tightly coordinated. Each DNA polymerase III holoenzyme has three sets of core subunits (yellow), linked together with a single clamp-loading complex, so one or two Okazaki fragments can be synthesized simultaneously, along with the leading strand.


(a)
Core
Leading strand
Clamp-loading complex with open β sliding clamp
DnaB
Lagging strand
RNA primer of previous Okazaki fragment
Primase
(b)
Primase
New RNA primer
(c)
(d)
β clamp left behind
(e)

Continuous synthesis on the leading strand proceeds as DNA is unwound by the DnaB helicase.

Okazaki fragment synthesis nears completion.

Primase binds to DnaB, synthesizes a new primer, then dissociates.

A new β clamp is loaded onto the new template primer by the clamp loader.

Synthesis of a new Okazaki fragment is completed on the lagging strand.

Lagging strand core subunits are transferred to the new template primer and its β clamp. The old β clamp is left behind.

The next β clamp is readied as Okazaki fragment synthesis is initiated.

FIGURE 25-12 DNA synthesis on the leading and lagging strands. Events at the replication fork are coordinated by a single DNA polymerase III dimer, in an integrated complex with DnaB helicase. This figure shows the replication process already underway; (a) through (e) are discussed in the text. Only two sets of polymerase core subunits rather than three are shown, to more clearly illustrate the cycling on the lagging strand. The lagging strand is looped so that DNA synthesis proceeds steadily on both the leading and lagging strand templates at the same time. Red arrows indicate the 3′ end of the two new strands and the direction of DNA synthesis. An Okazaki fragment is being synthesized on the lagging strand. The subunit colors and the functions of the clamp-loading complex are explained in Figure 25-13.

DNA polymerase III uses one set of its core subunits (the core polymerase) to synthesize the leading strand continuously, while the other two sets of core subunits cycle from one Okazaki fragment to the next on the looped lagging strand. In vitro, a DNA polymerase III holoenzyme with only two sets of core subunits can synthesize both leading and lagging strands. However, a third set of core subunits increases the efficiency of lagging strand synthesis as well as the processivity of the overall replisome.

DnaB helicase, bound in front of DNA polymerase III, unwinds the DNA at the replication fork (Fig. 25-12a) as it travels along the lagging strand template in the 5′→3′ direction. DnaG primase occasionally associates with DnaB helicase and synthesizes a short RNA primer (Fig. 25-12b). A new β sliding clamp is then positioned at the primer by the clamp-loading complex of DNA polymerase III (Fig. 25-12c). When synthesis of an Okazaki fragment has been completed, replication halts, and the core subunits of DNA polymerase III dissociate from their

TABLE 25-4 Proteins of the *E. coli* Replisome

Protein	M_r	Number of subunits	Function
SSB	75,600	4	Binding to single-stranded DNA
Helicase (DnaB protein)	300,000	6	DNA unwinding
Primase (DnaG protein)	60,000	1	RNA primer synthesis
DNA polymerase III	1,065,400	17	New strand elongation
DNA polymerase I	103,000	1	Filling of gaps; excision of primers
DNA ligase	74,000	1	Ligation
DNA gyrase (DNA topoisomerase II)	400,000	4	Supercoiling

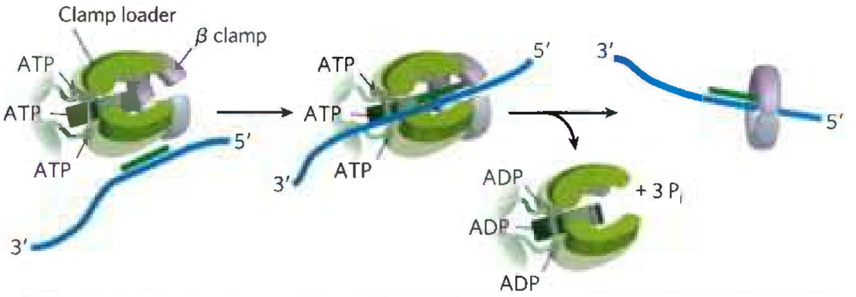


FIGURE 25-13 The DNA polymerase III clamp loader. The five subunits of the clamp-loading (γ) complex are the δ and δ' subunits and the amino-terminal domain of each of the three τ subunits (see Fig. 25-8). The complex binds to three molecules of ATP and to a dimeric β clamp. This binding forces the β clamp open at one of its two subunit interfaces. Hydrolysis of the bound ATP allows the β clamp to close again around the DNA.

β sliding clamp (and from the completed Okazaki fragment) and associate with the new clamp (Fig. 25-12d, e). This initiates synthesis of a new Okazaki fragment. Two sets of core subunits may be engaged in the synthesis of two different Okazaki fragments at the same time. The proteins acting at the replication fork are summarized in **Table 25-4**.

The clamp-loading complex of DNA polymerase III, consisting of parts of the three τ subunits along with the δ and δ' subunits, is also an AAA+ ATPase. This complex binds to ATP and to the new β sliding clamp. The binding imparts strain on the dimeric clamp, opening up the ring at one subunit interface (**Fig. 25-13**). The newly primed lagging strand is slipped into the ring through the resulting break. The clamp loader then hydrolyzes ATP, releasing the β sliding clamp and allowing it to close around the DNA.

The replisome promotes rapid DNA synthesis, adding ~1,000 to 2,000 nucleotides to each strand (leading and lagging). Once an Okazaki fragment has been completed, its RNA primer is removed by DNA polymerase I or RNase H1 and replaced with DNA by the polymerase; the remaining nick is sealed by DNA ligase (**Fig. 25-14**).

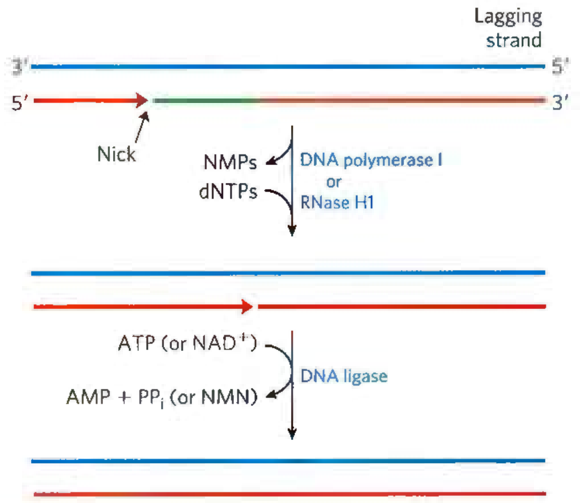


FIGURE 25-14 Final steps in the synthesis of lagging strand segments. RNA primers in the lagging strand are removed by the 5′→3′ exonuclease activity of DNA polymerase I or RNase H1, and then replaced with DNA by DNA polymerase I. The remaining nick is sealed by DNA ligase. The role of ATP or NAD$^+$ is shown in Figure 25-15.

DNA ligase catalyzes the formation of a phosphodiester bond between a 3′ hydroxyl at the end of one DNA strand and a 5′ phosphate at the end of another strand. The phosphate must be activated by adenylylation. DNA ligases isolated from viruses and eukaryotes use ATP for this purpose. DNA ligases from bacteria are unusual in that many use NAD$^+$—a cofactor that usually functions in hydride transfer reactions (see Fig. 13-24)—as the source of the AMP activating group (**Fig. 25-15**). DNA ligase is another enzyme of DNA metabolism that has become an important reagent in recombinant DNA experiments (see Fig. 9-1).

Termination Eventually, the two replication forks of the circular *E. coli* chromosome meet at a terminus region

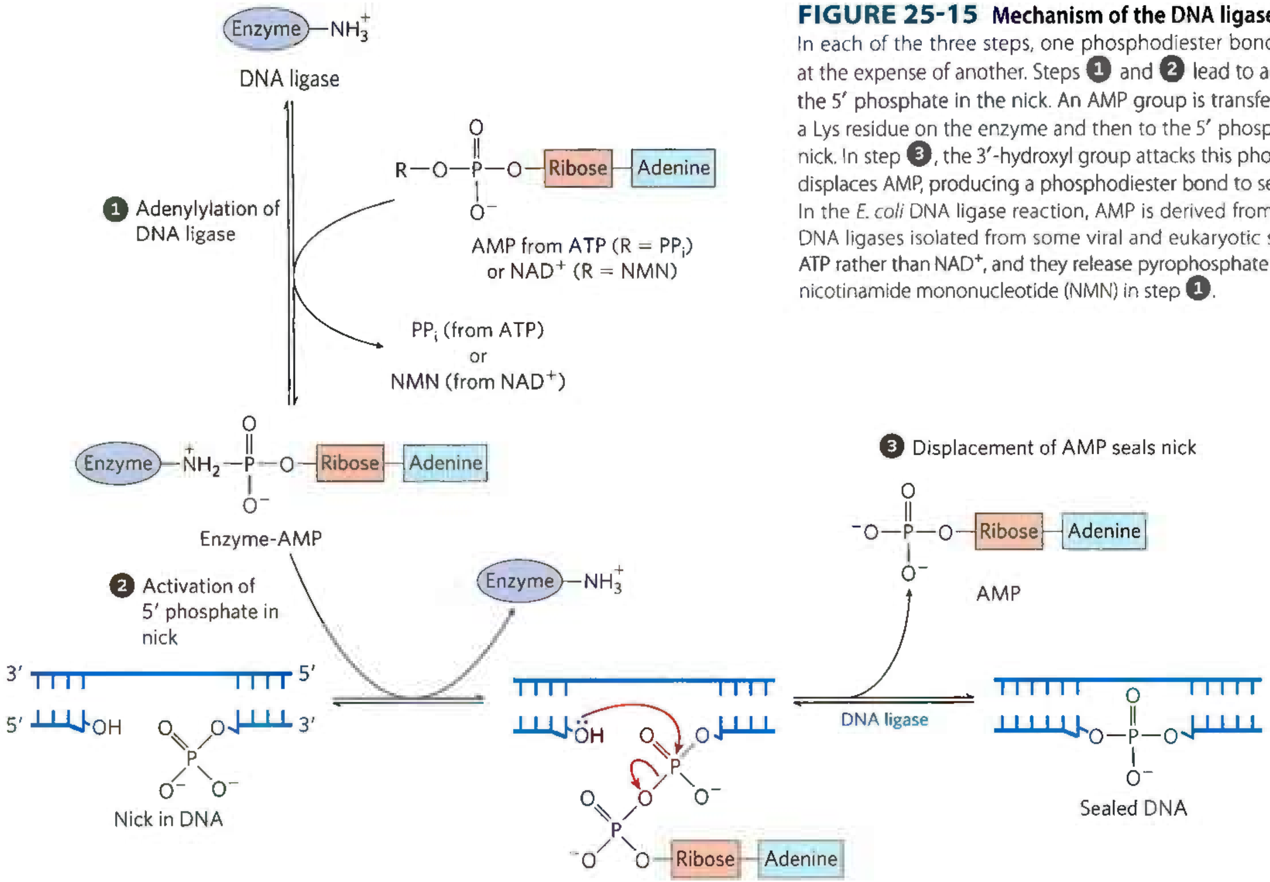

Enzyme—NH_3^+
DNA ligase
R—O—P—O—Ribose—Adenine
① Adenylylation of DNA ligase
AMP from ATP (R = PP_i) or NAD^+ (R = NMN)
PP_i (from ATP) or NMN (from NAD^+)
Enzyme—NH_2^+—P—O—Ribose—Adenine
Enzyme-AMP
② Activation of 5′ phosphate in nick
Enzyme—NH_3^+
3′ 5′
5′ OH 3′
Nick in DNA
OH
Ribose Adenine
③ Displacement of AMP seals nick
AMP
DNA ligase
Sealed DNA

FIGURE 25-15 **Mechanism of the DNA ligase reaction.** In each of the three steps, one phosphodiester bond is formed at the expense of another. Steps ① and ② lead to activation of the 5′ phosphate in the nick. An AMP group is transferred first to a Lys residue on the enzyme and then to the 5′ phosphate in the nick. In step ③, the 3′-hydroxyl group attacks this phosphate and displaces AMP, producing a phosphodiester bond to seal the nick. In the *E. coli* DNA ligase reaction, AMP is derived from NAD^+. The DNA ligases isolated from some viral and eukaryotic sources use ATP rather than NAD^+, and they release pyrophosphate rather than nicotinamide mononucleotide (NMN) in step ①.

containing multiple copies of a 20 bp sequence called Ter (**Fig. 25-16**). The Ter sequences are arranged on the chromosome to create a trap that a replication fork can enter but cannot leave. The Ter sequences function as binding sites for the protein Tus (terminus utilization substance). The Tus-Ter complex can arrest a replication fork from only one direction. Only one Tus-Ter complex functions per replication cycle—the complex first encountered by either replication fork. Given that opposing replication forks generally halt when they collide, Ter sequences would not seem to be essential, but they may prevent overreplication by one fork in the event that the other is delayed or halted by an encounter with DNA damage or some other obstacle.

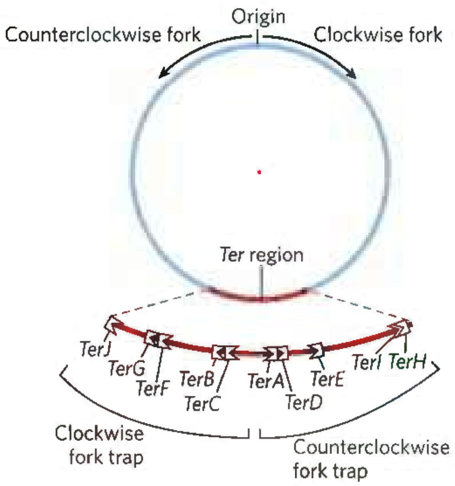

Origin
Counterclockwise fork
Clockwise fork
Ter region
TerJ
TerG
TerF
TerB
TerC
TerA
TerD
TerE
TerI
TerH
Clockwise fork trap
Counterclockwise fork trap

FIGURE 25-16 **Termination of chromosome replication in *E. coli*.** The Ter sequences (*TerA* through *TerJ*) are positioned on the chromosome in two clusters with opposite orientations. The overall Ter region encompasses about 9% of the circular chromosome.

So, when either replication fork encounters a functional Tus-Ter complex, it halts; the other fork halts when it meets the first (arrested) fork. The final few hundred base pairs of DNA between these large protein complexes are then replicated (by an as yet unknown mechanism), completing two topologically interlinked (catenated) circular chromosomes (**Fig. 25-17**). DNA circles linked in this way are known as **catenanes**. Separation of the catenated circles in *E. coli* requires topoisomerase IV (a type II topoisomerase). The separated chromosomes then segregate into daughter cells at cell division. The terminal phase of replication of other circular chromosomes, including many of the DNA viruses that infect eukaryotic cells, is similar.

Replication in Eukaryotic Cells Is Similar but More Complex

The DNA molecules in eukaryotic cells are considerably larger than those in bacteria and are organized into

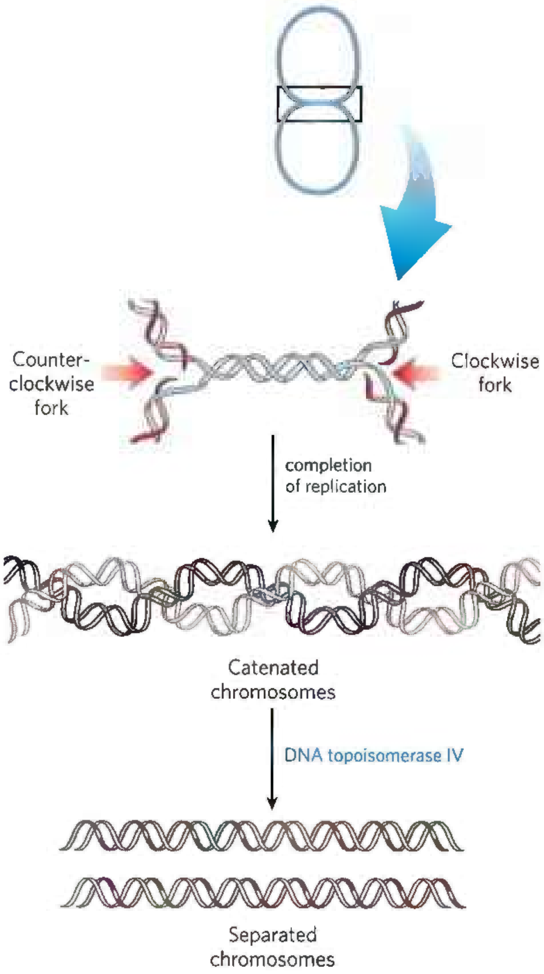


FIGURE 25-17 Role of topoisomerases in replication termination. Replication of the DNA separating opposing replication forks leaves the completed chromosomes joined as catenanes, or topologically interlinked circles. The circles are not covalently linked, but because they are interwound and each is covalently closed, they cannot be separated—except by the action of topoisomerases. In *E. coli*, a type II topoisomerase known as DNA topoisomerase IV plays the primary role in separating catenated chromosomes, transiently breaking both DNA strands of one chromosome and allowing the other chromosome to pass through the break.

complex nucleoprotein structures (chromatin; p. 898). The essential features of DNA replication are the same in eukaryotes and bacteria, and many of the protein complexes are functionally and structurally conserved. However, eukaryotic replication is regulated and coordinated with the cell cycle, and must function within the complexities of chromatin structure.

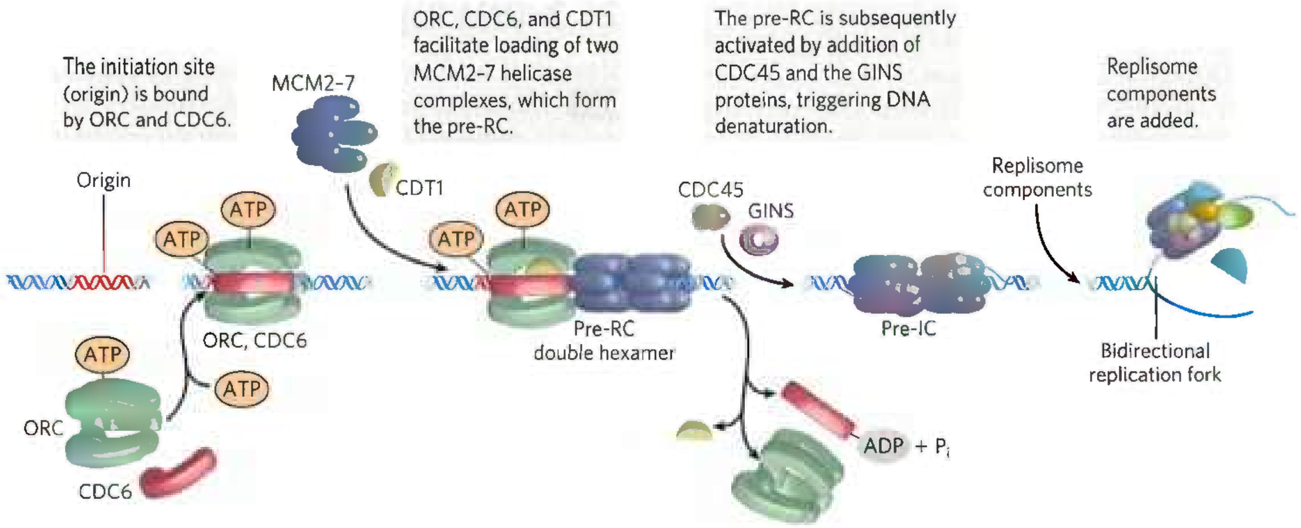
Origins of replication have a well-characterized structure in some lower eukaryotes, but they are much less defined in higher eukaryotes. In both cases, replication begins in short nucleosome-free regions. Yeast (*S. cerevisiae*) has about 400 defined replication origins called autonomously replicating sequences (ARSs), or **replicators**. Yeast replicators span ~150 bp and contain several essential, conserved sequences. There are about 30,000 to 50,000 replication origins in human chromosomes. The replication origins of vertebrates in general may be defined by some aspect of DNA secondary structure, as yet unknown.

Regulation ensures that all cellular DNA is replicated once per cell cycle. Much of this regulation involves proteins called cyclins and the cyclin-dependent kinases (CDKs) with which they form complexes (see Section 12.8). The cyclins are rapidly destroyed by ubiquitin-dependent proteolysis at the end of the M phase (mitosis), and the absence of cyclins allows the establishment of **prereplicative complexes (pre-RCs)** on replication initiation sites. In rapidly growing cells, the pre-RC forms at the end of M phase. In slow-growing cells, it does not form until the end of G1. Formation of the pre-RC renders the cell competent for replication, an event sometimes called **licensing**.

As in bacteria, the key event in the initiation of replication in all eukaryotes is the loading of the replicative helicase, a heterohexameric complex of **minichromosome maintenance (MCM) proteins** (MCM2 to MCM7). The ring-shaped MCM2–7 helicase functions in some ways like the bacterial DnaB helicase, although it translocates 3′→5′ along the leading strand template. It is loaded onto the DNA in steps (**Fig. 25-18**). The origin is recognized and bound first by another six-protein complex, called **ORC (origin recognition complex)**, followed by the protein CDC6 (cell division cycle), which recruits CDT1 (CDC10-dependent transcript 1). Together, they facilitate the loading of two inactive MCM2–7 complexes (the pre-RC). The ORC-CDC6 complex and CTD1 dissociate, leaving behind the pre-RC. ORC has five AAA+ ATPase domains among its subunits and is functionally analogous to the bacterial DnaA. The yeast CDC6 is yet another AAA+ ATPase that forms a complex with the ORC subunits. Following pre-RC formation, another set of proteins, CDC45 and the GINS, bind to and activate the MCM2–7 helicase, triggering DNA denaturation. (GINS refers to the first letters of the numbers 5-1-2-3 in Japanese, *go-ichi-ni-san*, providing a somewhat cryptic callout to the four protein subunits of the complex: SLD5, PSF1, PSF2, and PSF3.) The replication proteins then bind to form a replisome, and bidirectional replication begins.

Commitment to replication requires the synthesis and activity of S-phase cyclin-CDK complexes (such as the cyclin E–CDK2 complex; see Fig. 12-36) and CDC7-DBF4. Both types of complexes help to activate replication by binding to and phosphorylating several subunits of the pre-RC. Other cyclins and CDKs function to inhibit the formation of more pre-RC complexes once replication has been initiated. For example, CDK2 binds to cyclin A as cyclin E levels decline during S phase, inhibiting CDK2 and preventing the licensing of additional pre-RC complexes.

The rate of movement of the replication fork in eukaryotes (~50 nucleotides/s) is only one-twentieth that observed in *E. coli*. At this rate, replication of an average


The initiation site (origin) is bound by ORC and CDC6.

ORC, CDC6, and CDT1 facilitate loading of two MCM2-7 helicase complexes, which form the pre-RC.

The pre-RC is subsequently activated by addition of CDC45 and the GINS proteins, triggering DNA denaturation.

Replisome components are added.

FIGURE 25-18 Assembly of a prereplicative complex at a eukaryotic replication origin. The initiation site (origin) is bound by ORC, CDC6, and CDT1. These proteins, many of them AAA+ ATPases, promote loading of two MCM2–7 helicase complexes, in a reaction analogous to the loading of the bacterial DnaB helicase by DnaC protein. The two loaded but inactive MCM2–7 complexes comprise the prereplicative complex, or pre-RC. The pre-RC is subsequently activated by addition of CDC45 and the GINS proteins, followed by addition of the replisome components. [Information from M. W. Parker et al., *Crit. Rev. Biochem. Mol. Biol.* 52:107, 2017.]

human chromosome proceeding from a single origin would take more than 500 hours, making the requirement for many origins evident.

Like bacteria, eukaryotes have several types of DNA polymerases. Some have been linked to particular functions, such as the replication of mitochondrial DNA. The replication of nuclear chromosomes primarily involves three multisubunit DNA polymerases. The highly processive **DNA polymerase ε** synthesizes the leading strand, and **DNA polymerase δ** synthesizes the lagging strand. Both enzymes have 3′→5′ proofreading exonuclease activities. **DNA polymerase α**, a DNA polymerase/primase, synthesizes RNA primers and also extends them by about 10 nucleotides of DNA to initiate synthesis of each Okazaki fragment on the lagging strand. One subunit of DNA polymerase α has a primase activity, and the largest subunit (M_r ~180,000) contains the polymerization activity. However, this polymerase has no proofreading 3′→5′ exonuclease activity, making it unsuitable for high-fidelity DNA replication.

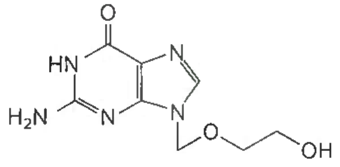
DNA polymerases ε and δ are associated with and stimulated by proliferating cell nuclear antigen (PCNA; M_r 29,000), a protein found in large amounts in the nuclei of proliferating cells. The three-dimensional structure of PCNA is remarkably similar to that of the β subunit of *E. coli* DNA polymerase III (Fig. 25-8b), although primary sequence homology is not evident. PCNA has a function analogous to that of the β subunit, forming a circular clamp that enhances the processivity of the two polymerases.

Two other protein complexes also function in eukaryotic DNA replication. RPA (replication protein A) is a single-stranded DNA–binding protein, equivalent in function to the *E. coli* SSB protein. RFC (replication factor C) is a clamp loader for PCNA and facilitates the assembly of active replication complexes. The subunits of the RFC complex have significant sequence similarity to the subunits of the bacterial clamp-loading (γ) complex.

Termination of replication on linear eukaryotic chromosomes occurs when replication forks operating from nearby origins converge. As in bacteria, there are successive steps of final replication, replisome dissociation, and decatenation of the DNA products. All of the steps are mediated by additional protein complexes, with some parts of the process still undefined.

Viral DNA Polymerases Provide Targets for Antiviral Therapy

Many DNA viruses encode their own DNA polymerases, and some of these have become targets for pharmaceuticals. For example, the DNA polymerase of the herpes simplex virus is inhibited by acyclovir, a compound developed by Gertrude Elion and George Hitchings (p. 836). Acyclovir consists of guanine attached to an incomplete ribose ring.



Acyclovir

It is phosphorylated by a virally encoded thymidine kinase; acyclovir binds to this viral enzyme with an affinity 200-fold greater than its binding to the cellular thymidine kinase. This ensures that phosphorylation occurs mainly in virus-infected cells. Cellular kinases convert the resulting acyclo-GMP to acyclo-GTP, which is both an inhibitor and a substrate of DNA polymerases; acyclo-GTP competitively inhibits the herpes DNA polymerase more strongly than cellular DNA polymerases. Because it lacks a 3′ hydroxyl, acyclo-GTP also acts as a chain terminator when incorporated into DNA. Thus viral replication is inhibited at several steps. ■

SUMMARY 25.1 *DNA Replication*

- Replication of DNA follows a set of universal rules. Replication is semiconservative, each strand acting as template for a new daughter strand. It is carried out in three identifiable phases: initiation, elongation, and termination. The process starts at a single origin in bacteria and usually proceeds bidirectionally. DNA is synthesized in the 5′→3′ direction by DNA polymerases. At the replication fork, the leading strand is synthesized continuously in the same direction as replication fork movement; the lagging strand is synthesized discontinuously as Okazaki fragments, which are subsequently ligated.
- Nucleases are enzymes that degrade DNA. Endonucleases cleave within a DNA polymer; exonucleases degrade DNA from the end of one strand (either 5′→3′ or 3′→5′).
- DNA polymerases are complex enzymes that synthesize DNA, and often possess additional activities, including exonuclease functions.
- DNA is replicated with very high fidelity. Accuracy is maintained by (1) base selection by the polymerase, (2) a 3′→5′ proofreading exonuclease activity that is part of many DNA polymerases, and (3) specific repair systems for mismatches left behind after replication.
- Most cells have several DNA polymerases. In *E. coli*, DNA polymerase III is the primary replication enzyme. DNA polymerase I is responsible for special functions during replication, recombination, and repair.
- Replication requires an array of enzymes and protein factors in addition to DNA polymerases. Many of these proteins belong to the AAA+ ATPase family.
- Replication initiation occurs when replicative helicases are loaded onto replication origins in stepwise fashion. Elongation is achieved by an active replisome—a supramolecular complex of nucleic acids and many proteins, including polymerases. Termination occurs when replisomes proceeding in opposite directions converge. It requires decatenation of the replication products when replication is complete.
- The major replicative DNA polymerases in eukaryotes are DNA polymerases ε and δ. DNA polymerase α synthesizes primers.
- Viral DNA replication is a drug target.

25.2 DNA Repair

Most cells have only one or two sets of genomic DNA. P1 Damaged proteins and RNA molecules can be quickly replaced by using information encoded in the DNA, but DNA molecules themselves are irreplaceable. Maintaining the integrity of the information in DNA is a cellular imperative, supported by an elaborate set of DNA repair systems. DNA can become damaged by a variety of processes, some spontaneous, others catalyzed by environmental agents (Chapter 8). Replication itself can very occasionally damage the information content in DNA when polymerase errors create mismatched base pairs (such as G paired with T).

The chemistry of DNA damage is diverse and complex. The cellular response to this damage includes enzymatic systems that catalyze some of the most interesting chemical transformations in DNA metabolism. We first examine the effects of alterations in DNA sequence and then consider specific repair systems.

Mutations Are Linked to Cancer

The best way to illustrate the importance of DNA repair is to consider the effects of *unrepaired* DNA damage (a lesion). P3 The most serious outcome is a change in the base sequence of the DNA, which, if replicated and transmitted to future generations of cells, becomes permanent. A permanent change in the nucleotide sequence of DNA is called a **mutation**. Mutations can involve the replacement of one base pair with another (substitution mutation) or the addition or deletion of one or more base pairs (insertion or deletion mutations). If the mutation affects nonessential DNA or if it has a negligible effect on the function of a gene, it is known as a **silent mutation**. P1 P3 Rarely, a mutation confers some biological advantage. Most nonsilent mutations, however, are neutral or deleterious.

In mammals there is a strong correlation between the accumulation of mutations and cancer. A simple test developed by Bruce Ames in the 1970s measures the potential of a given chemical compound to promote certain easily detected mutations in a specialized bacterial strain (**Fig. 25-19**). Few of the chemicals that we encounter in daily life score as mutagens in this test. However, of the compounds known to be carcinogenic from extensive animal trials, more than 90% are also found to be mutagenic in the Ames test. Because of this strong correlation between mutagenesis and carcinogenesis, the Ames test for bacterial mutagens is still widely used as a rapid and inexpensive screen for potential human carcinogens.

The genomic DNA in a typical mammalian cell accumulates many thousands of lesions during a 24-hour period. However, as a result of DNA repair, fewer than 1 in 1,000 become a mutation. DNA is a relatively stable molecule, but in the absence of repair systems, the cumulative effect of many infrequent but damaging reactions would make life impossible. ■

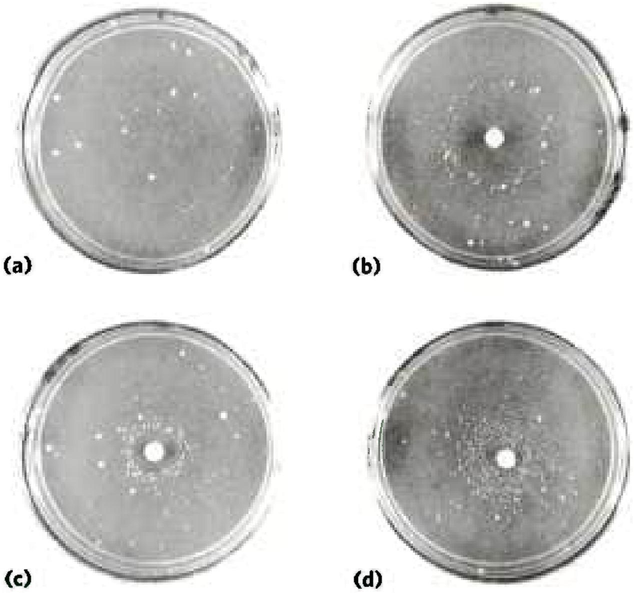


FIGURE 25-19 Ames test for carcinogens, based on their mutagenicity. A strain of *Salmonella typhimurium* having a mutation that inactivates an enzyme of the histidine biosynthetic pathway is plated on a histidine-free medium. Few cells grow. (a) The few small colonies of *S. typhimurium* that do grow on a histidine-free medium carry spontaneous mutations that permit the histidine biosynthetic pathway to operate. Three identical nutrient plates (b), (c), and (d) have been inoculated with an equal number of cells. Each plate then receives a disk of filter paper containing progressively lower concentrations of a mutagen. The mutagen greatly increases the rate of back-mutation and hence the number of colonies. The clear areas around the filter paper indicate where the concentration of mutagen is so high that it is lethal to the cells. As the mutagen diffuses away from the filter paper, it is diluted to sublethal concentrations that promote back-mutation. Mutagens can be compared on the basis of their effect on mutation rate. Because many compounds undergo a variety of chemical transformations after entering cells, compounds are sometimes tested for mutagenicity after first incubating them with a liver extract. Some substances have been found to be mutagenic only after this treatment. [Bruce N. Ames, University of California, Berkeley, Department of Biochemistry and Molecular Biology.]

TABLE 25-5 Types of DNA Repair Systems in *E. coli*

Enzymes/proteins	Type of damage
Mismatch repair	
Dam methylase	Mismatches
MutH, MutL, MutS proteins	
DNA helicase II	
SSB	
DNA polymerase III	
Exonuclease I	
Exonuclease VII	
RecJ nuclease	
Exonuclease X	
DNA ligase	
Base-excision repair	
DNA glycosylases	Abnormal bases (uracil, hypoxanthine, xanthine); alkylated bases; in some other organisms, pyrimidine dimers
AP endonucleases	
DNA polymerase I	
DNA ligase	
Nucleotide-excision repair	
ABC excinuclease	DNA lesions that cause large structural change (e.g., pyrimidine dimers)
DNA polymerase I	
DNA ligase	
Direct repair	
DNA photolyases	Pyrimidine dimers
O^6-Methylguanine-DNA methyltransferase	O^6-Methylguanine
AlkB protein	1-Methylguanine, 3-methylcytosine

All Cells Have Multiple DNA Repair Systems

The number and diversity of repair systems reflect both the importance of DNA repair to cell survival and the diverse sources of DNA damage (**Table 25-5**). Some common types of lesions, such as pyrimidine dimers (see Fig. 8-30), can be repaired by several distinct systems. Nearly 200 genes in the human genome encode proteins dedicated to DNA repair. In many cases, the loss of function of one of these proteins results in genomic instability and an increased occurrence of oncogenesis (**Box 25-1**).

P2 Many DNA repair processes also seem to be extraordinarily inefficient energetically—an exception to the pattern observed in the vast majority of metabolic pathways, where every ATP is generally accounted for and used optimally. When the integrity of the genetic information is at stake, the amount of chemical energy invested in a repair process seems almost irrelevant.

Accurate DNA repair is possible largely because the DNA molecule consists of two complementary strands. Damaged DNA in one strand can be removed and replaced, without introducing mutations, by using the undamaged complementary strand as a template. We consider here the principal types of repair systems, beginning with those that repair the rare nucleotide mismatches that are left behind by replication.

Mismatch Repair Correction of the rare mismatches left after replication in *E. coli* improves the overall fidelity of replication by an additional factor of 10^2 to 10^3. The mismatches are nearly always corrected to reflect the information in the old (template) strand, which the repair system can distinguish from the newly synthesized strand by the presence of methyl group tags on the template DNA. The mismatch repair system of *E. coli* includes at least 10 protein components (Table 25-5) that function either in strand discrimination or in the repair process itself. The functions of many of these were first worked out by Paul Modrich and colleagues in the 1980s.

The strand discrimination mechanism has not been determined for most bacteria or eukaryotes, but it is well understood for *E. coli* and some closely related bacterial species. In these bacteria, strand discrimination is based on the action of Dam methylase, which, as you will recall, methylates DNA at the N^6 position of all adenines within (5′)GATC sequences. Immediately after passage of the replication fork, there is a short period (a few seconds or minutes) during which the template strand is methylated

BOX 25-1 MEDICINE

DNA Repair and Cancer

Human cancers develop when genes that regulate normal cell division (oncogenes and tumor suppressor genes; see Chapter 12) fail to function, are activated at the wrong time, or are altered. As a consequence, cells may grow out of control and form a tumor. The genes controlling cell division can be damaged by spontaneous mutation or overridden by the invasion of a tumor virus (Chapter 26). Not surprisingly, alterations in DNA repair genes that result in a higher rate of mutation can greatly increase an individual's susceptibility to cancer. Defects in the genes encoding the proteins involved in nucleotide-excision repair, mismatch repair, recombinational repair, and error-prone translesion DNA synthesis have all been linked to human cancers. Clearly, DNA repair can be a matter of life and death.

Nucleotide-excision repair requires a larger number of proteins in humans than in bacteria, although the overall pathways are very similar. Genetic defects that inactivate nucleotide-excision repair have been associated with several genetic diseases, the best-studied of which is xeroderma pigmentosum (XP). Because nucleotide-excision repair is the sole repair pathway for pyrimidine dimers in humans, people with XP are extremely sensitive to light and readily develop sunlight-induced skin cancers. Most people with XP also have neurological abnormalities, presumably because of their inability to repair certain lesions caused by the high rate of oxidative metabolism in neurons. Defects in the genes encoding any of at least seven different protein components of the nucleotide-excision repair system can result in XP, giving rise to seven different genetic groups, denoted XPA to XPG. Note that XPC and XPE are parts of complexes that recognize damaged DNA, whereas XPA, XPB, XPD, XPF, and XPG are all components of a much larger multisubunit complex that represents the human excinuclease depicted in Figure 25-24. These proteins are involved in making the DNA incisions and removing the 29mer segment of DNA.

Most microorganisms have redundant pathways for the repair of cyclobutane pyrimidine dimers — making use of DNA photolyases and sometimes base-excision repair as alternatives to nucleotide-excision repair — but humans and other placental mammals do not. This lack of a backup for nucleotide-excision repair for removing pyrimidine dimers has led to speculation that early mammals were small, furry, nocturnal animals with little need to repair UV damage. However, mammals do have a pathway for the translesion bypass of cyclobutane pyrimidine dimers, which involves DNA polymerase η. This enzyme preferentially inserts two A residues opposite a T–T pyrimidine dimer, minimizing mutations. People with a genetic condition in which DNA polymerase η function is missing exhibit an XP-like illness known as XP-variant, or XP-V. Clinical manifestations of XP-V are similar to those of the classic XP diseases, although mutation levels are higher in XP-V when cells are exposed to UV light. Apparently, the nucleotide-excision repair system works in concert with DNA polymerase η in normal human cells, repairing and/or bypassing pyrimidine dimers as needed to keep cell growth and DNA replication going. Exposure to UV light introduces a heavy load of pyrimidine dimers, and some must be bypassed by translesion synthesis to keep replication on track. When one system is missing, it is partly compensated for by the other. A loss of DNA polymerase η activity leads to stalled replication forks and bypass of UV lesions by different, more mutagenic, translesion synthesis (TLS) polymerases. As when other DNA repair systems are absent, the resulting increase in mutations often leads to cancer.

One of the most common inherited cancer-susceptibility syndromes is hereditary nonpolyposis colon cancer (HNPCC). This syndrome has been traced to defects in mismatch repair. Human and other eukaryotic cells have several proteins analogous to the bacterial MutL and MutS proteins (see Fig. 25-21). Defects in at least five different mismatch repair genes can give rise to HNPCC. The most prevalent are defects in the *hMLH1* (*h*uman *M*ut*L* *h*omolog *1*) and *hMSH2* (*h*uman *M*ut*S* *h*omolog *2*) genes. In individuals with HNPCC, cancer generally develops at an early age, with colon cancers being most common.

Most human breast and ovarian cancer occurs in women with no known predisposition. However, about 10% of cases are associated with inherited defects in two genes, *BRCA1* and *BRCA2*. Human BRCA1 and BRCA2 are large proteins (1,834 and 3,418 amino acid residues, respectively) that interact with a wide range of other proteins involved in transcription, chromosome maintenance, DNA repair, and control of the cell cycle. BRCA2 has been implicated in the recombinational DNA repair of double-strand breaks. One of the key roles of BRCA2 is to load the human RecA homolog, called Rad51, onto DNA at the sites of double-strand breaks. BRCA1 has as yet imperfectly defined roles in the repair of double-strand breaks, transcription, and some other processes of DNA metabolism. Women with defects in either the *BRCA1* or *BRCA2* gene have a high (~70%) chance of developing breast cancer.

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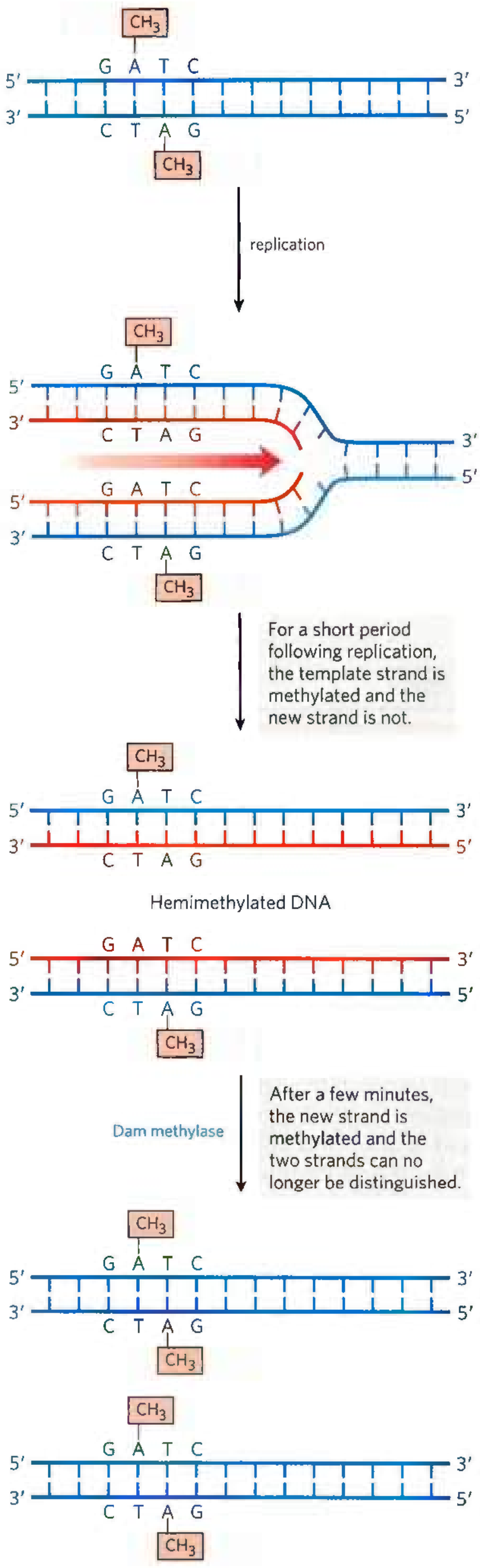
but the newly synthesized strand is not (**Fig. 25-20**). The transient unmethylated state of GATC sequences in the newly synthesized strand permits the new strand to be distinguished from the template strand. Replication mismatches in the vicinity of a hemimethylated GATC sequence are then repaired according to the information in the methylated parent (template) strand. If both strands are methylated at a GATC sequence, few mismatches are repaired; if neither strand is methylated, repair occurs but does not favor either strand. The methyl-directed mismatch repair system of *E. coli* efficiently repairs mismatches up to 1,000 bp from a hemimethylated GATC sequence.

How is the mismatch correction process directed by relatively distant GATC sequences? **Figure 25-21** illustrates one mechanism. MutS scans the DNA and forms a clamplike complex upon encountering a lesion. The complex binds to all mismatched base pairs (except C–C). MutL protein forms a complex with MutS protein, and the MutSL complex slides along the DNA to find a hemimethylated GATC sequence. MutH binds to MutL, and the MutSLH complex moves in either direction at random along the DNA. MutH has a site-specific endonuclease activity that is inactive until the complex encounters a hemimethylated GATC sequence. At this site, MutH catalyzes cleavage of the unmethylated strand on the 5′ side of the G in GATC, which marks the strand for repair. Further steps in the pathway depend on where the mismatch is located relative to this cleavage site (**Fig. 25-22**).

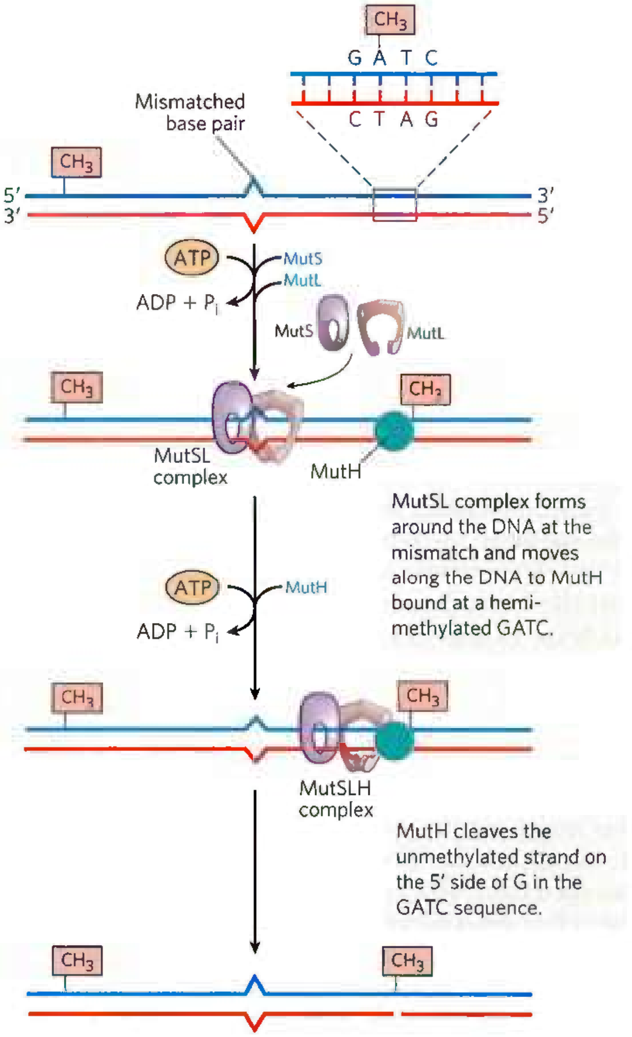
When the mismatch is on the 5′ side of the cleavage site (Fig. 25-22, right side), the unmethylated strand is unwound and degraded in the 3′→5′ direction from the


CH_3
G A T C
5′
3′
3′
5′
C T A G
CH_3
replication
CH_3
G A T C
5′
3′
C T A G
3′
5′
G A T C
5′
3′
C T A G
CH_3
CH_3
G A T C
5′
3′
3′
5′
C T A G
Hemimethylated DNA
G A T C
5′
3′
3′
5′
C T A G
CH_3
Dam methylase
CH_3
G A T C
5′
3′
3′
5′
C T A G
CH_3
CH_3
G A T C
5′
3′
3′
5′
C T A G
CH_3

For a short period following replication, the template strand is methylated and the new strand is not.

After a few minutes, the new strand is methylated and the two strands can no longer be distinguished.

FIGURE 25-20 Methylation and mismatch repair. Methylation of DNA strands can serve to distinguish parent (template) strands from newly synthesized strands in *E. coli* DNA, a function that is critical to mismatch repair. The methylation occurs at the N^6 of adenines in (5′)GATC sequences. This sequence is a palindrome, present in opposite orientations on the two strands.


CH_3
G A T C
C T A G
Mismatched base pair
CH_3
5′
3′
3′
5′
ATP
ADP + P_i
MutS
MutL
MutS
MutL
CH_3
CH_3
MutSL complex
MutH
ATP
ADP + P_i
MutH
CH_3
CH_3
MutSLH complex
CH_3
CH_3

MutSL complex forms around the DNA at the mismatch and moves along the DNA to MutH bound at a hemimethylated GATC.

MutH cleaves the unmethylated strand on the 5′ side of G in the GATC sequence.

FIGURE 25-21 A model for the early steps of methyl-directed mismatch repair. Recognition of the sequence (5′)GATC and of the mismatch are specialized functions of the MutH and MutS proteins, respectively.

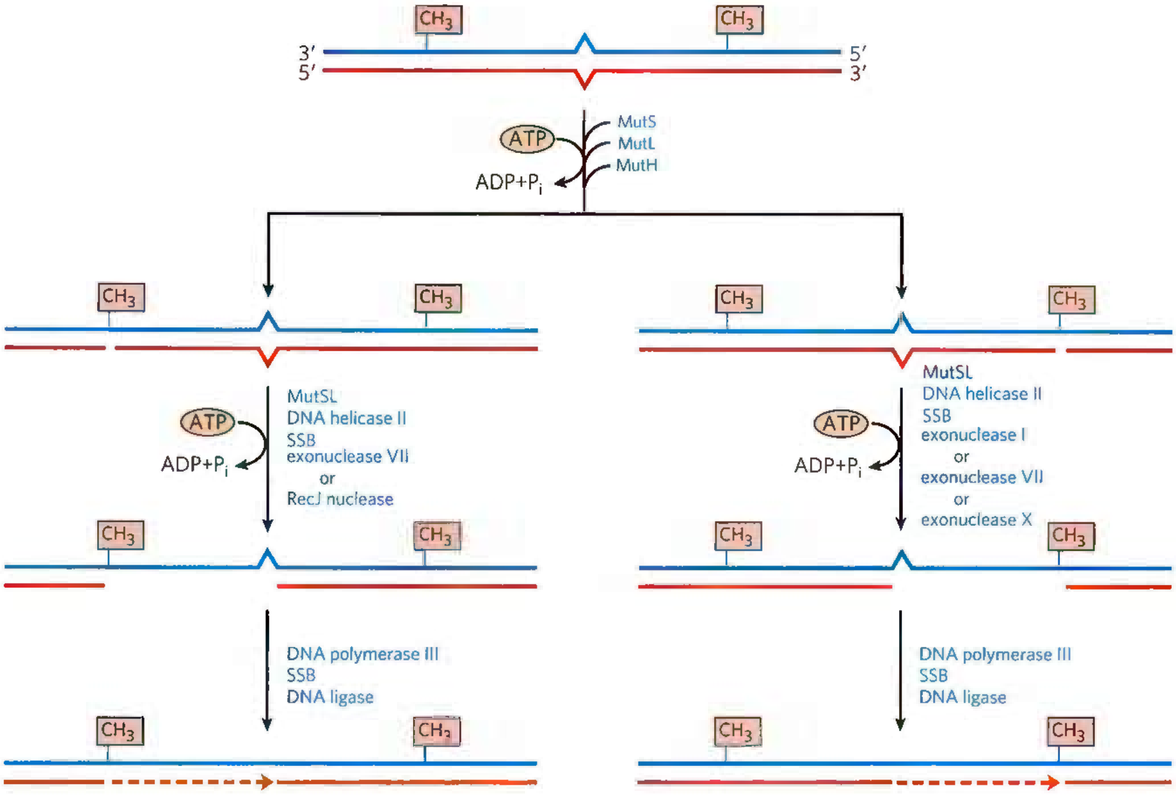


FIGURE 25-22 Completion of methyl-directed mismatch repair. The combined action of DNA helicase II, SSB, and one of four different exonucleases removes a segment of the new strand between the MutH cleavage site and a point just beyond the mismatch. The particular exonuclease depends on the location of the cleavage site relative to the mismatch, as shown by the alternative pathways here. The resulting gap is filled in (dashed line) by DNA polymerase III, and the nick is sealed by DNA ligase (not shown).

cleavage site through the mismatch, and this segment is replaced with new DNA. This process requires the combined action of DNA helicase II (also called UvrD helicase), SSB, exonuclease I or exonuclease X (both of which degrade strands of DNA in the 3′→5′ direction) or exonuclease VII (which degrades single-stranded DNA in either direction), DNA polymerase III, and DNA ligase. The pathway for repair of mismatches on the 3′ side of the cleavage site is similar (Fig. 25-22, left), except that the exonuclease is either exonuclease VII or RecJ nuclease (which degrades single-stranded DNA in the 5′→3′ direction).

P2 Mismatch repair is particularly costly for *E. coli* in terms of energy expended. The mismatch may occur 1,000 or more base pairs from the GATC sequence. The degradation and replacement of a strand segment of this length require an enormous investment in activated deoxynucleotide precursors to repair a *single* mismatched base. This again underscores the importance to the cell of genomic integrity.

Eukaryotic cells also have mismatch repair systems, with several proteins structurally and functionally analogous to the bacterial MutS and MutL (but not MutH) proteins. Alterations in human genes encoding proteins of this type produce some of the most common inherited cancer-susceptibility syndromes (see Box 25-1), further demonstrating the value to the organism of DNA repair systems. The main MutS homologs in most eukaryotes, from yeast to humans, are MSH2 (MutS homolog), MSH3, and MSH6. Heterodimers of MSH2 and MSH6 generally bind to single base-pair mismatches, and they bind less well to slightly longer mispaired loops. In many organisms, the longer mismatches (2 to 6 bp) may be bound instead by a heterodimer of MSH2 and MSH3, or are bound by both types of heterodimers in tandem. Homologs of MutL, predominantly a heterodimer of MLH1 (MutL homolog) and PMS1 (post-meiotic segregation), bind to and stabilize the MSH complexes. Many details of the subsequent events in eukaryotic mismatch repair remain to be worked out. In particular, we do not know how newly synthesized DNA strands are identified, although research reveals that this process does not involve GATC sequences.

Base-Excision Repair Every cell has a class of enzymes called **DNA glycosylases** that recognize particularly common DNA lesions (such as the products of cytosine and adenine deamination; see Fig. 8-29a) and remove the affected base by cleaving the *N*-glycosyl bond. The repair pathway is called **base-excision repair**, as the first step involves only the removal of the base rather than an entire nucleotide. The cleavage creates an apurinic or apyrimidinic site in the DNA, commonly referred to as an **AP site** or **abasic site**. Each DNA glycosylase is generally specific for one type of lesion.

Uracil DNA glycosylases, for example, found in most cells, specifically remove from DNA the uracil that results from spontaneous deamination of cytosine. Mutant cells that lack this enzyme have a high rate of G≡C to A=T mutations. This glycosylase does not remove uracil residues from RNA or thymine residues from DNA. The capacity to distinguish thymine from uracil, the product of cytosine deamination—necessary for the selective repair of the latter—may be one reason why DNA evolved to contain thymine instead of uracil (p. 280).

Most bacteria have just one type of uracil DNA glycosylase, whereas humans have at least four types, with different specificities—an indicator of the importance of removing uracil from DNA. The most abundant human uracil glycosylase, UNG, is associated with the replisome, where it eliminates the occasional U residue inserted in place of a T during replication. The deamination of C residues is 100-fold faster in single-stranded DNA than in double-stranded DNA, and humans have an enzyme, hSMUG1, that removes any U residues occurring in single-stranded DNA during replication or transcription. Two other human DNA glycosylases, TDG and MBD4, remove either U or T residues paired with G, which are generated by deamination of cytosine or 5-methylcytosine, respectively.

Other DNA glycosylases recognize and remove a variety of damaged bases, including formamidopyrimidine and 8-hydroxyguanine (both arising from purine oxidation), hypoxanthine (from adenine deamination), and alkylated bases such as 3-methyladenine and 7-methylguanine. Glycosylases that recognize other lesions, including pyrimidine dimers, have also been identified in some classes of organisms. Remember that AP sites also arise from slow, spontaneous hydrolysis of the *N*-glycosyl bonds in DNA (see Fig. 8-29b).

Once an AP site has been formed by a DNA glycosylase, another type of enzyme must repair it. The repair is *not* made by simply inserting a new base and re-forming the *N*-glycosyl bond. Instead, the deoxyribose 5′-phosphate left behind is removed and replaced with a new nucleotide. This process begins with one of the **AP endonucleases**, enzymes that cut the DNA strand containing the AP site. The position of the incision relative to the AP site (5′ or 3′ to the site) depends on the type of AP endonuclease. A segment of DNA including the AP site is then removed, DNA polymerase I replaces the DNA, and DNA ligase seals the remaining nick (**Fig. 25-23**). In eukaryotes, nucleotide replacement is carried out by specialized polymerases, as described below.

Nucleotide-Excision Repair DNA lesions that cause large distortions in the helical structure of DNA generally are repaired by the nucleotide-excision system, a repair pathway critical to the survival of all free-living organisms. In nucleotide-excision repair (**Fig. 25-24**),

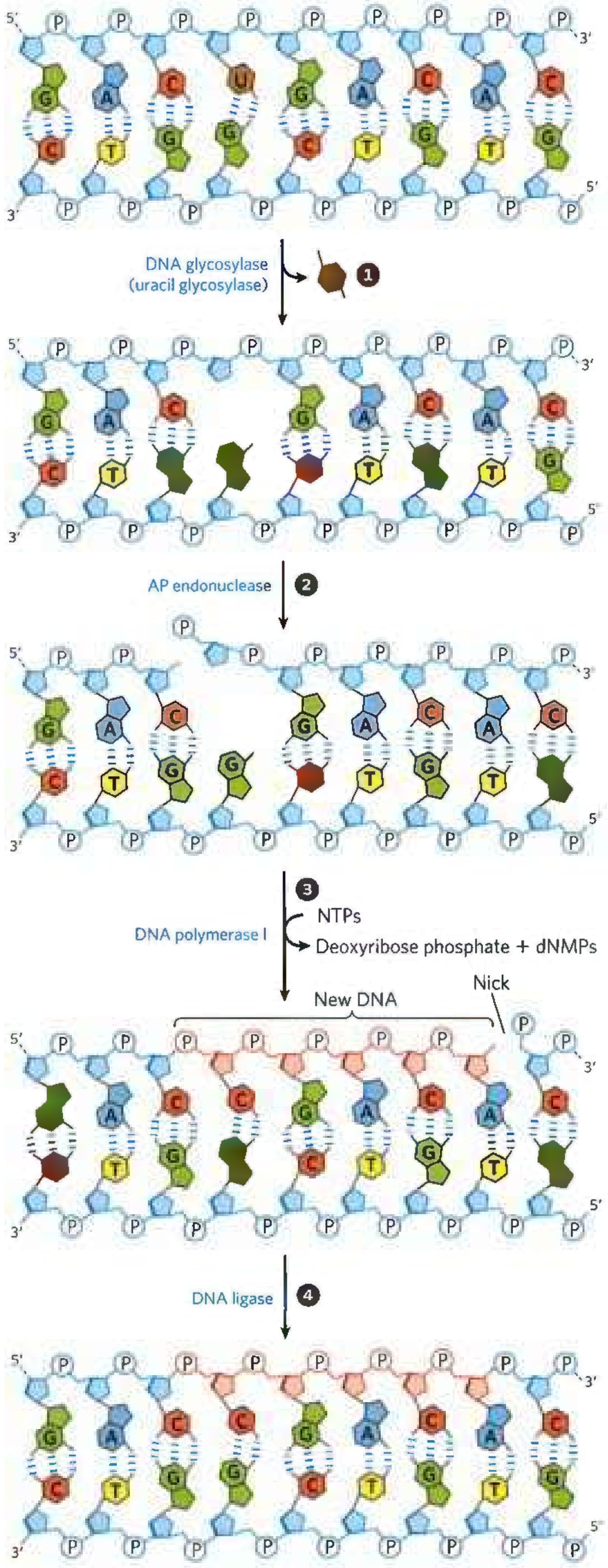


FIGURE 25-23 DNA repair by the base-excision repair pathway. ① A DNA glycosylase recognizes a damaged base (in this case, a uracil) and cleaves between the base and deoxyribose in the backbone. ② An AP endonuclease cleaves the phosphodiester backbone near the AP site. ③ DNA polymerase I initiates repair synthesis from the free 3′ hydroxyl at the nick, removing (with its 5′→3′ exonuclease activity) and replacing a portion of the damaged strand. ④ The nick remaining after DNA polymerase I has dissociated is sealed by DNA ligase.

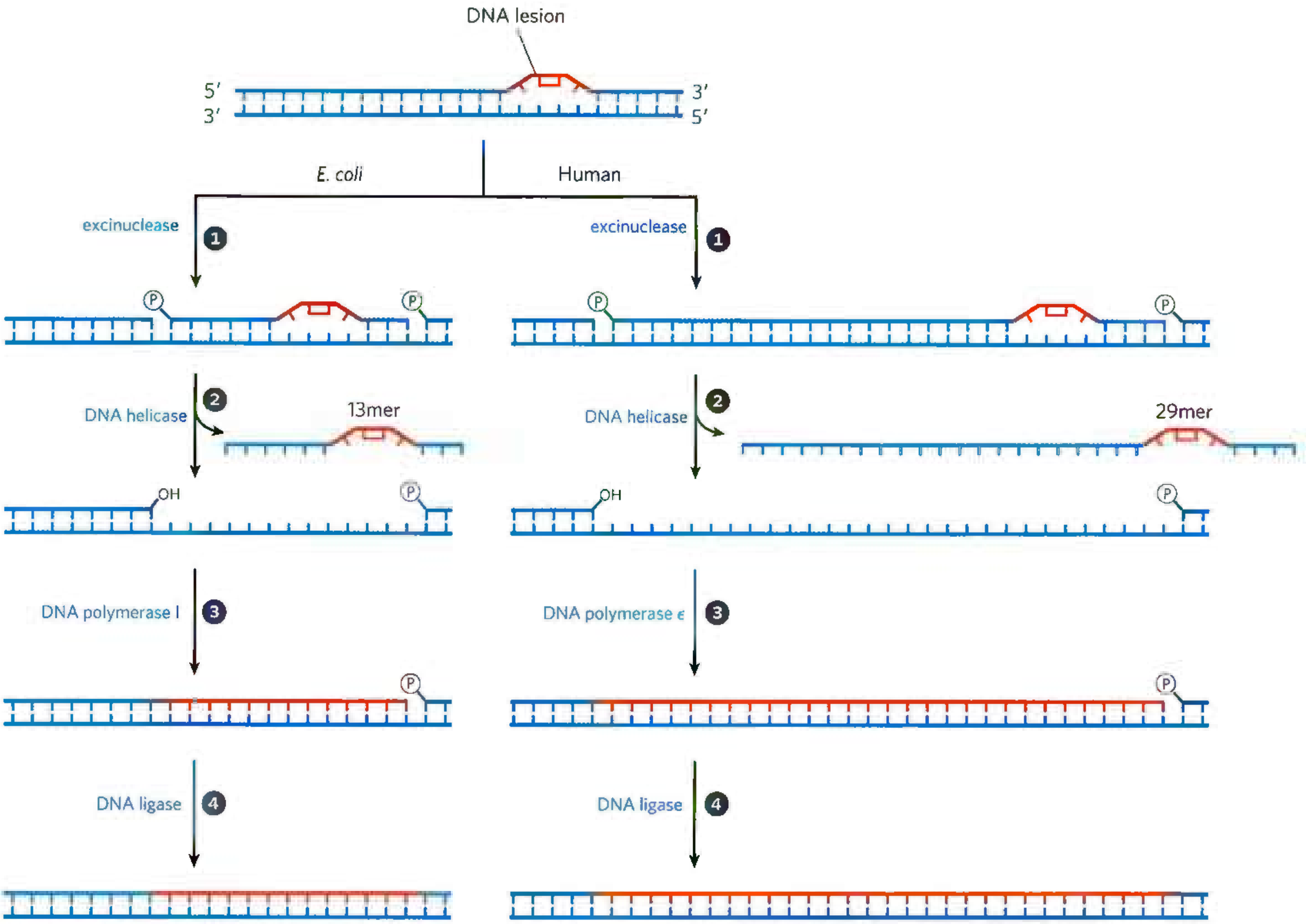


FIGURE 25-24 Nucleotide-excision repair in *E. coli* and humans. The general pathway of nucleotide-excision repair is similar in all organisms. ① An excinuclease binds to DNA at the site of a bulky lesion and cleaves the damaged DNA strand on either side of the lesion. ② The DNA segment — of 13 nucleotides (13mer) or 29 nucleotides (29mer) — is removed with the aid of a helicase. ③ The gap is filled in by DNA polymerase, and ④ the remaining nick is sealed with DNA ligase. [Information from a figure provided by Aziz Sancar.]

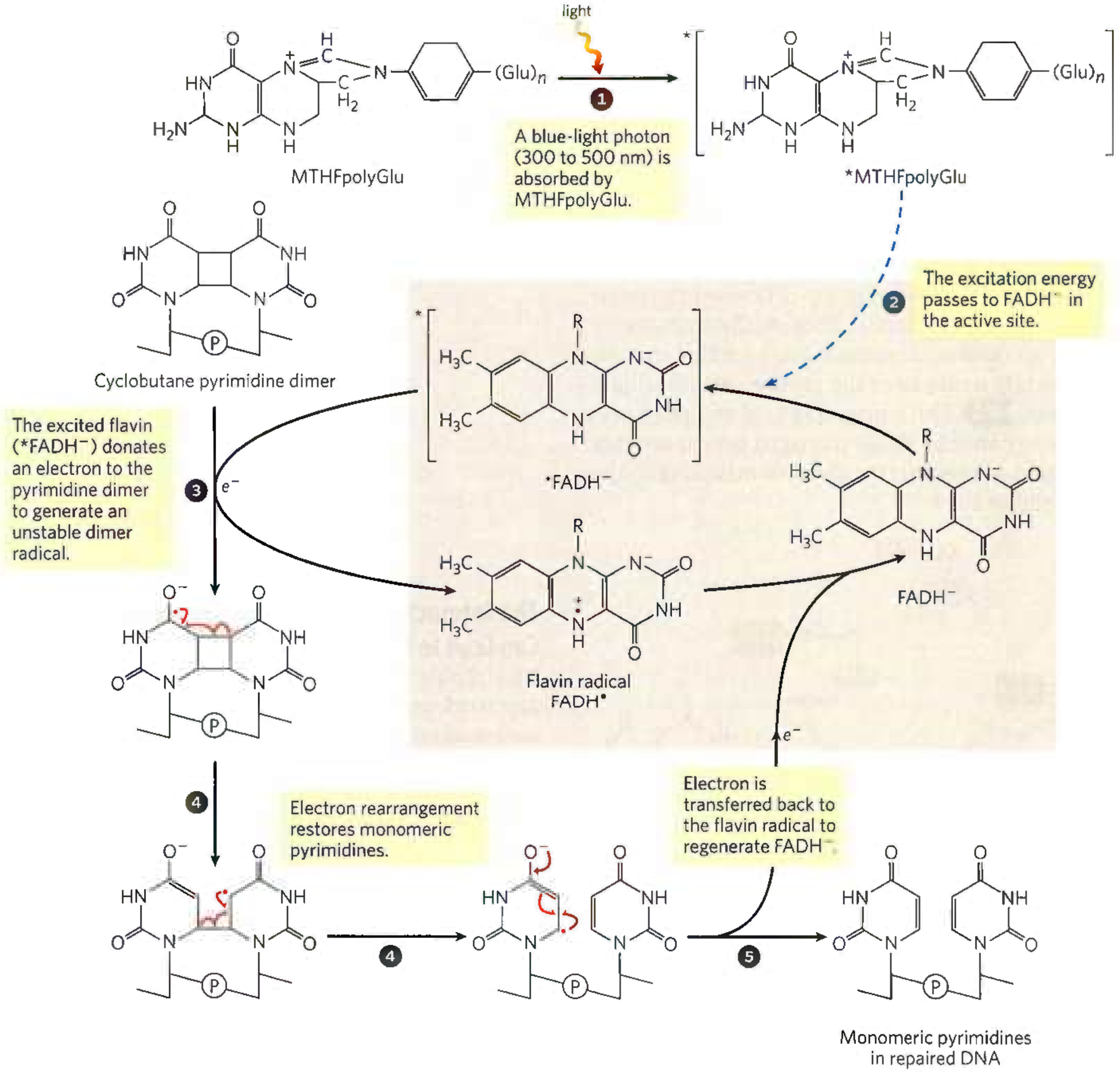
a multisubunit enzyme (excinuclease) hydrolyzes two phosphodiester bonds, one on either side of the distortion caused by the lesion. In *E. coli* and other bacteria, the enzyme system hydrolyzes the fifth phosphodiester bond on the 3′ side and the eighth phosphodiester bond on the 5′ side to generate a fragment of 12 to 13 nucleotides (depending on whether the lesion involves one or two bases). In humans and other eukaryotes, the enzyme system hydrolyzes the sixth phosphodiester bond on the 3′ side and the twenty-second phosphodiester bond on the 5′ side, producing a fragment of 27 to 29 nucleotides. Following the dual incision, the excised oligonucleotides are released from the duplex and the resulting gap is filled — by DNA polymerase I in *E. coli* and DNA polymerase ε in humans. DNA ligase seals the nick.

In *E. coli*, the key enzymatic complex is the ABC excinuclease, which has three protein components, UvrA (M_r 104,000), UvrB (M_r 78,000), and UvrC (M_r 68,000). The term "excinuclease" is used to describe the unique capacity of this enzyme complex to catalyze two specific endonucleolytic cleavages, distinguishing this activity from that of standard endonucleases. A dimeric UvrA protein (an ATPase) scans the DNA and binds to the site of a lesion. A UvrB protein can bind to UvrA either before or after an encounter with the lesion. At the lesion, the UvrA dimer dissociates, leaving a tight UvrB-DNA complex. UvrC protein then binds to UvrB, and UvrB makes an incision at the fifth phosphodiester bond on the 3′ side of the lesion. This is followed by a UvrC-mediated incision at the eighth phosphodiester bond on the 5′ side. The resulting fragment, consisting of 12 to 13 nucleotides, is removed by UvrD helicase. The short gap thus created is filled in by DNA polymerase I and DNA ligase. This pathway (Fig. 25-24, left) is a primary repair route for many types of lesions, including cyclobutane pyrimidine dimers, 6-4 photoproducts (see Fig. 8-30), and several other types of base adducts, including benzo[*a*]pyrene-guanine, which is formed in DNA by exposure to cigarette smoke. The nucleolytic activity of the ABC excinuclease is novel in the sense that two cuts are made in the DNA.

The mechanism of eukaryotic excinucleases is quite similar to that of the bacterial enzyme, although at least 16 polypeptides with no similarity to the *E. coli* excinuclease subunits are required for the dual incision. Some of the nucleotide-excision repair and base-excision repair in eukaryotes is closely tied to transcription (see Chapter 26). Genetic deficiencies in nucleotide-excision repair in humans give rise to a variety of serious diseases (see Box 25-1).

Direct Repair Several types of damage are repaired without removing a base or nucleotide. The best-characterized example is direct photoreactivation of cyclobutane pyrimidine dimers, a reaction promoted by **DNA photolyases**. Pyrimidine dimers result from a UV-induced reaction. Through a mechanism worked out by Aziz Sancar and colleagues, photolyases use energy derived from absorbed light to reverse the damage (**Fig. 25-25**). Photolyases generally contain two cofactors that serve as light-absorbing agents, or chromophores: in all organisms, one is $FADH_2$; in *E. coli* and yeast, the other is a folate. The reaction mechanism entails the generation of free radicals. DNA photolyases are not found in humans and other placental mammals.

Additional examples are seen in the repair of nucleotides with alkylation damage. The modified nucleotide O^6-methylguanine forms in the presence of alkylating agents and is a common and highly mutagenic lesion. It tends to pair with thymine rather than cytosine during replication, and therefore causes G≡C to A=T mutations (**Fig. 25-26**).



① A blue-light photon (300 to 500 nm) is absorbed by MTHFpolyGlu.

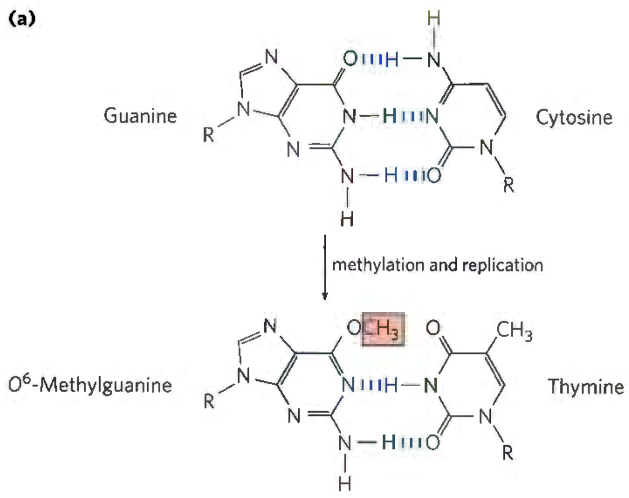
② The excitation energy passes to FADH⁻ in the active site.

③ The excited flavin (*FADH⁻) donates an electron to the pyrimidine dimer to generate an unstable dimer radical.

④ Electron rearrangement restores monomeric pyrimidines.

⑤ Electron is transferred back to the flavin radical to regenerate FADH⁻.

MECHANISM FIGURE 25-25 Repair of pyrimidine dimers with photolyase. Energy derived from absorbed light is used to reverse the photoreaction that caused the lesion. The two chromophores in *E. coli* photolyase (M_r 54,000), N^5,N^{10}-methenyltetrahydrofolylpolyglutamate (MTHFpolyGlu) and FADH⁻, perform complementary functions. MTHFpolyGlu functions as a photoantenna to absorb blue-light photons. The excitation energy passes to FADH⁻, and the excited flavin (*FADH⁻) donates an electron to the pyrimidine dimer, leading to the rearrangement as shown.


(a)
Guanine
Cytosine
methylation and replication
O^6-Methylguanine
Thymine
CH_3
R

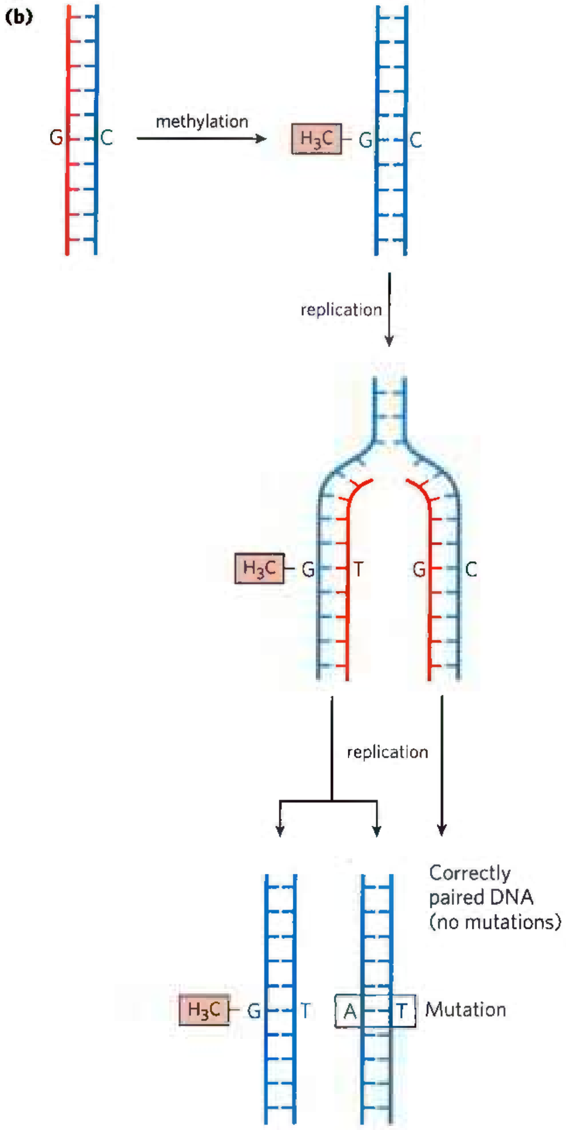
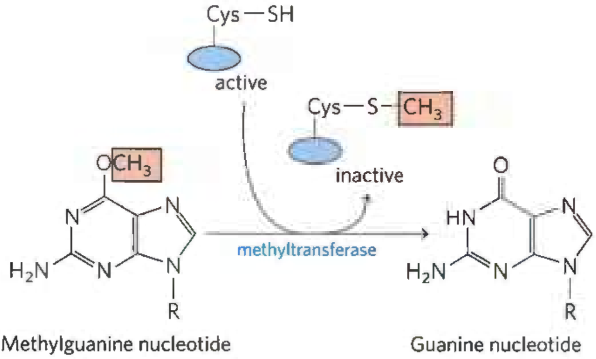

(b)
G
C
methylation
H_3C
replication
T
replication
Correctly paired DNA (no mutations)
A
Mutation

FIGURE 25-26 Example of how DNA damage results in mutations. (a) The methylation product O^6-methylguanine pairs with thymine rather than cytosine residues. (b) If not repaired, this leads to a G≡C to A═T mutation after replication.

Direct repair of O^6-methylguanine is carried out by O^6-methylguanine-DNA methyltransferase, a protein that catalyzes transfer of the methyl group of O^6-methylguanine to one of its own Cys residues. This methyltransferase is not strictly an enzyme, because a single methyl transfer event permanently methylates the protein, inactivating it in this pathway. **P2** The consumption of an entire protein molecule to correct a single damaged base is another vivid illustration of the priority given to maintaining the integrity of cellular DNA.


Cys—SH
active
Cys—S—CH_3
inactive
methyltransferase
O^6-Methylguanine nucleotide
Guanine nucleotide
H_2N
R

A very different but equally direct mechanism is used to repair 1-methyladenine and 3-methylcytosine. The amino groups of A and C residues are sometimes methylated when the DNA is single-stranded, and the methylation directly affects proper base pairing. In *E. coli*, oxidative demethylation of these alkylated nucleotides is mediated by the AlkB protein, a member of the α-ketoglutarate-Fe^{2+}–dependent dioxygenase superfamily (**Fig. 25-27**). (See Box 4-2 for a description of proline hydroxylation, catalyzed by another member of this enzyme family.)

The Interaction of Replication Forks with DNA Damage Can Lead to Error-Prone Translesion DNA Synthesis

The repair pathways considered to this point generally work only for lesions in double-stranded DNA, the undamaged strand providing the correct genetic information to restore the damaged strand to its original state. However, in certain types of lesions, such as double-strand breaks, double-strand cross-links, or lesions in a single-stranded DNA, the complementary strand is itself damaged or is absent. **P3** Double-strand breaks and lesions in single-stranded DNA most often arise when a replication fork encounters an unrepaired DNA lesion (**Fig. 25-28**). Such lesions and DNA cross-links can also result from ionizing radiation and oxidative reactions.

At a stalled bacterial replication fork, there are two avenues for repair. In the absence of a second strand, the information required for accurate repair must come from a separate, homologous chromosome. The repair system thus involves homologous genetic recombination. This recombinational DNA repair is considered in

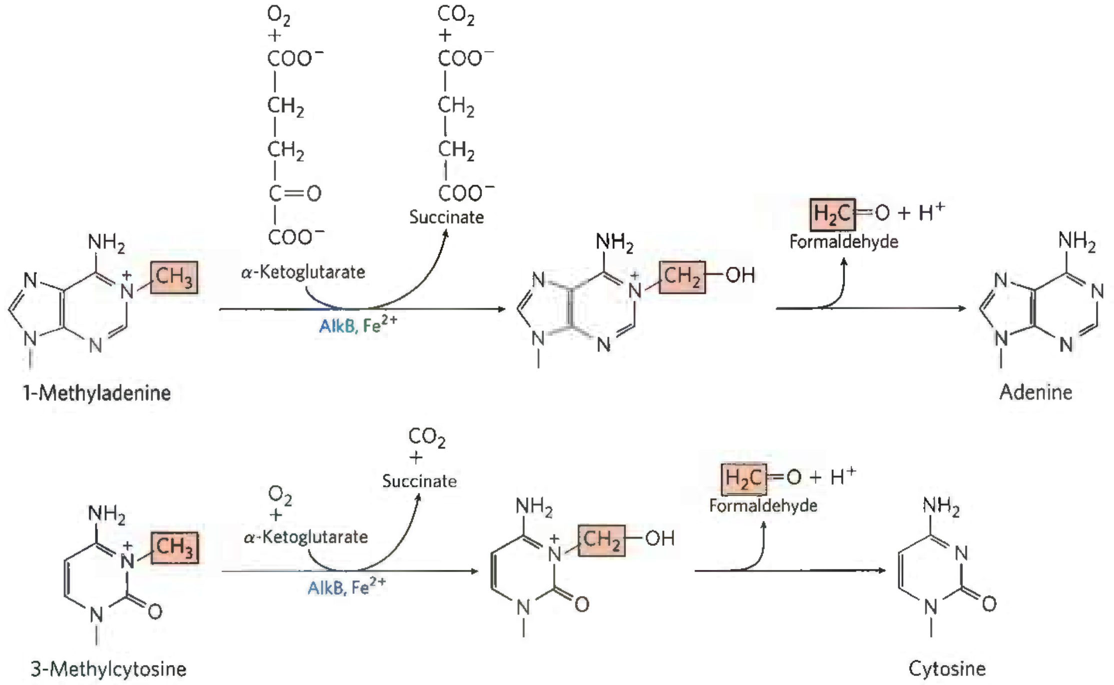

O2 + COO⁻ CH2 CH2 C=O COO⁻
α-Ketoglutarate
CO2 + COO⁻ CH2 CH2 COO⁻
Succinate
AlkB, Fe2+
NH2
CH3
CH2—OH
H2C=O + H+
Formaldehyde
1-Methyladenine
Adenine
O2 + α-Ketoglutarate
CO2 + Succinate
AlkB, Fe2+
3-Methylcytosine
Cytosine

FIGURE 25-27 Direct repair of alkylated bases by AlkB. The AlkB protein is an α-ketoglutarate-Fe^{2+}–dependent hydroxylase (see Box 4-2). It catalyzes the oxidative demethylation of 1-methyladenine and 3-methylcytosine residues.

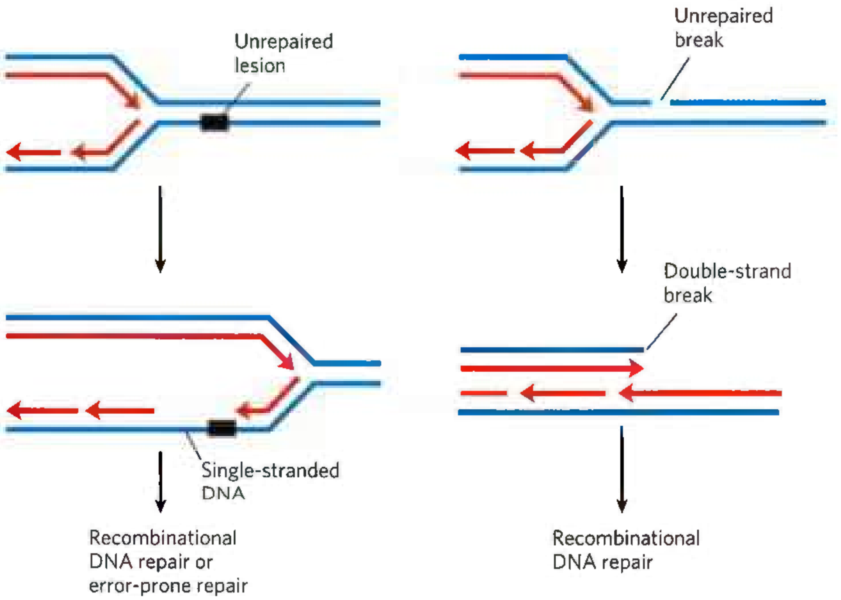

Unrepaired lesion
Unrepaired break
Double-strand break
Single-stranded DNA
Recombinational DNA repair or error-prone repair
Recombinational DNA repair

FIGURE 25-28 DNA damage and its effect on DNA replication. If the replication fork encounters an unrepaired lesion or strand break, the DNA polymerase sometimes disengages and re-initiates downstream. The lesion remains in an unreplicated, single-stranded gap that is left behind the replication fork (left). In other cases, a replication fork may encounter a lesion that is actively undergoing repair such that a transient break is present in one of the template strands. When the replication fork encounters it, the single-strand break becomes a double-strand break (right). In each case, the damage to one strand cannot be repaired by mechanisms described earlier in this chapter, because the complementary strand required to direct accurate repair is damaged or absent. There are at least two possible avenues for repair: recombinational DNA repair or, when lesions are unusually numerous, error-prone repair. The latter mechanism involves translesion DNA polymerases such as DNA polymerase V, encoded by the *umuC* and *umuD* genes and activated by the RecA protein that can replicate, albeit inaccurately, over many types of lesions. The repair mechanism is "error-prone" because mutations often result.

detail in Section 25.3. Under some conditions, a second repair pathway, **error-prone translesion DNA synthesis** (often abbreviated TLS), becomes available. When this pathway is active, DNA repair is significantly less accurate, and a high mutation rate can result. In bacteria, error-prone translesion DNA synthesis is part of a cellular stress response to extensive DNA damage known, appropriately enough, as the **SOS response**.

Some of the 40 or more SOS proteins, such as the UvrA and UvrB proteins involved in the error-free nucleotide-excision repair already described, are normally present in the cell but are induced to higher levels as part of the SOS response. Additional SOS proteins participate in the pathway for error-prone repair; these include the UmuC and UmuD proteins (*unmu*table; lack of the *umu* gene eliminates error-prone repair). The UmuD protein is cleaved in an SOS-regulated process to a shorter form called UmuD′, which forms a complex with UmuC and a protein called RecA (described in Section 25.3) to create a specialized DNA polymerase, DNA polymerase V (UmuD′$_2$UmuCRecA), which can replicate past many of the DNA lesions that would normally block replication. Proper base pairing is often impossible at the site of such a lesion, so this translesion replication is error-prone.

Given the emphasis on the importance of genomic integrity throughout this chapter, the existence of a system that increases the rate of mutation may seem incongruous. However, we can think of this system as a desperation strategy. The *umuC* and *umuD* genes are fully induced only late in the SOS response, and they are not activated for translesion synthesis initiated by UmuD cleavage unless the levels of DNA damage are particularly high and all replication forks are blocked. The mutations resulting from DNA polymerase V–mediated replication kill some cells and create deleterious mutations in others, but this is the biological price a species pays to overcome an otherwise insurmountable barrier to replication, as it permits at least a few mutant daughter cells to survive. P3 The resultant mutations contribute to evolution.

Yet another DNA polymerase, DNA polymerase IV, is also induced during the SOS response. Replication by DNA polymerase IV, a product of the *dinB* gene, is also highly error-prone. The bacterial DNA polymerases IV and V (Table 25-1) are part of a family of TLS polymerases found in all organisms. These enzymes lack a proofreading exonuclease and have a more open active site than other DNA polymerases, one that accommodates damaged template nucleotides. With these enzymes, the fidelity of base selection during replication may be reduced by a factor of 10^2, lowering overall replication fidelity to one error in ~1,000 nucleotides.

P4 Mammals have many low-fidelity DNA polymerases of the TLS polymerase family. However, the presence of these enzymes does not necessarily translate into an unacceptable mutational burden, because most of the enzymes also have specialized functions in DNA repair. DNA polymerase η (eta), for example, found in all eukaryotes, promotes translesion synthesis primarily across cyclobutane T–T dimers. Few mutations result, because the enzyme preferentially inserts two A residues across from the linked T residues. Several other low-fidelity polymerases, including DNA polymerases β, ι (iota), and λ, have specialized roles in eukaryotic base-excision repair. Each of these enzymes has a 5′-deoxyribose phosphate lyase activity in addition to its polymerase activity. After base removal by a glycosylase and backbone cleavage by an AP endonuclease, these polymerases remove the abasic site (a 5′-deoxyribose phosphate) and fill in the very short gap. The frequency of mutation due to DNA polymerase η activity is minimized by the short lengths (often one nucleotide) of DNA synthesized.

What emerges from research into cellular DNA repair systems is a picture of a DNA metabolism that maintains genomic integrity with multiple and often redundant systems. Most of the major DNA repair systems occur in all organisms. P4 These repair systems are often integrated with the DNA replication systems and are complemented by recombination systems, which we turn to next.

SUMMARY 25.2 ***DNA Repair***

- Mutations are genomic changes that alter the information in DNA. When mutations occur in genes encoding enzymes involved in DNA repair, the loss of function can lead to cancer.
- Cells have many systems for DNA repair. Major repair systems present in all organisms include mismatch repair, base excision repair, nucleotide-excision repair, and direct repair.
- In bacteria, TLS DNA polymerases respond to very heavy DNA damage with error-prone translesion DNA synthesis. In eukaryotes, similar polymerases have specialized roles in DNA repair that minimize the introduction of mutations.

25.3 DNA Recombination

The rearrangement of genetic information within and among DNA molecules encompasses a variety of processes, collectively placed under the heading of genetic recombination. The practical applications of DNA rearrangements in altering the genomes of increasing numbers of organisms are now being explored (Chapter 9).

Barbara McClintock, 1902–1992

[AP Photo.]

Genetic recombination events fall into at least three general classes. **Homologous genetic recombination** (also called general recombination) involves genetic exchanges between any two DNA molecules (or segments of the same molecule) that share an extended region of nearly identical sequence. The actual sequence of bases is irrelevant, as long as it is similar in the two DNAs. In **site-specific recombination**, the exchanges occur only at a *particular* DNA sequence. **DNA transposition** is distinct from both other classes in that it usually involves a short segment of DNA with the remarkable capacity to move from one location in a chromosome to another. These "jumping genes" were first observed in maize in the 1940s by Barbara McClintock. There are also unusual genetic rearrangements for which no mechanism or purpose has yet been proposed. Here we focus on the three general classes.

Homologous genetic recombination is largely a pathway to repair double-strand breaks in DNA. An alternative process for double-strand break repair that does not entail recombination, called **nonhomologous end joining (NHEJ)**, is also described here. Genetic recombination systems have functions as varied as their mechanisms. They include roles in specialized DNA repair systems, specialized activities in DNA replication, regulation of expression of certain genes, facilitation of proper chromosome segregation during eukaryotic cell division, maintenance of genetic diversity, and implementation of programmed genetic rearrangements during embryonic development. In most cases, genetic recombination is closely integrated with other processes in DNA metabolism, and this becomes a theme of our discussion.

Bacterial Homologous Recombination Is a DNA Repair Function

P4 In bacteria, homologous genetic recombination is primarily a DNA repair process, and in this context (as noted in Section 25.2) it is referred to as **recombinational DNA repair**. It is usually directed at the reconstruction of replication forks that have stalled or collapsed at the site of DNA damage. Homologous genetic recombination can also occur during conjugation (mating), when chromosomal DNA is transferred from one bacterial cell (donor) to another (recipient). Recombination during conjugation, although rare in wild bacterial populations, contributes to genetic diversity.

P4 When a replication fork encounters DNA damage, many pathways may resolve the conflict. A common feature of the DNA repair pathways illustrated in Figures 25-21 to 25-24 is that they introduce a transient break into one of the DNA strands. If a replication fork encounters a damaged site under repair near a break in one of the template strands, one arm of the replication fork becomes disconnected by a double-strand break and the fork collapses (**Fig. 25-29**). The end of that break is processed by degrading the 5′-ending strand. The resulting 3′ single-stranded extension is bound by a recombinase

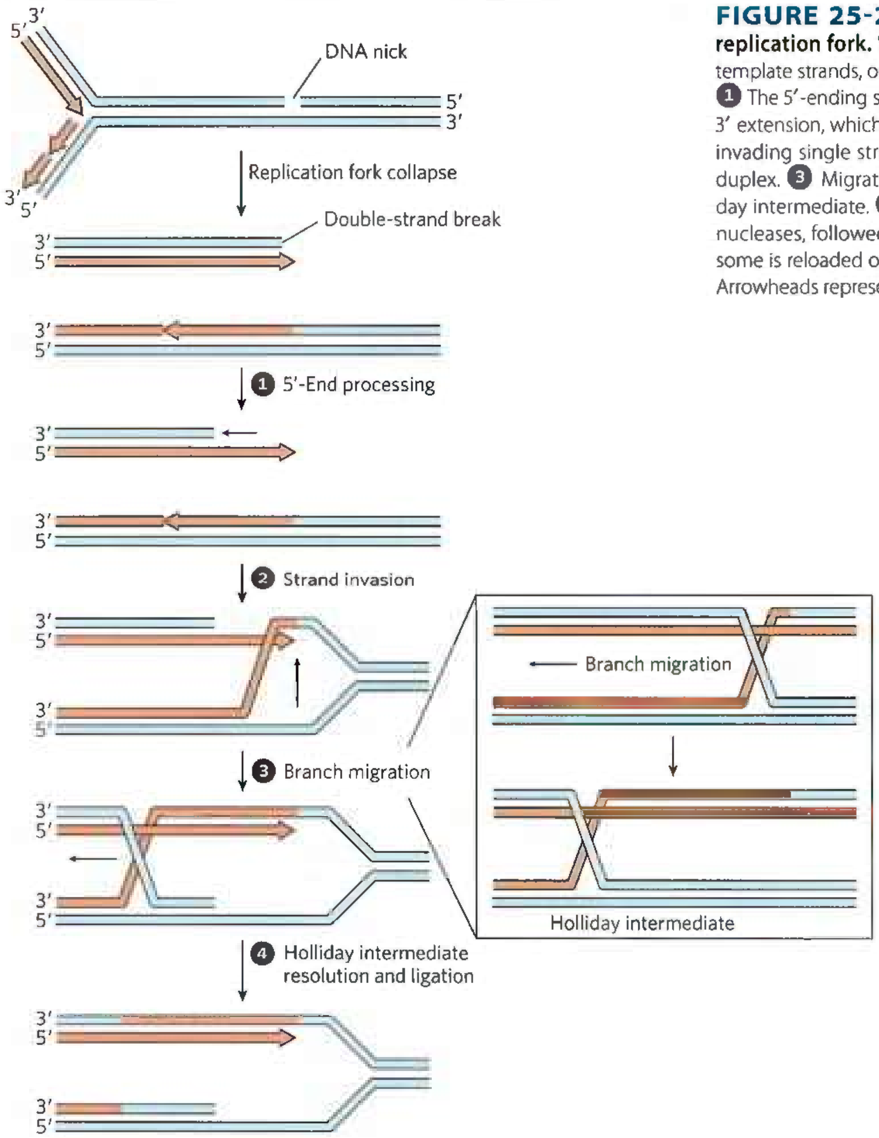
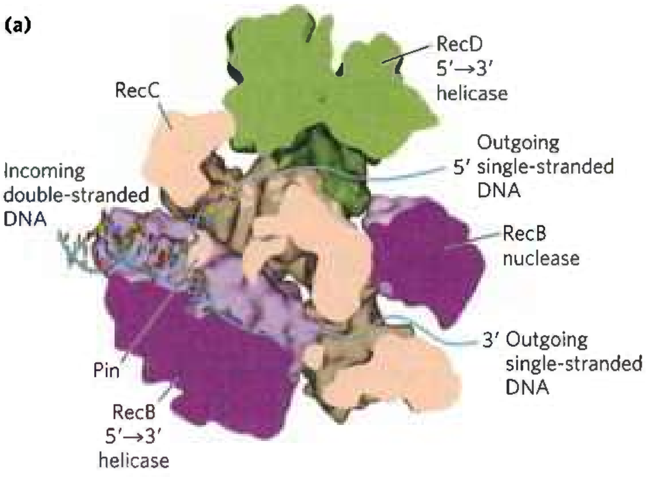
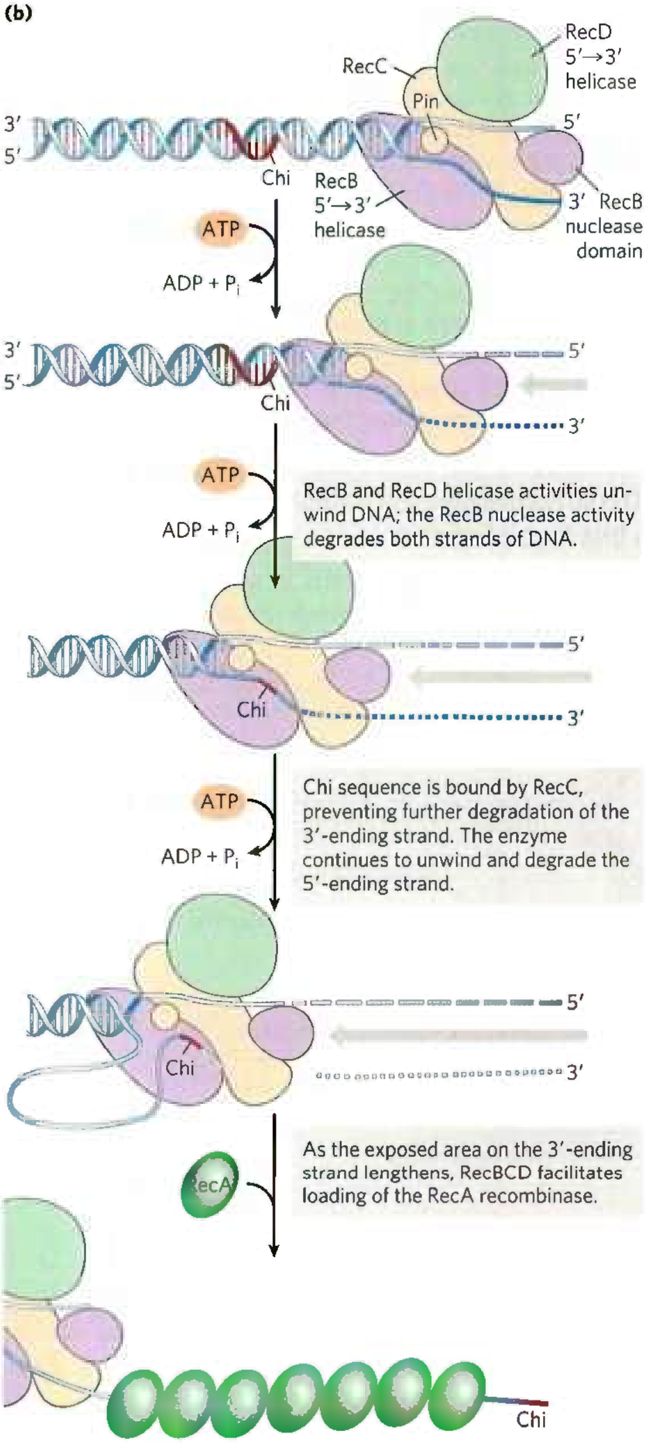


FIGURE 25-29 Recombinational DNA repair at a collapsed replication fork. When a replication fork encounters a break in one of the template strands, one arm of the fork is lost and the replication fork collapses. ① The 5′-ending strand at the break is degraded to create a single-stranded 3′ extension, which is then used in ② a strand invasion process, pairing the invading single strand with its complementary strand within the adjacent duplex. ③ Migration of the branch (shown in the box) can create a Holliday intermediate. ④ Cleavage of the Holliday intermediate by specialized nucleases, followed by ligation, restores a viable replication fork. The replisome is reloaded onto this structure (not shown), and replication continues. Arrowheads represent 3′ ends.

(a)



(b)



RecB and RecD helicase activities unwind DNA; the RecB nuclease activity degrades both strands of DNA.

Chi sequence is bound by RecC, preventing further degradation of the 3′-ending strand. The enzyme continues to unwind and degrade the 5′-ending strand.

As the exposed area on the 3′-ending strand lengthens, RecBCD facilitates loading of the RecA recombinase.

FIGURE 25-30 The RecBCD helicase/nuclease. (a) A cutaway view of the RecBCD enzyme structure as it is bound to DNA. The subunits are shown in different colors; the DNA is entering from the left, and the unwound DNA strands (not part of the solved structure) are shown exiting to the right. A bulbous protein structure called a pin, part of the RecC subunit, facilitates the separation of strands. (b) Activities of the RecBCD enzyme at a DNA end. [(a) Data from PDB ID 1W36, M. R. Singleton et al., *Nature* 432:187, 2004.]

that uses it to promote strand invasion: the 3′ end invades the intact duplex DNA connected to the other arm of the fork and pairs with its complementary sequence. This creates a branched DNA structure (a point where three DNA segments come together). The DNA branch can be moved in a process called **branch migration** to create an X-like crossover structure known as a **Holliday intermediate**, named after researcher Robin Holliday, who first postulated its existence. The Holliday intermediate is cleaved, or "resolved," by a special class of nucleases. The overall process reconstructs the replication fork.

In *E. coli*, the DNA end-processing is promoted by the RecBCD nuclease/helicase. The RecBCD enzyme binds to linear DNA at a free (broken) end and moves inward along the double helix, unwinding and degrading the DNA in a reaction coupled to ATP hydrolysis (**Fig. 25-30**). The RecB and RecD subunits are helicase motors, with RecB moving 3′→5′ along one strand, and RecD moving 5′→3′ along the other strand. The activity of the enzyme is altered when it interacts with a sequence referred to as **chi**, (5′)GCTGGTGG, which binds tightly to a site on the RecC subunit. From that point, degradation of the strand with a 3′ terminus is greatly reduced, but degradation of the 5′-terminal strand is increased. This process creates a single-stranded DNA with a 3′ end, which is used during subsequent steps in recombination. The 1,009 chi sequences scattered throughout the *E. coli* genome enhance the frequency of recombination about 5- to 10-fold within 1,000 bp of each chi site. The enhancement declines as the distance from chi increases. Sequences that enhance recombination frequency have also been identified in several other organisms.

The bacterial recombinase is the RecA protein. RecA is unusual among the proteins of DNA metabolism in that its active form is an ordered, helical filament of up to several thousand subunits that assemble cooperatively on DNA (**Fig. 25-31**). This filament usually forms on single-stranded DNA, such as that produced by the RecBCD enzyme. Its formation is not as straightforward as shown in Figure 25-31, because the single-stranded DNA–binding protein (SSB) is normally present and

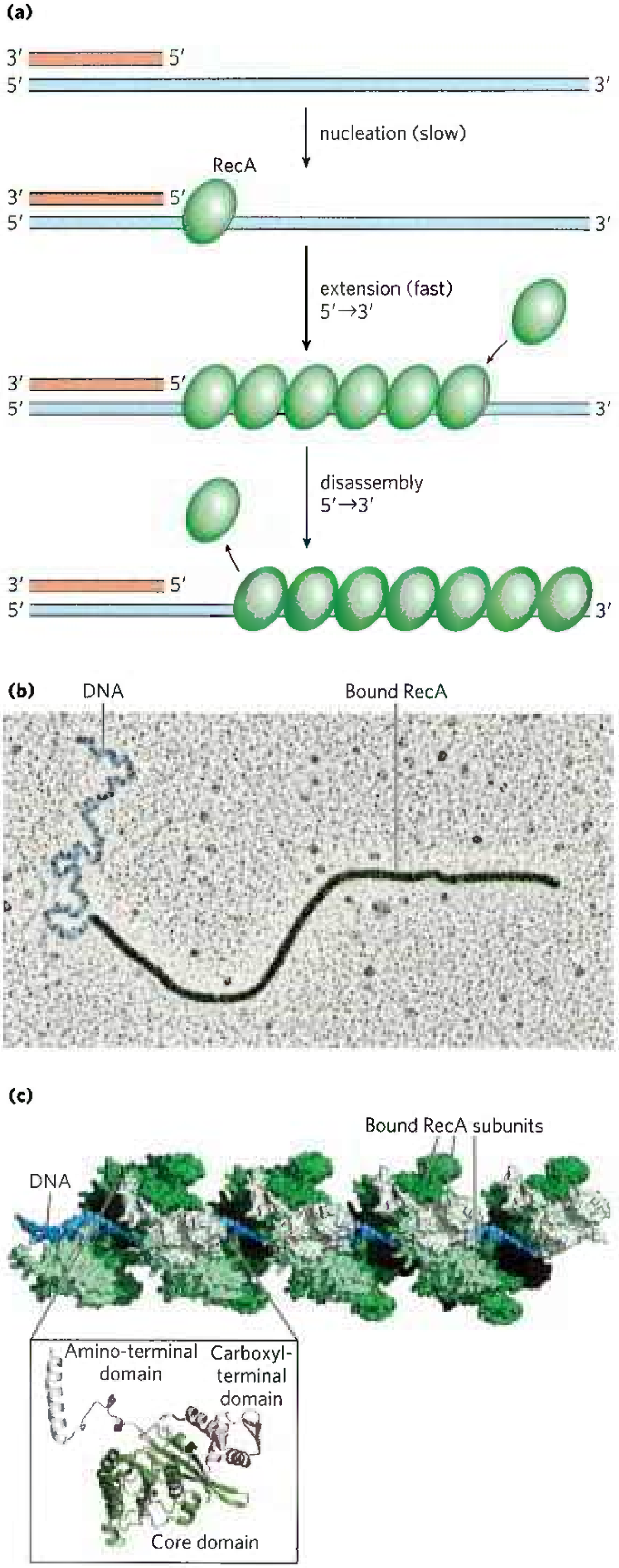


FIGURE 25-31 RecA protein filaments. RecA and other recombinases in this class function as filaments of nucleoprotein. (a) Filament formation proceeds in discrete nucleation and extension steps. Nucleation is the addition of the first few RecA subunits. Extension occurs by adding RecA subunits so that the filament grows in the 5′→3′ direction. When disassembly occurs, subunits are subtracted from the trailing end. (b) Colorized electron micrograph of a RecA filament bound to DNA. (c) Segment of a RecA filament with four helical turns (24 RecA subunits). Notice the bound double-stranded DNA in the center. The core domain of RecA is structurally related to the motor domains of helicases. [(b) By permission of the Estate of Ross Inman. Special thanks to Kim Voss. (c) Data from PDB ID 3CMX, Z. Chen et al., *Nature* 453:489, 2008.]

specifically impedes the binding of the first few subunits to DNA (filament nucleation). The RecBCD enzyme acts directly as a RecA loader, facilitating the nucleation of a RecA filament on single-stranded DNA that is coated with SSB. The filaments assemble and disassemble predominantly in a 5′→3′ direction. Many other bacterial proteins regulate the formation and disassembly of RecA filaments, including an alternative set of RecA loading proteins called RecF, RecO, and RecR. RecA protein promotes the central steps of homologous recombination, including the DNA strand invasion step of Figure 25-29, as well as other strand exchange reactions occurring in vitro. Once a Holliday intermediate has been created via branch migration, it can be cleaved by specialized nucleases such as the bacterial RuvC protein (**Fig. 25-32**), and nicks are sealed by DNA ligase. A viable replication fork structure is thus reconstructed, as outlined in Figure 25-29.

After the recombination steps are completed, the replication fork reassembles in a process called **origin-independent restart of replication**. Different combinations of four proteins (PriA, PriB, PriC, and DnaT) act with DnaC in several pathways to load DnaB helicase onto the reconstructed replication fork. The DnaG primase then synthesizes an RNA primer, and DNA polymerase III reassembles on DnaB to restart DNA synthesis. Complexes that include some combination of the PriA, PriB, PriC, and DnaT, along with DnaB, DnaC, and DnaG proteins, are called **replication restart primosomes**. In this way, the process of recombination is tightly intertwined with replication. One process of DNA metabolism supports the other.

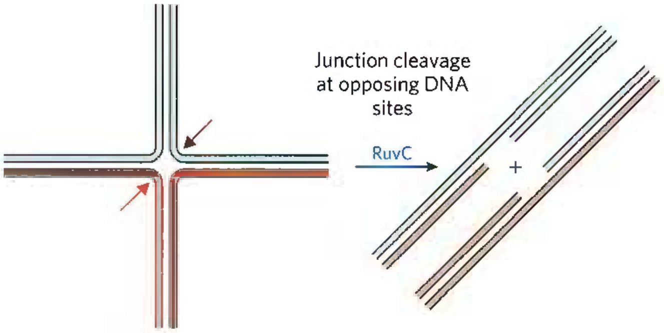
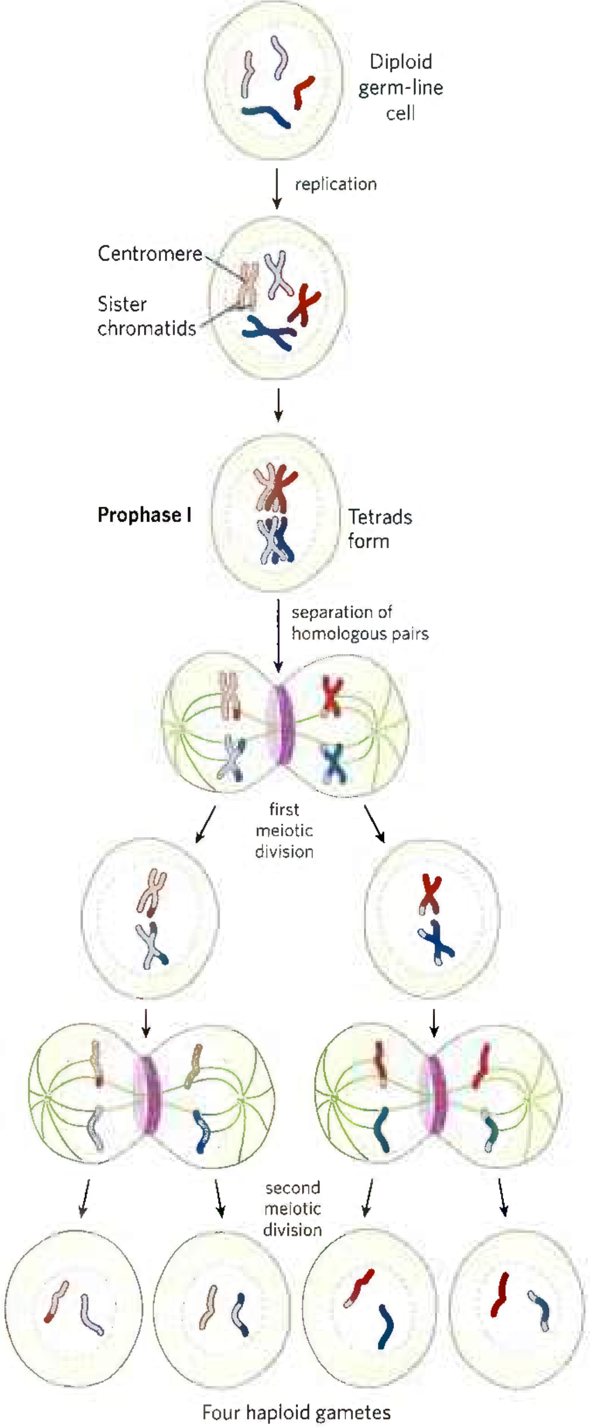


FIGURE 25-32 Resolution of a Holliday intermediate by the RuvC protein. RuvC is a specialized nuclease that binds to the RuvAB complex and cleaves the Holliday intermediate on opposing sides of the crossover junction (red arrows), so that two contiguous DNA arms remain in each product.

Eukaryotic Homologous Recombination Is Required for Proper Chromosome Segregation during Meiosis

In eukaryotes, homologous genetic recombination has roles in replication and cell division, including the repair of stalled replication forks. Recombination occurs with the highest frequency during **meiosis**, the process by which diploid germ-line cells with two sets of chromosomes divide to produce haploid gametes (sperm cells or ova) in animals (haploid spores in plants) — each gamete having only one member of each chromosome pair (**Fig. 25-33**).

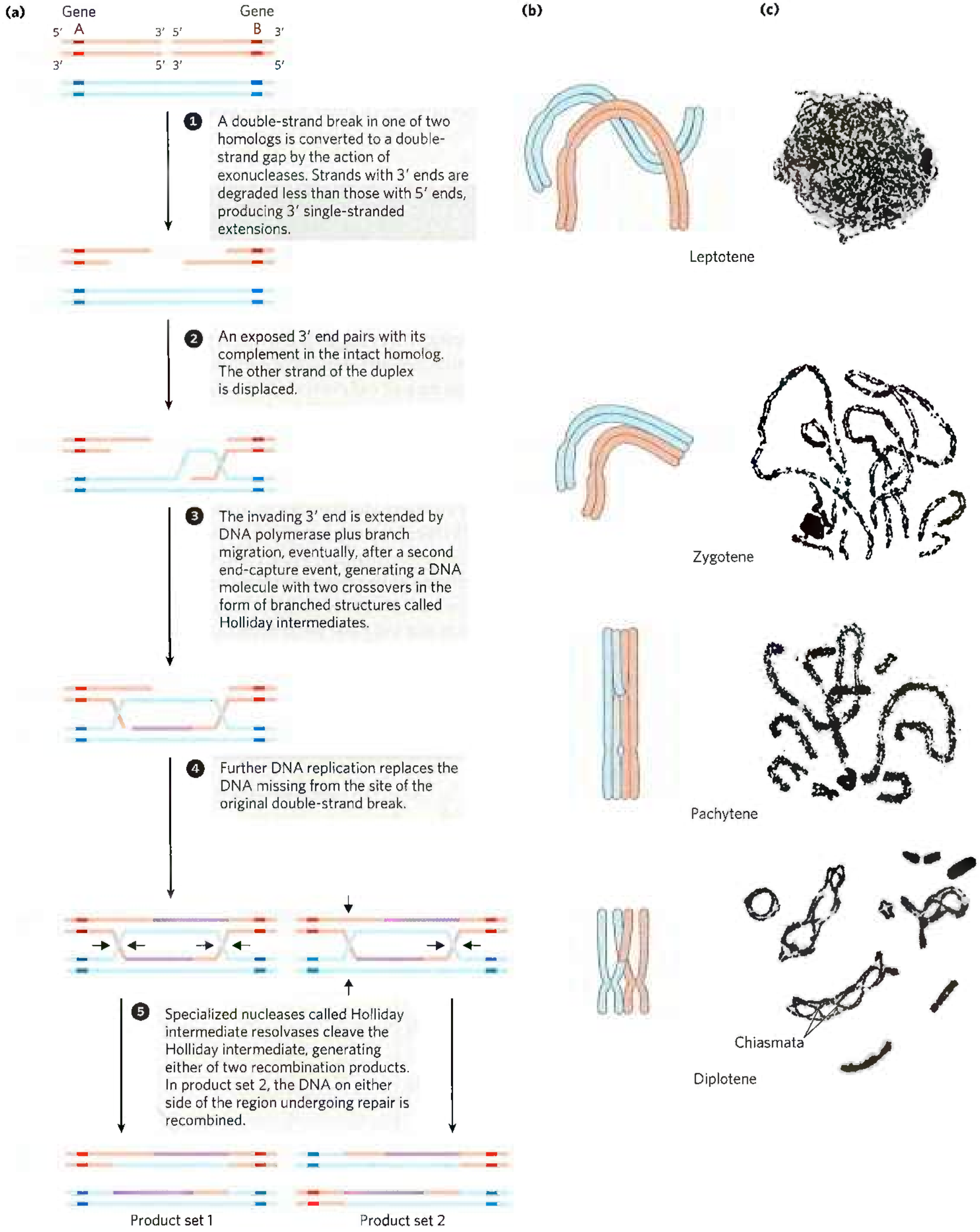


Meiosis begins with replication of the DNA in the germ-line cell so that each DNA molecule is present in four copies. Each set of four homologous chromosomes (tetrad) exists as two pairs of sister chromatids, and the sister chromatids remain associated at their centromeres. The cell then goes through two rounds of cell division without an intervening round of DNA replication. In the first cell division, the two pairs of sister chromatids are segregated into daughter cells. In the second cell division, the two chromosomes in each sister chromatid pair are segregated into new daughter cells. In each division, the chromosomes to be segregated are drawn into the daughter cells by spindle fibers attached to opposite poles of the dividing cell. The two successive divisions reduce the DNA content to the haploid level in each gamete.

Proper chromosome segregation into daughter cells requires that physical links exist between the homologous chromosomes to be segregated. As the spindle fibers attach to the centromeres of chromosomes and start to pull, the links between homologous chromosomes create tension. This tension, sensed by cellular mechanisms not yet understood, signals that this pair of chromosomes or sister chromatids is properly aligned for segregation. Once the tension is sensed, the links are gradually dissolved and segregation proceeds. If improper spindle fiber attachment occurs (e.g., if the centromeres of a chromosome pair are attached to the same cellular pole), a cellular kinase senses the lack of tension and activates a system that removes the spindle attachments, allowing the cell to try again.

During the second meiotic division, the centromeric attachments between sister chromatids, augmented by cohesins deposited during replication (see Fig. 24-33), provide the physical links that are needed to guide segregation. However, during the first meiotic cell division, the two pairs of sister chromatids to be segregated are not related by a recent replication event and are not linked by cohesins or any other physical association. Instead, the homologous pairs of sister chromatids are aligned and new links are created by recombination, a process involving the breakage and rejoining of DNA (**Fig. 25-34**).

FIGURE 25-33 Meiosis in animal germ-line cells. The chromosomes of a hypothetical diploid germ-line cell (four chromosomes; two homologous pairs) replicate and are held together at their centromeres. Each replicated double-stranded DNA molecule is called a chromatid (sister chromatid). In prophase I, just before the first meiotic division, the two homologous sets of chromatids align to form tetrads, held together by covalent links at homologous junctions (chiasmata). Crossovers occur within the chiasmata (see Fig. 25-34). These transient associations between homologs ensure that the two tethered chromosomes segregate properly in the next step, when attached spindle fibers pull them toward opposite poles of the dividing cell in the first meiotic division. The products of this division are two daughter cells, each with two pairs of different sister chromatids. The pairs now line up across the equator of the cell in preparation for separation of the chromatids (now called chromosomes). The second meiotic division produces four haploid daughter cells that can serve as gametes. Each has two chromosomes, half the number of the diploid germ-line cell. The chromosomes have re-sorted and recombined.



❶ A double-strand break in one of two homologs is converted to a double-strand gap by the action of exonucleases. Strands with 3′ ends are degraded less than those with 5′ ends, producing 3′ single-stranded extensions.

❷ An exposed 3′ end pairs with its complement in the intact homolog. The other strand of the duplex is displaced.

❸ The invading 3′ end is extended by DNA polymerase plus branch migration, eventually, after a second end-capture event, generating a DNA molecule with two crossovers in the form of branched structures called Holliday intermediates.

❹ Further DNA replication replaces the DNA missing from the site of the original double-strand break.

❺ Specialized nucleases called Holliday intermediate resolvases cleave the Holliday intermediate, generating either of two recombination products. In product set 2, the DNA on either side of the region undergoing repair is recombined.

FIGURE 25-34 Recombination during prophase I in meiosis. (a) A model of double-strand break repair for homologous genetic recombination. The two homologous chromosomes (one shown in red, the other blue) involved in this recombination event have identical or very nearly identical sequences. Each of the two genes shown has different alleles on the two chromosomes. The steps are described in the text. (b) Crossing over occurs during prophase of meiosis I. The several stages of prophase I are aligned with the recombination processes shown in (a). Double-strand breaks are introduced and processed in the leptotene stage. The strand invasion and completion of crossover occur later. As homologous sequences in the two pairs of sister chromatids are aligned in the zygotene stage, synaptonemal complexes form and strand invasion occurs. The homologous chromosomes are tightly aligned by the pachytene stage. (c) Homologous chromosomes of a grasshopper, viewed at successive stages of meiotic prophase I. The chiasmata become visible in the diplotene stage. [(c) B. John, *Meiosis*, Figs 2.1a, 2.2a, 2.2b, 2.3a, Cambridge University Press, 1990. Reprinted with the permission of Cambridge University Press.]

This exchange, also referred to as crossing over, can be observed with the light microscope. Crossing over links the two pairs of sister chromatids together at points called chiasmata (singular, chiasma). Also during crossing over, genetic material is exchanged between the pairs of sister chromatids. These exchanges increase genetic diversity in the resulting gametes. The importance of meiotic recombination to proper chromosome segregation is well illustrated by the physiological and societal consequences of their failure (**Box 25-2**).

BOX 25-2 **MEDICINE**

Why Proper Segregation of Chromosomes Matters

When chromosomal alignment and recombination are not correct and complete in meiosis I, segregation of chromosomes can go awry. One result may be aneuploidy, a condition in which a cell has the wrong number of chromosomes. The haploid products of meiosis (gametes or spores) may have no copies or two copies of a chromosome. When a gamete having two copies of a chromosome joins with a gamete having one copy of a chromosome during fertilization, cells in the resulting embryo have three copies of that chromosome (they are trisomic).

In *S. cerevisiae*, aneuploidy resulting from errors in meiosis occurs at a rate of about 1 in 10,000 meiotic events. In fruit flies, the rate is about 1 in a few thousand. Rates of aneuploidy in mammals are considerably higher. In mice, the rate is 1 in 100, and it is even higher in other mammals. The rate of aneuploidy in fertilized human eggs has been estimated as 10% to 30%; this is almost certainly an underestimate. Most of these aneuploid cells are monosomies (they have a single copy of a chromosome) or trisomies. Most trisomies are lethal, and many result in miscarriage long before the pregnancy is detected. Almost all monosomies are fatal in the early stages of fetal development. Aneuploidy is the leading cause of pregnancy loss. The few trisomic fetuses that survive to birth generally have three copies of chromosome 13, 18, or 21 (trisomy 21 is Down syndrome). Abnormal complements of the sex chromosomes are also found in the human population. The societal consequences of aneuploidy in humans are considerable. Aneuploidy is the leading genetic cause of developmental and mental disabilities. At the heart of these high rates is a feature of meiosis in female mammals that has special significance for the human species.

In a human male, germ-line cells begin to undergo meiosis at puberty, and each meiotic event requires a relatively short time. In contrast, meiosis in the germ-line cells of human females is a highly protracted process. The production of an egg begins before a female is born, with the onset of meiosis in the fetus, at 12 to 13 weeks of gestation. Meiosis is initiated in all the developing fetal germ-line cells over a period of a few weeks. The cells proceed through much of meiosis I. Chromosomes line up and generate crossovers, continuing just beyond the pachytene stage (see Fig. 25-34)—and then the process stops. The chromosomes enter an arrested phase called the dictyate stage, with the crossovers in place, a kind of suspended animation where they remain as the female matures—so, typically remaining in this stage for anywhere from about 13 to 50 years. At sexual maturity, individual germ-line cells continue through the two meiotic cell divisions to produce egg cells.

Between the onset of the dictyate stage and the final completion of meiosis, something may happen that disrupts or damages the crossovers linking homologous chromosomes in the germ-line cells. As a woman ages, the rate of trisomy in the egg cells she produces increases, dramatically so as she approaches menopause (Fig. 1). There are many hypotheses on why this occurs, and several different factors may play a role. However, most of the hypotheses are centered on recombination crossovers in meiosis I and their stability over the protracted dictyate stage.

It is not yet clear what medical steps could be taken to reduce the incidence of aneuploidy in women of child-bearing age. What is revealed is the inherent importance of recombination and generation of crossovers in human meiosis.

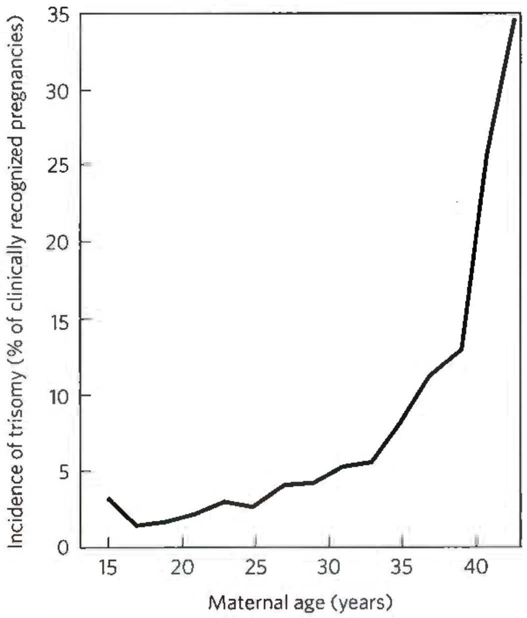


FIGURE 1 The increasing incidence of human trisomy with increasing age of the mother. [Data from T. Hassold and P. Hunt, *Nat. Rev. Genet.* 2:280, 2001, Fig. 6.]

A likely pathway for homologous recombination during meiosis is outlined in Figure 25-34a. The model has four key features. First, homologous chromosomes align. Second, a double-strand break is created in a DNA molecule, and the exposed ends are processed by an exonuclease, leaving a single-stranded extension with a free 3′-hydroxyl group at the broken end (step ①). Third, the exposed 3′ ends invade the intact duplex DNA of the homolog, and this is followed by branch migration and/or replication to create a pair of Holliday intermediates (steps ② to ④). Fourth, cleavage of the two crossovers creates either of two pairs of complete recombinant products (step ⑤). Notice the similarity of these steps to the bacterial recombinational repair processes outlined in Figure 25-29. The DNA strand invasion in eukaryotes is catalyzed by RecA-like recombinases called Rad51 and Dmc1. Loading of Rad51 onto DNA is promoted by Rad51 loading protein BRCA2 (analogous to the bacterial RecF, RecO, and RecR proteins).

In this **double-strand break repair model** for recombination, the 3′ ends are used to initiate the genetic exchange. Once paired with the complementary strand on the intact homolog, a region of hybrid DNA is created that contains complementary strands from two different parent DNAs (the product of step ② in Fig. 25-34a). Each of the 3′ ends can then act as a primer for DNA replication. Meiotic homologous recombination can vary in many details from one species to another, but most of the steps outlined above are generally present in some form. There are two ways to resolve the Holliday intermediate with a RuvC-like nuclease so that the two products carry genes in the same linear order as in the substrates—the original, unrecombined chromosomes (step ⑤). If cleaved one way, the DNA flanking the region containing the hybrid DNA is not recombined; if cleaved the other way, the flanking DNA is recombined. Both outcomes are observed in vivo.

The homologous recombination illustrated in Figure 25-34 is an elaborate process that is essential to accurate chromosome segregation. Its molecular consequences for the generation of genetic diversity are subtle. To understand how this process contributes to diversity, we should keep in mind that the two homologous chromosomes that undergo recombination are not necessarily *identical*. The linear array of genes may be the same, but the base sequences in some of the genes may differ slightly (in different alleles). In a human, for example, one chromosome may contain the allele for hemoglobin A (normal hemoglobin) while the other contains the allele for hemoglobin S (the sickle cell mutation). The difference may consist of no more than one base pair among millions.

Crossing over is not an entirely random process, and "hot spots" have been identified on many eukaryotic chromosomes. However, the assumption that crossing over can occur with equal probability at almost any point along the length of two homologous chromosomes remains a reasonable approximation in many cases, and it is this assumption that permits the mapping of genes on a particular chromosome. The frequency of homologous recombination in any region separating two points on a chromosome is roughly proportional to the distance between the points, and this allows determination of the relative positions of different genes and the distances between those genes. The independent assortment of unlinked genes on different chromosomes (**Fig. 25-35**)

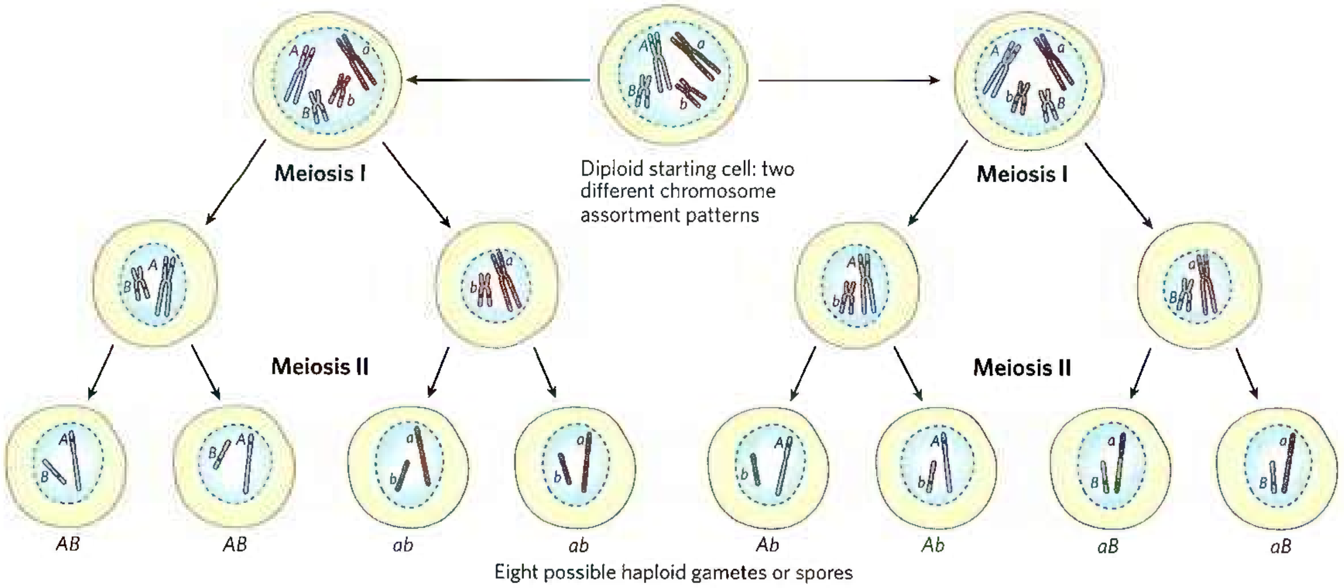

Meiosis I
Diploid starting cell: two different chromosome assortment patterns
Meiosis I
Meiosis II
Meiosis II
AB
AB
ab
ab
Ab
Ab
aB
aB
Eight possible haploid gametes or spores

FIGURE 25-35 The contribution of independent assortment to genetic diversity. In this example, the two chromosomes have already been replicated to create two pairs of sister chromatids. Blue and red distinguish the sister chromatids of each pair. One gene on each chromosome is highlighted, with different alleles (*A* or *a*, *B* or *b*) in the homologs. Independent assortment can lead to gametes with any combination of the alleles present on the two different chromosomes. Crossing over (not shown here; see Fig. 25-34) would also contribute to genetic diversity in a typical meiotic sequence.

makes another major contribution to the genetic diversity of gametes. These genetic realities guide many of the modern applications of genomics, such as defining haplotypes (see Fig. 9-26) or searching for disease genes in the human genome (see Fig. 9-30).

As in bacteria, this recombination process is used to repair double-strand breaks that arise anywhere in the genome. In eukaryotes, these systems operate in the context of chromatin, rendering additional complexities to their regulation and damage detection mechanisms (**Box 25-3**). **P3** Homologous recombination thus serves at least three identifiable functions in eukaryotes: (1) it contributes to the repair of several types of DNA damage; (2) it provides, in eukaryotic cells, a transient physical link between chromatids that promotes the orderly segregation of chromosomes at the first meiotic cell division; and (3) it enhances genetic diversity in a population.

Some Double-Strand Breaks Are Repaired by Nonhomologous End Joining

Double-strand breaks sometimes occur when recombinational DNA repair is not feasible, such as during phases of the cell cycle when no replication is occurring and no sister chromatids are present. At these times, another path is needed to avoid the cell death that would result from a broken chromosome. That alternative is provided by **nonhomologous end joining (NHEJ)**. The broken chromosome ends are simply processed and ligated back together.

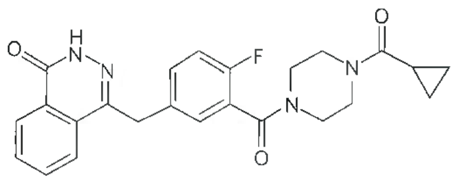
Nonhomologous end joining is an important pathway for double-strand break repair in all eukaryotes and has also been detected in some bacteria. The importance of NHEJ increases with genomic complexity, and the process accounts for most double-strand break

BOX 25-3 MEDICINE

How a DNA Strand Break Gets Attention

Each human chromosome contains many millions of DNA base pairs, all bound up in an elaborate chromatin structure (Chapter 24). If a strand break occurs somewhere in the DNA, how do the many proteins needed for its repair actually find it? The answer lies, at least in part, in a protein called poly-ADP ribose polymerase 1, or PARP1. PARP1 is a first responder, scanning the DNA for DNA damage and in particular for single-strand breaks. When it finds such sites, it binds and synthesizes an elaborate branched poly-ADP ribose polymer from an NAD precursor (Fig. 1). The polymers are attached to the PARP1 enzyme and also linked to some nearby proteins through Glu, Asp, or Lys residues. The resulting structure is a kind of signal, marking the chromosomal location of damage. A large number of DNA repair proteins bind to and are thus recruited to the poly-ADP ribose polymers, effecting DNA repair. If PARP1 activity is absent, repair is compromised and the number of single-strand breaks in all chromosomes increases. When the chromosome is replicated, the single-strand breaks become double-strand breaks (see Fig. 25-29).

As we saw in Box 25-1, many malignant tumors have a defect in a DNA repair pathway. For example, breast or ovarian cancer is often associated with defects in double-strand break repair (e.g., in the genes encoding BRCA1 or BRCA2 or other proteins in the pathway). In these cells, the further loss of PARP1 activity is especially toxic, as single-strand breaks build up and chromosomes become broken during replication. This has led to the development of PARP1 inhibitors as a treatment for tumors in which double-strand break repair is defective. The first such pharmaceutical agent, olaparib, was approved for use in the United States in 2014. Many more PARP1 inhibitors have since been approved or are undergoing clinical trials. The effects have often been dramatic. For women with breast or ovarian tumors displaying deficiencies in BRCA1 or BRCA2 that have responded to more traditional therapies, subsequent maintenance treatment with PARP1 inhibitors has led to a fourfold increase in progression-free survival. PARP1 inhibitors are also showing promise for use with other breast and ovarian tumors, as well as other types of tumors, most of which have DNA repair deficiencies of some kind. As research continues, the use of PARP inhibitors is becoming an important part of the standard of care for a growing list of cancers.



Olaparib

repair outside meiosis in mammals. In yeast, most double-strand breaks are repaired by recombination, and only a few by NHEJ. NHEJ is a mutagenic process, and a smaller genome, such as that of yeast, has relatively little tolerance for the loss of information. The small genomic alterations may be tolerable in mammalian somatic cells, because they are balanced by the undamaged information on the homolog in each diploid cell, and in these non-germ-line cells the mutations are not inherited. In vertebrates, a loss of the genes encoding NHEJ function can produce a predisposition to cancer.

Unlike homologous recombinational repair, NHEJ does not conserve the original DNA sequence. The pathway in eukaryotes is illustrated in **Figure 25-36**. The reaction is initiated at the broken ends of a double-strand break by the binding of a heterodimer consisting of the proteins Ku70 and Ku80 ("KU" being the initials of the individual with scleroderma whose serum autoantibodies were used to identify this protein complex; the numbers refer to the approximate molecular weights of the subunits). The Ku proteins are conserved in almost all eukaryotes and act as a kind of molecular scaffold to assemble the other protein components. Ku70-Ku80 interacts with another protein complex containing a protein kinase called DNA-PKcs and a nuclease known as Artemis. Once the complex is assembled, the two broken DNA ends are synapsed (held together). DNA-PKcs autophosphorylates in several locations and also phosphorylates Artemis. Artemis, when phosphorylated, acquires an endonuclease function that can remove 5′ or 3′ single-stranded extensions or hairpins that might be present at the ends. The DNA ends are then separated with the aid of a helicase, and strands from the two different ends are annealed at locations

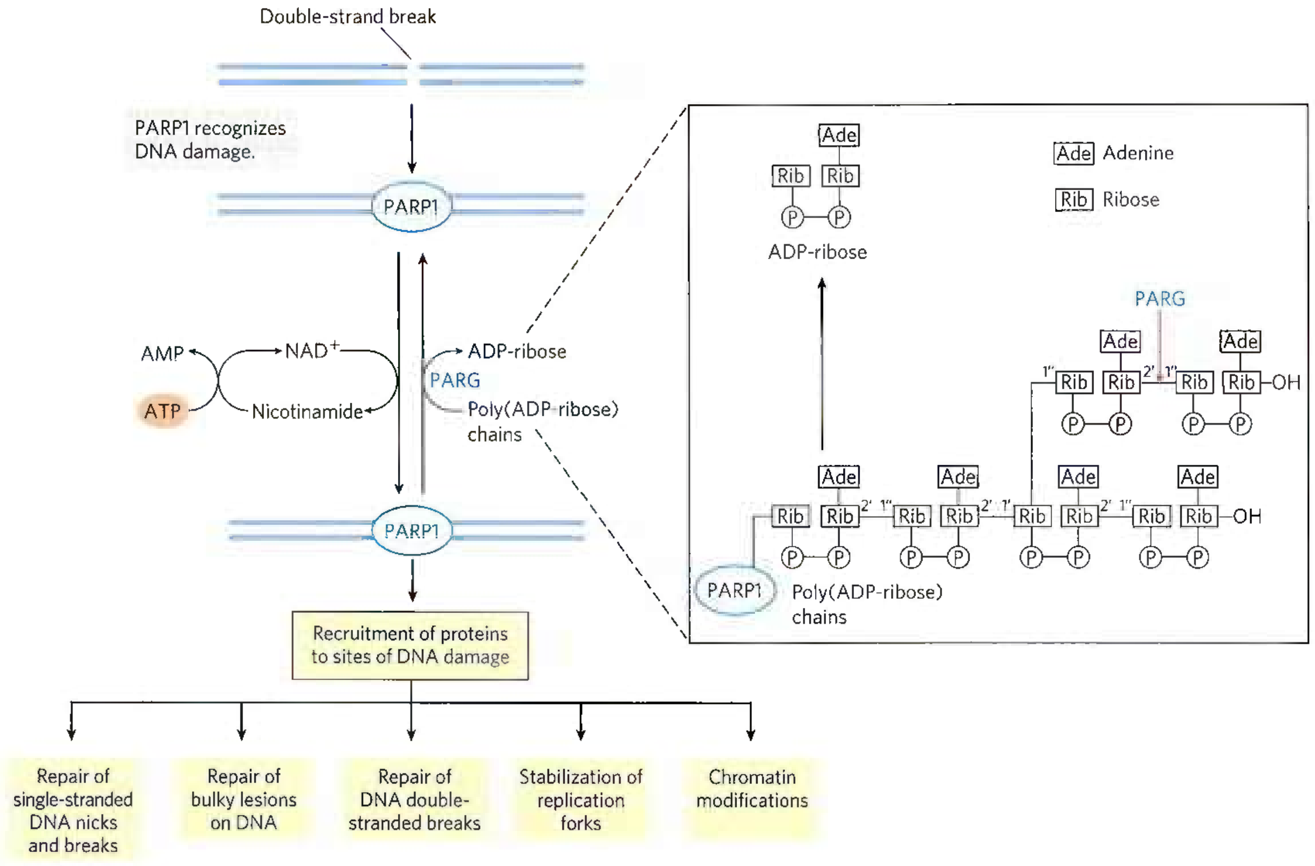

Double-strand break
PARP1 recognizes DNA damage.
PARP1
AMP
ATP
NAD+
Nicotinamide
ADP-ribose
PARG
Poly(ADP-ribose) chains
PARP1
Recruitment of proteins to sites of DNA damage
Repair of single-stranded DNA nicks and breaks
Repair of bulky lesions on DNA
Repair of DNA double-stranded breaks
Stabilization of replication forks
Chromatin modifications
Ade
Rib
P
ADP-ribose
Ade Adenine
Rib Ribose
PARG
2′
1″
OH
PARP1
Poly(ADP-ribose) chains

FIGURE 1 The activity and function of poly-ADP ribose polymerase in detecting DNA strand breaks and other types of damage. [Information from A. R. Chaudhuri and A. Nussenzweig, *Nat. Rev. Mol. Cell Biol.* 18:610, 2017, Fig. 1.]

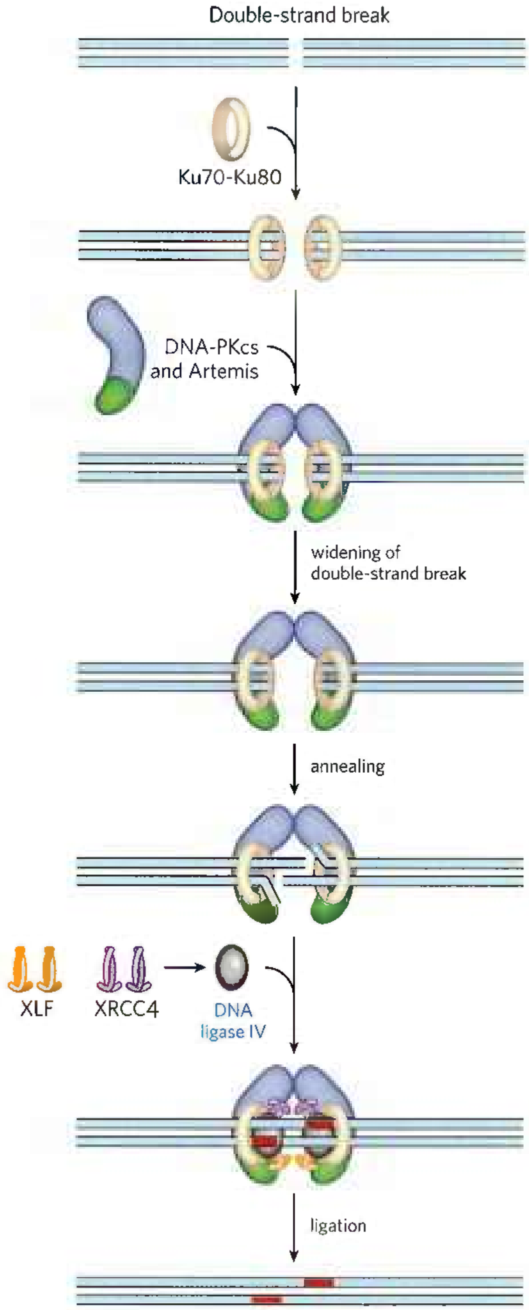


FIGURE 25-36 Nonhomologous end joining. The Ku70-Ku80 complex is the first to bind the DNA ends, followed by a complex including DNA-PKcs and the nuclease Artemis. These proteins then recruit a complex consisting of XRCC4, XLF, and DNA ligase IV. Either of two DNA polymerases, Pol μ or Pol λ (not shown), subsequently extends the annealed DNA strands, as needed, before ligation. [Information from J. M. Sekiguchi and D. O. Ferguson, *Cell* 124:260, 2006, Fig. 1.]

where short regions of complementarity are encountered. Artemis cleaves any unpaired DNA segments that are created. Small DNA gaps are filled by a DNA polymerase, Pol μ or Pol λ. Finally, the nicks are sealed by a protein complex consisting of XRCC4 (*x-r*ay *c*ross *c*omplementation group), XLF (*X*RCC4-*l*ike *f*actor), and DNA ligase IV.

DNA ends are not joined randomly by NHEJ. Instead, when a double-strand break occurs, the ends are generally constrained by the structure of chromatin and thus remain close together. Very rare events linking end sequences that are normally far apart in the chromosome, or are on different chromosomes, may be responsible for occasional dramatic and usually deleterious genomic rearrangements.

Site-Specific Recombination Results in Precise DNA Rearrangements

Homologous genetic recombination can involve any two homologous sequences. The second general type of recombination, site-specific recombination, is a very different type of process: recombination is limited to specific sequences. Recombination reactions of this type occur in virtually every cell, filling specialized roles that vary greatly from one species to another. Examples include regulation of the expression of certain genes and promotion of programmed DNA rearrangements in embryonic development or in the replication cycles of some viral and plasmid DNAs. Each site-specific recombination system consists of an enzyme called a recombinase and a short (20 to 200 bp), unique DNA sequence where the recombinase acts (the recombination site). One or more auxiliary proteins may regulate the timing or outcome of the reaction.

There are two general classes of site-specific recombination systems, which rely on either Tyr or Ser residues in the active site. In vitro studies of many site-specific recombination systems in the tyrosine class have elucidated some general principles, including the fundamental reaction pathway (**Fig. 25-37a**). Several of these enzymes have been crystallized, revealing structural details of the reaction. A separate recombinase recognizes and binds to each of two recombination sites on two different DNA molecules or within the same DNA. One DNA strand in each site is cleaved at a specific point within the site, and the recombinase becomes covalently linked to the DNA at the cleavage site through a phosphotyrosine bond (step ❶). The transient protein-DNA linkage preserves the phosphodiester bond that is lost in cleaving the DNA, so high-energy cofactors such as ATP are unnecessary in subsequent steps. The cleaved DNA strands are rejoined to new partners to form a Holliday intermediate, with new phosphodiester bonds created at the expense of the protein-DNA linkage (step ❷). An isomerization then occurs (step ❸), and the process is repeated at a second point within each of the two recombination sites (steps ❹ and ❺). In systems that employ an active-site Ser residue, both strands of each recombination site are cut concurrently and rejoined to new partners without the Holliday intermediate. In both types of systems, the exchange is always reciprocal and precise, regenerating the recombination sites when the reaction is complete. We can view a recombinase as a site-specific endonuclease and ligase in one package.

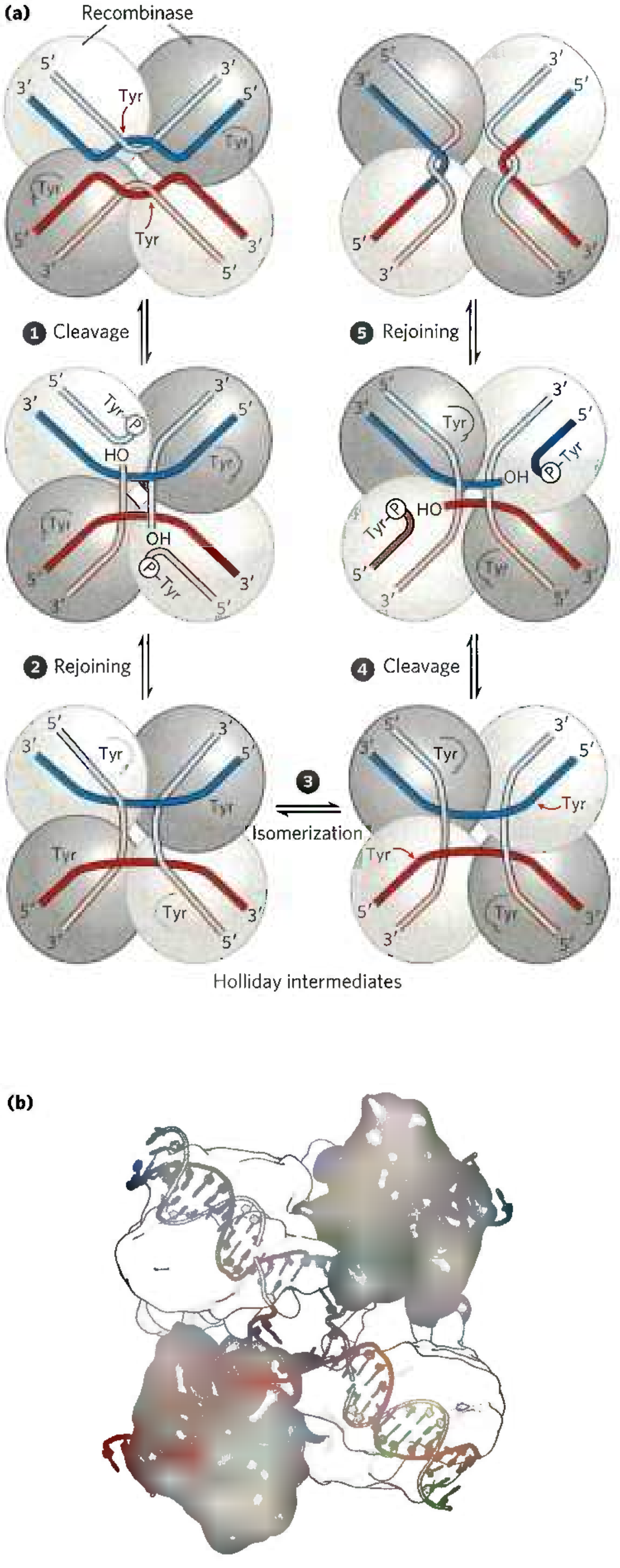


FIGURE 25-37 A site-specific recombination reaction. (a) The reaction shown here is for a common class of site-specific recombinases called integrase-class recombinases (named after bacteriophage λ integrase, the first recombinase characterized). These enzymes use Tyr residues as nucleophiles at the active site. The reaction is carried out within a tetramer of identical subunits. Recombinase subunits bind to a specific sequence, the recombination site. Two dimeric complexes, each bound to a single site in the DNA, come together to form the tetrameric complex shown here. ① One strand in each DNA is cleaved at particular points in the sequence. The nucleophile is the —OH group of an active-site Tyr residue, and the product of rejoining ② is a covalent phosphotyrosine link between protein and DNA. After isomerization ③, the cleaved strands join to new partners, producing a Holliday intermediate. Steps ④ and ⑤ complete the reaction by a process similar to the first two steps. The original sequence of the recombination site is regenerated after recombining the DNA flanking the site. These steps occur within a complex of multiple recombinase subunits that sometimes includes other proteins not shown here. (b) Surface contour model of a four-subunit integrase-class recombinase called the FLP recombinase, bound to a Holliday intermediate (shown with light blue and dark blue helix strands). The protein has been rendered transparent so that the bound DNA is visible. Another group of recombinases, called the resolvase/invertase family, use a Ser residue as nucleophile at the active site. [(b) Data from PDB ID 1P4E, P. A. Rice and Y. Chen, *J. Biol. Chem.* 278:24,800, 2003.]

The sequences of the recombination sites recognized by site-specific recombinases are partially asymmetric (nonpalindromic), and the two recombining sites align in the same orientation during the recombinase reaction. The outcome depends on the location and orientation of the recombination sites (**Fig. 25-38**). If the two sites are on the same DNA molecule, the reaction either inverts or deletes the intervening DNA, determined by whether the recombination sites have the opposite or the same orientation, respectively. If the sites are on different DNAs, the recombination is intermolecular; if one or both DNAs are circular, the result is an insertion. Some recombinase systems are highly specific for one of these reaction types and act only on sites with particular orientations.

Complete chromosomal replication can require site-specific recombination. Recombinational DNA repair of a circular bacterial chromosome, while essential, sometimes generates deleterious byproducts. The resolution of a Holliday intermediate at a replication fork by a nuclease such as RuvC, followed by completion of replication, can give rise to one of two products: the usual two monomeric chromosomes or a contiguous dimeric chromosome (**Fig. 25-39**). In the latter case, the covalently linked chromosomes cannot be segregated to daughter cells at cell division, and the dividing cells become "stuck." A specialized site-specific recombination system in *E. coli*, the XerCD system, converts the dimeric chromosomes to monomeric chromosomes so that cell division can proceed. The reaction is a site-specific deletion (Fig. 25-38b). **P4** This is another example of the close coordination between DNA recombination processes and other aspects of DNA metabolism.

Transposable Genetic Elements Move from One Location to Another

We now consider the third general type of recombination system: recombination that allows the movement of transposable elements, or **transposons**. These segments of DNA, found in virtually all cells, move, or "jump," from

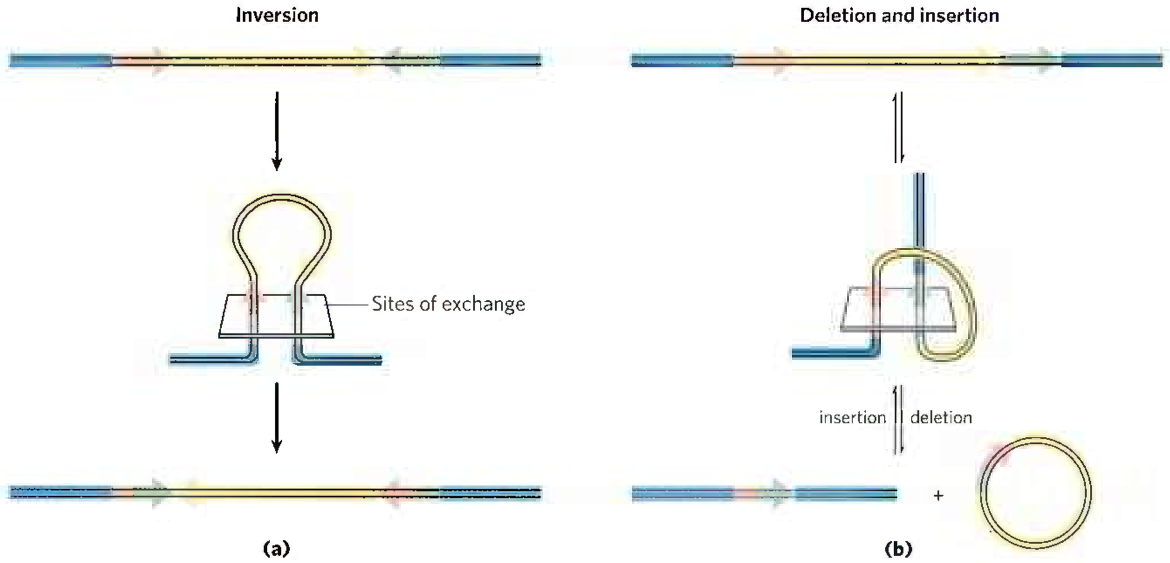


FIGURE 25-38 Effects of site-specific recombination. The outcome of site-specific recombination depends on the location and orientation of the recombination sites (red and green) in a double-stranded DNA molecule. Orientation here (shown by arrowheads) refers to the order of nucleotides in the recombination site, *not* the $5' \rightarrow 3'$ direction. (a) Recombination sites with opposite orientation in the same DNA molecule. The result is an inversion. (b) Recombination sites with the same orientation, either on one DNA molecule, producing a deletion, or on two DNA molecules, producing an insertion.

one place on a chromosome (the donor site) to another on the same or a different chromosome (the target site). DNA sequence homology is not usually required for this movement, called **transposition**; the new location is determined more or less randomly. Insertion of a transposon in an essential gene could kill the cell, so transposition is tightly regulated and usually very infrequent. Transposons are perhaps the simplest of molecular parasites, adapted to replicate passively within the chromosomes of host cells. In some cases they carry genes that are useful to the host cell, and thus exist in a kind of symbiosis with the host.

Bacteria have two classes of transposons. **Insertion sequences** (simple transposons) contain only the sequences required for transposition and the genes for the proteins (transposases) that promote the process. **Complex transposons** contain one or more genes in addition to those needed for transposition. These extra genes might, for example, confer resistance to antibiotics and thus enhance the survival chances of the host cell. The spread of antibiotic-resistance elements among disease-causing bacterial populations that is rendering some antibiotics ineffectual (p. 887) is mediated to a large degree by transposition. ■

Bacterial transposons vary in structure, but most have short repeated sequences at each end that serve as binding sites for the transposase. When transposition occurs, a short sequence at the target site (5 to 10 bp) is duplicated to form an additional short repeated sequence that flanks each end of the inserted transposon (**Fig. 25-40**). These duplicated segments result from the cutting mechanism used to insert a transposon into the DNA at a new location.

There are two general pathways for transposition in bacteria. In direct (or simple) transposition (**Fig. 25-41**,

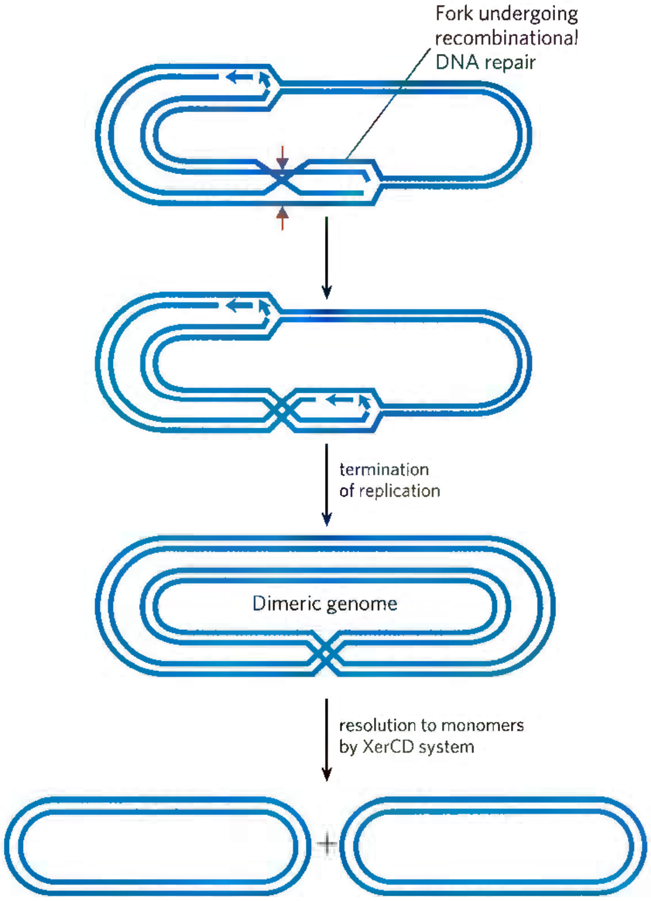

Fork undergoing recombinational DNA repair
termination of replication
Dimeric genome
resolution to monomers by XerCD system
+

FIGURE 25-39 DNA deletion to undo a deleterious effect of recombinational DNA repair. The resolution of a Holliday intermediate during recombinational DNA repair (if cut at the points indicated by the red arrows) can generate a contiguous dimeric chromosome. A specialized site-specific recombinase in *E. coli*, XerCD, converts the dimer to monomers, allowing chromosome segregation and cell division to proceed.

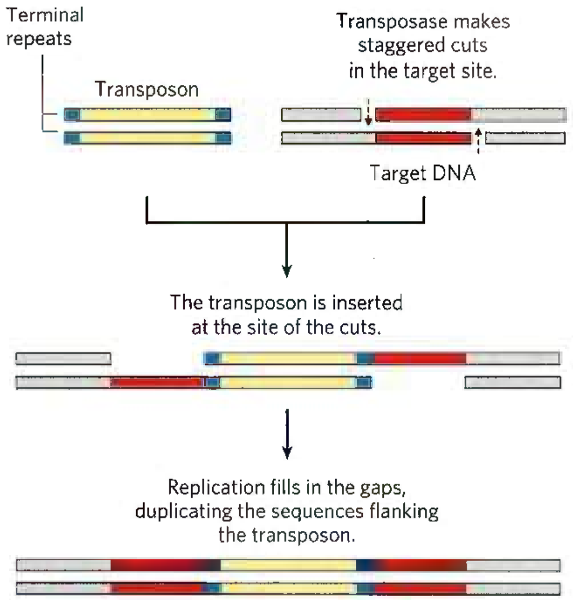

Terminal repeats
Transposon
Transposase makes staggered cuts in the target site.
Target DNA
The transposon is inserted at the site of the cuts.
Replication fills in the gaps, duplicating the sequences flanking the transposon.

FIGURE 25-40 Duplication of the DNA sequence at a target site when a transposon is inserted. The sequences duplicated following transposon insertion are shown in red. These sequences are generally only a few base pairs long, so their size relative to that of a typical transposon is greatly exaggerated in this drawing.

left), cuts on each side of the transposon excise it, and the transposon moves to a new location. This leaves a double-strand break in the donor DNA that must be repaired. At the target site, a staggered cut is made (as in Fig. 25-40), the transposon is inserted into the break, and DNA replication fills in the gaps to duplicate the target-site sequence. In replicative transposition (Fig. 25-41, right), the entire transposon is replicated, leaving a copy behind at the donor location. A **cointegrate** is an intermediate in this process, consisting of the donor region covalently linked to DNA at the target site. Two complete copies of the transposon are present in the cointegrate, both having the same relative orientation in the DNA. In some well-characterized transposons, the cointegrate intermediate is converted to products by site-specific recombination, in which specialized recombinases promote the required deletion reaction.

Eukaryotes also have transposons, structurally similar to bacterial transposons, and some use similar transposition mechanisms. In other cases, however, the mechanism of transposition seems to involve an RNA intermediate. Evolution of these transposons is intertwined with the evolution of certain classes of RNA viruses. Both are described in the next chapter. As illustrated in Figure 9-25, nearly half of the human genome is made up of various types of transposable elements.

Immunoglobulin Genes Assemble by Recombination

Some DNA rearrangements are a programmed part of development in eukaryotic organisms. An important example is the generation of complete immunoglobulin genes from separate gene segments in vertebrate genomes.

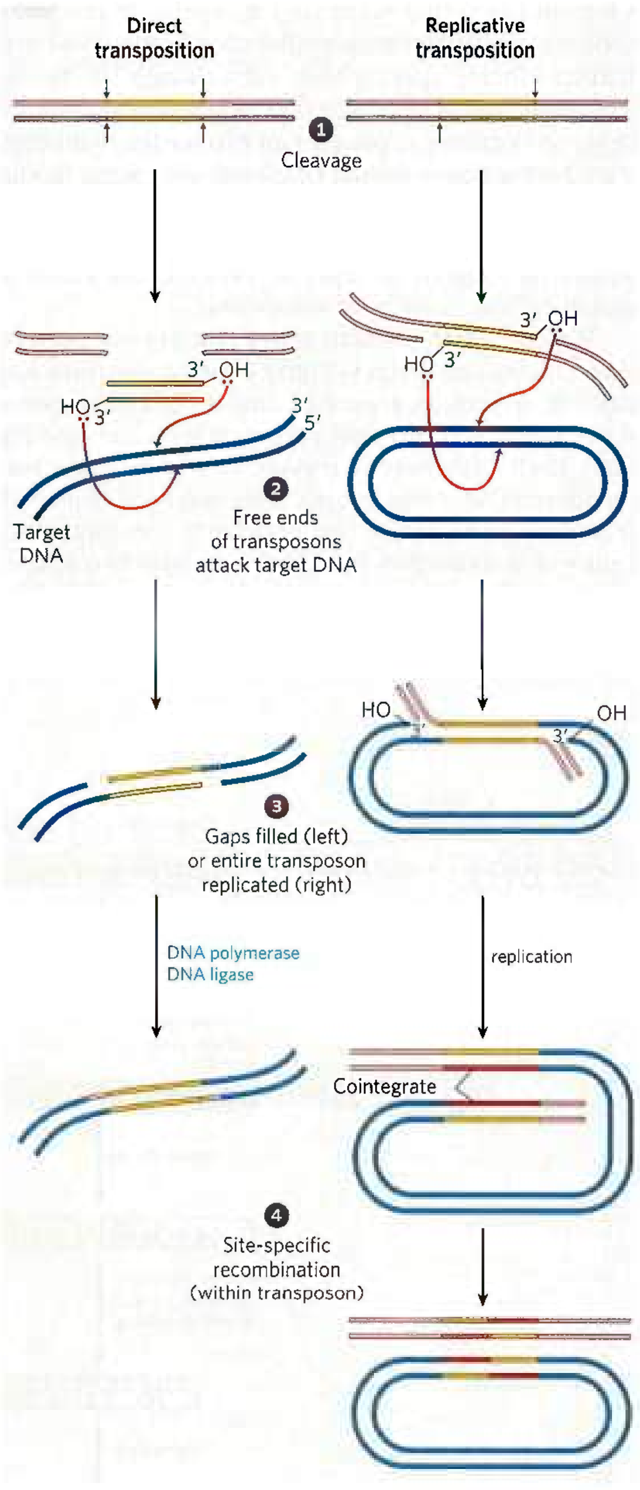

Direct transposition
Replicative transposition
①
Cleavage
3′ OH
HO 3′
3′
5′
3′ OH
HO 3′
Target DNA
②
Free ends of transposons attack target DNA
HO
OH
3′
③
Gaps filled (left) or entire transposon replicated (right)
DNA polymerase
DNA ligase
replication
Cointegrate
④
Site-specific recombination (within transposon)

FIGURE 25-41 Two general pathways for transposition: direct (simple) and replicative. ① The DNA is first cleaved on each side of the transposon, at the sites indicated by arrows. ② The liberated 3′-hydroxyl groups at the ends of the transposon act as nucleophiles in a direct attack on phosphodiester bonds in the target DNA. The target phosphodiester bonds are staggered (not directly across from each other) in the two DNA strands. ③ The transposon is now linked to the target DNA. In direct transposition (left), replication fills in gaps at each end to complete the process. In replicative transposition (right), the entire transposon is replicated to create a cointegrate intermediate. ④ The cointegrate is often resolved later, with the aid of a separate site-specific recombination system. The cleaved host DNA left behind after direct transposition is either repaired by DNA end joining or degraded (not shown); the latter outcome can be lethal to the organism.

A human (like other mammals) is capable of producing *millions* of different immunoglobulins (antibodies) with distinct binding specificities, even though the human genome contains only ~20,000 genes. Recombination allows an organism to produce an extraordinary diversity of antibodies from a limited DNA-coding capacity. Studies of the recombination mechanism reveal a close relationship to DNA transposition and suggest that this system for generating antibody diversity may have evolved from an ancient cellular invasion by transposons.

We can use the human genes that encode proteins of the immunoglobulin G (IgG) class to illustrate how antibody diversity is generated. Immunoglobulins consist of two heavy and two light polypeptide chains (see Fig. 5-20). Each chain has two regions: a variable region, with a sequence that differs greatly from one immunoglobulin to another, and a region that is virtually constant within a class of immunoglobulins. There are also two distinct families of light chains, kappa and lambda, which differ somewhat in the sequences of their constant regions. For all three types of polypeptide chains (heavy chain, and kappa and lambda light chains), diversity in the variable regions is generated by a similar mechanism. The genes for these polypeptides are divided into segments, and the genome contains clusters with multiple versions of each segment. The joining of one version of each gene segment creates a complete gene.

Figure 25-42 depicts the organization of the DNA encoding the kappa light chains of human IgG and shows how a mature kappa light chain is generated. In undifferentiated cells, the coding information for this polypeptide chain is separated into three segments. The V (variable) segment encodes the first 95 amino acid residues of the variable region, the J (joining) segment encodes the remaining 12 residues of the variable region, and the C segment encodes the constant region. The genome contains 40 different V segments, 5 different J segments, and 1 C segment.

As a stem cell in the bone marrow differentiates to form a mature B lymphocyte, one V segment and one J segment are brought together by a specialized recombination system (Fig. 25-42). During this programmed

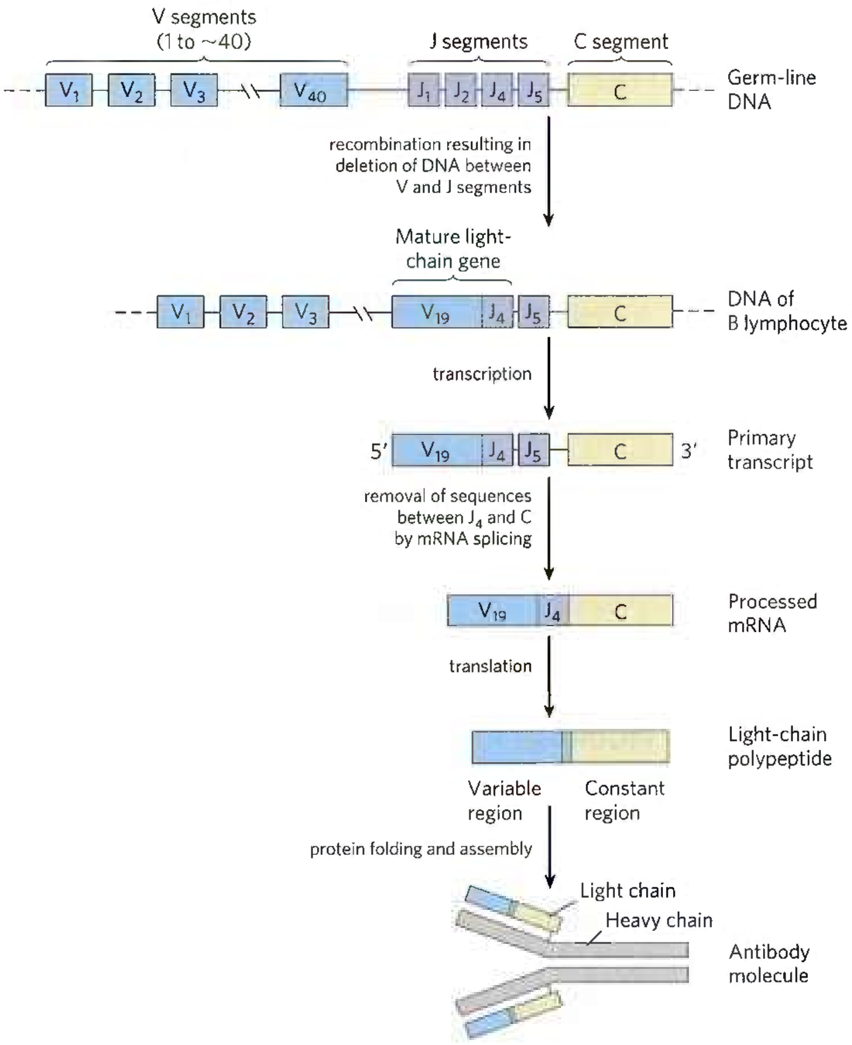

V segments (1 to ~40)
J segments
C segment
V_1
V_2
V_3
V_{40}
J_1
J_2
J_4
J_5
C
Germ-line DNA
recombination resulting in deletion of DNA between V and J segments
Mature light-chain gene
V_{19}
DNA of B lymphocyte
transcription
5′
3′
Primary transcript
removal of sequences between J_4 and C by mRNA splicing
Processed mRNA
translation
Light-chain polypeptide
Variable region
Constant region
protein folding and assembly
Light chain
Heavy chain
Antibody molecule

FIGURE 25-42 Recombination of the V and J gene segments of the human IgG kappa light chain. At the top is shown the arrangement of IgG-coding sequences in a stem cell of the bone marrow. Recombination deletes the DNA between a particular V segment and a J segment. Transcription and RNA splicing, as described in Chapter 26, produces the light-chain polypeptide. The light chain can combine with any of 5,000 possible heavy chains to produce an antibody molecule.

DNA deletion, the intervening DNA is discarded. There are about 40 × 5 = 200 possible V–J combinations. The recombination process is not as precise as the site-specific recombination described earlier, so additional variation occurs in the sequence at the V–J junction. This increases the overall variation by a factor of at least 2.5, so the cells can generate about 2.5 × 200 = 500 different V–J combinations. The final joining of the V–J combination to the C region is accomplished by an RNA-splicing reaction after transcription, a process described in Chapter 26.

The recombination mechanism for joining the V and J segments is illustrated in **Figure 25-43**. Just beyond each V segment and just before each J segment lie recombination signal sequences (RSSs). These are bound by proteins called RAG1 and RAG2 (products of the *r*ecombination *a*ctivating *g*ene). The RAG proteins catalyze the formation of a double-strand break between the signal sequences and the V (or J) segments to be joined. The V and J segments are then joined with the aid of a second complex of proteins.

The genes for the heavy chains and the lambda light chains form by similar processes. Heavy chains have more gene segments than light chains, with more than 5,000 possible combinations. Because any heavy chain can combine with any light chain to generate an immunoglobulin, each human has at least 500 × 5,000 = 2.5 × 10^6 possible IgGs. And additional diversity is generated by high mutation rates (of unknown mechanism) in the V sequences during B-lymphocyte differentiation. Each mature B lymphocyte produces only one type of antibody, but the range of antibodies produced by the B lymphocytes of an individual organism is clearly enormous.

Did the immune system evolve in part from ancient transposons? The mechanism for generation of the double-strand breaks by RAG1 and RAG2 mirrors several reaction steps in transposition (Fig. 25-43). In addition, the deleted DNA, with its terminal RSSs, has a sequence structure found in most transposons. In the test tube, RAG1 and RAG2 can associate with this deleted DNA and insert it, transposonlike, into other DNA molecules (probably a rare reaction in B lymphocytes). Although we cannot know for certain, the properties of the immunoglobulin gene rearrangement system suggest an intriguing origin in which the distinction between host and parasite has become blurred by evolution.

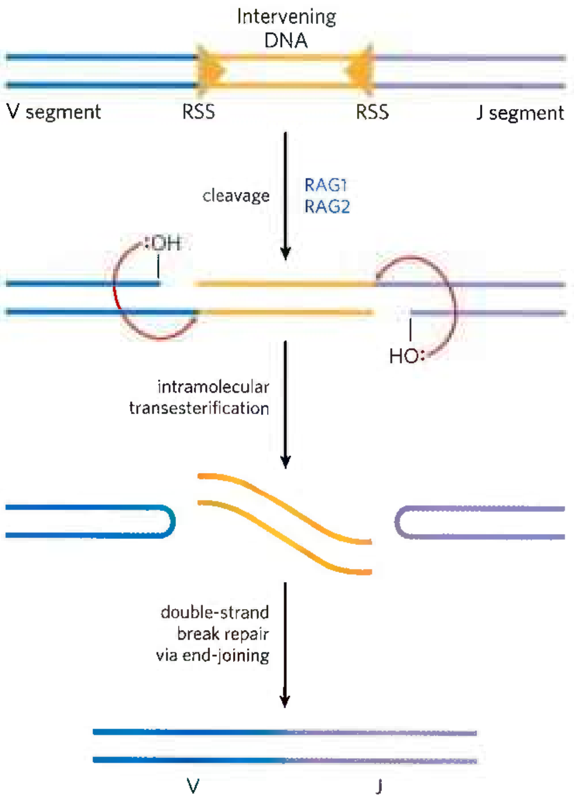

Intervening DNA
V segment
RSS
RSS
J segment
cleavage
RAG1
RAG2
:OH
HO:
intramolecular transesterification
double-strand break repair via end-joining
V
J

FIGURE 25-43 Mechanism of immunoglobulin gene rearrangement. The RAG1 and RAG2 proteins bind to the recombination signal sequences (RSSs) and cleave one DNA strand between the RSS and the V (or J) segments to be joined. The liberated 3′ hydroxyl then acts as a nucleophile, attacking a phosphodiester bond in the other strand to create a double-strand break. The resulting hairpin bends on the V and J segments are cleaved, and the ends are covalently linked by a complex of proteins specialized for end-joining repair of double-strand breaks.

SUMMARY 25.3 *DNA Recombination*

- DNA sequences are rearranged in recombination reactions, usually in processes tightly coordinated with DNA replication or repair.
- Homologous genetic recombination can take place between any two DNA molecules that share sequence homology. In bacteria, recombination serves mainly as a DNA repair process, focused on reactivating stalled or collapsed replication forks or on the general repair of double-strand breaks.
- In eukaryotes, recombination is essential to ensure accurate chromosome segregation during the first meiotic cell division. It also helps to create genetic diversity in the resulting gametes.
- Nonhomologous end joining provides an alternative mechanism for the repair of double-strand breaks, especially in eukaryotic cells.
- Site-specific recombination occurs only at specific target sequences, and this process can also involve a Holliday intermediate. Recombinases cleave the DNA at specific points and ligate the strands to new partners. This type of recombination is found in virtually all cells, and its many functions include DNA integration and regulation of gene expression.
- In almost all cells, transposons use recombination to move within or between chromosomes.
- In vertebrates, a programmed recombination reaction related to transposition joins immunoglobulin gene segments to form immunoglobulin genes during B-lymphocyte differentiation.

KEY TERMS

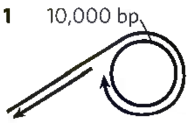
Terms in bold are defined in the glossary.

template	915
semiconservative replication	915
replication fork	916
origin	916
Okazaki fragment	916
leading strand	916
lagging strand	916
nucleases	916
exonucleases	916
endonucleases	916
DNA polymerases	916
DNA polymerase I	916
primer	917
primer terminus	917
processivity	917
proofreading	919
DNA polymerase III	919
replisome	921
helicases	921
topoisomerases	922
primases	922
DNA ligases	922
DNA unwinding element (DUE)	922
AAA+ ATPases	922
catenane	927
prereplicative complex (pre-RC)	928
licensing	928
minichromosome maintenance (MCM) protein	928
ORC (origin recognition complex)	928
DNA polymerase ε	929
DNA polymerase δ	929
DNA polymerase α	929
mutation	930
DNA glycosylases	934
base-excision repair	934
AP site	935
abasic site	935
AP endonucleases	935
DNA photolyases	937
error-prone translesion DNA synthesis	939
SOS response	939
homologous genetic recombination	940
site-specific recombination	940
DNA transposition	940
recombinational DNA repair	941
branch migration	942
Holliday intermediate	942
replication restart primosome	943
meiosis	944
double-strand break repair model	947
nonhomologous end joining (NHEJ)	948
transposon	951
transposition	952
insertion sequence	952

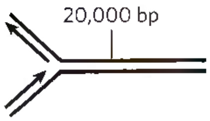
PROBLEMS

1. DNA Replication An investigator adds DNA polymerase III holoenzyme, DNA primase (DnaG), single-stranded DNA-binding protein (SSB), an ATP-dependent DNA ligase, and the replicative helicase (DnaB) to each of the DNA substrates shown, along with ATP and all four dNTPs. The length of DNA in the circle in structure **1** is 10,000 bp. The linear (unbranched) part of structure **2** is 20,000 bp long.

1



2



(a) If precursor (dNTP) concentrations are not limiting, Okazaki fragments are 2,000 nucleotides long, and replication proceeds at 1,000 nucleotides/s for 30 seconds, which DNA substrate will generate a longer replication product? **(b)** For structure **1**, draw the product of this 30 second replication. **(c)** Draw the expected product if DnaG were left out of the reaction. **(d)** Draw the expected product if DNA ligase were instead left out of the reaction.

2. Heavy Isotope Analysis of DNA Replication A researcher switches a culture of *E. coli* growing in a medium containing $^{15}NH_4Cl$ to a medium containing $^{14}NH_4Cl$ for three generations (an eightfold increase in population). What is the molar ratio of hybrid DNA (^{15}N–^{14}N) to light DNA (^{14}N–^{14}N) at this point?

3. Replication of the *E. coli* Chromosome The *E. coli* chromosome contains 4,641,652 bp.

(a) How many turns of the double helix must be unwound during replication of the *E. coli* chromosome?

(b) Using the data in this chapter, how long would it take to replicate the *E. coli* chromosome at 37 °C if two replication forks proceeded from the origin? Assume replication occurs at a rate of 1,000 bp/s. Under some conditions, *E. coli* cells can divide every 20 min. How might this be possible?

(c) In the replication of the *E. coli* chromosome, about how many Okazaki fragments would be formed? What factors are required to link together newly synthesized Okazaki fragments in the lagging strand?

4. Base Composition of DNAs Made from Single-Stranded Templates Predict the base composition of the total DNA synthesized by DNA polymerase on templates provided by an equimolar mixture of the two complementary strands of bacteriophage ϕX174 DNA (a circular DNA molecule). The base composition of one strand is A, 24.7%; G, 24.1%; C, 18.5%; and T, 32.7%. What assumption is necessary to answer this problem?

5. DNA Replication Kornberg and his colleagues incubated soluble extracts of *E. coli* with a mixture of dATP, dTTP, dGTP, and dCTP, all labeled with ^{32}P in the α-phosphate group. After a time, they treated the incubation mixture with trichloroacetic acid, which precipitates the DNA but not the nucleotide precursors. They then collected the precipitate and determined the extent of precursor incorporation into DNA from the amount of radioactivity present in the precipitate.

(a) If any one of the four nucleotide precursors were omitted from the incubation mixture, would radioactivity be found in the precipitate? Explain.

(b) Would ^{32}P be incorporated into the DNA if only dTTP were labeled? Explain.

(c) Would radioactivity be found in the precipitate if ^{32}P labeled the β phosphate or γ phosphate rather than the α phosphate of the deoxyribonucleotides? Explain.

6. The Chemistry of DNA Replication All DNA polymerases synthesize new DNA strands in the 5′→3′ direction. In some respects, replication of the antiparallel strands of duplex DNA would be simpler if there were also a second type of polymerase, one that synthesized DNA in the 3′→5′ direction. The two types of polymerase could, in principle, coordinate DNA synthesis without the complicated mechanics required for lagging strand replication. However, no such 3′→5′-synthesizing enzyme has been found. Suggest two possible mechanisms for 3′→5′ DNA synthesis. Pyrophosphate should be one product of both proposed reactions. Could one or both mechanisms be supported in a cell? Why or why not? (Hint: You may suggest the use of DNA precursors not actually present in extant cells.)

7. Activities of DNA Polymerases You are characterizing a new DNA polymerase. When you incubate the enzyme with ^{32}P-labeled DNA and no dNTPs, you observe the release of $[^{32}P]$dNMPs. The addition of unlabeled dNTPs prevents this release. Explain the reactions that most likely underlie these observations. What would you expect to observe if you added pyrophosphate instead of dNTPs?

8. Leading and Lagging Strands Prepare a table that lists the names and compares the functions of the precursors, enzymes, and other proteins needed to make the leading strand versus the lagging strand during DNA replication in *E. coli*.

9. Function of DNA Ligase Some *E. coli* mutants contain defective DNA ligase. When researchers expose these mutants to ^{3}H-labeled thymine and then sediment the DNA produced on an alkaline sucrose density gradient, two radioactive bands appear. One corresponds to a high molecular weight fraction, the other to a low molecular weight fraction. Explain.

10. Fidelity of Replication of DNA What factors promote the fidelity of replication during synthesis of the leading strand of DNA? Would you expect the lagging strand to be made with the same fidelity? Give reasons for your answers.

11. Importance of DNA Topoisomerases in DNA Replication DNA unwinding, such as that occurring in replication, affects the superhelical density of DNA. In the absence of topoisomerases, the DNA would become overwound ahead of a replication fork as the DNA is unwound behind it. A bacterial replication fork will stall when the superhelical density (σ) of the DNA ahead of the fork reaches +0.14 (see Chapter 24).

An investigator initiates bidirectional replication at the origin of a 6,000 bp plasmid in vitro, in the absence of topoisomerases. The plasmid initially has a σ of -0.06. How many base pairs will be unwound and replicated by each replication fork before the forks stall? Assume that both forks travel at the same rate and that each includes all components necessary for elongation except topoisomerase.

12. The Ames Test In a nutrient medium that lacks histidine, a thin layer of agar containing ~10^9 *Salmonella typhimurium* histidine auxotrophs (mutant cells that require histidine to survive) produces ~13 colonies over a two-day incubation period at 37 °C (see Fig. 25-19). How do these colonies arise in the absence of histidine? When investigators repeat the experiment in the presence of 0.4 μg of 2-aminoanthracene, the number of colonies produced over two days exceeds 10,000. What does this indicate about 2-aminoanthracene? What can you surmise about its carcinogenicity?

13. DNA Repair Mechanisms Vertebrate and plant cells often methylate cytosine in DNA to form 5-methylcytosine (see Fig. 8-5a). In these same cells, a specialized repair system recognizes G–T mismatches and repairs them to G≡C base pairs. How might this repair system be advantageous to the cell? (Explain in terms of the presence of 5-methylcytosine in the DNA.)

14. The Energetic Cost of Mismatch Repair In an *E. coli* cell, DNA polymerase III makes a rare error and inserts a G opposite an A residue at a position 650 bp away from the nearest GATC sequence. The mismatch repair system accurately repairs the mismatch. How many phosphodiester bonds derived from deoxynucleotides (dNTPs) does this repair expend? This process also uses ATP molecules. Which enzyme(s) consume the ATP?

15. DNA Repair in People with Xeroderma Pigmentosum The condition known as xeroderma pigmentosum (XP) arises from mutations in at least seven different human genes (see Box 25-1). The deficiencies are generally in genes encoding enzymes involved in some part of the pathway for human nucleotide-excision repair. The various types of XP are denoted A through G (XPA, XPB, etc.), with a few additional variants lumped under the label XPV.

Investigators irradiate cultures of fibroblasts from healthy individuals and from patients with XPG with ultraviolet light. After isolating and denaturing the DNA, they characterize the resulting single-stranded DNA by analytical ultracentrifugation.

(a) Samples from the normal fibroblasts show a significant reduction in the average molecular weight of the single-stranded DNA after irradiation, but samples from the XPG fibroblasts show no such reduction. Why might this be?

(b) If you assume that a nucleotide-excision repair system is operative in fibroblasts, which step might be defective in the cells from the patients with XPG? Explain.

16. DNA Repair and Cancer Many pharmaceuticals used for tumor chemotherapy are DNA damaging agents. What is the rationale behind actively damaging DNA to address tumors? Why do such treatments often have a greater effect on a tumor than on healthy tissue?

17. Direct Repair Cells normally repair the lesion O^6-meG by directly transferring the methyl group to the protein O^6-methylguanine-DNA methyltransferase. For the nucleotide sequence AAC(O^6-meG)TGCAC, with a damaged (methylated) G residue, what would be the sequence of both strands of double-stranded DNA resulting from replication in each of the situations listed?

(a) Replication occurs before repair.

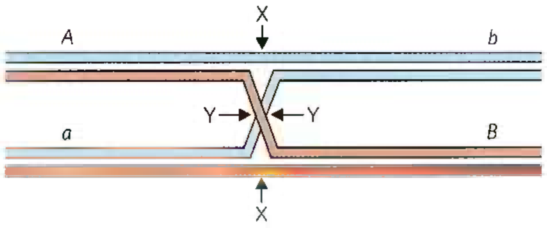
(b) Replication occurs after repair.

(c) Two rounds of replication occur, followed by repair.

18. Strand Invasion in Recombination A key step in many homologous recombination reactions is strand invasion (see step ② in Fig. 25-29). In almost every case, strand invasion proceeds with a single strand that has a free 3′ end rather than a 5′ end. What DNA metabolic advantage is inherent with the use of a free 3′ end for strand invasion?

19. Holliday Intermediates How does the formation of Holliday intermediates in homologous genetic recombination differ from their formation in site-specific recombination?

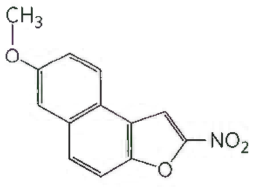
20. Cleavage of Holliday Intermediates A Holliday intermediate forms between two homologous chromosomes, at a point between genes *A* and *B*, as shown. The chromosomes have different alleles of the two genes (*A* and *a*, *B* and *b*). Where would the Holliday intermediate have to be cleaved (points X and/or Y) to generate a chromosome that would carry **(a)** an *Ab* genotype or **(b)** an *ab* genotype?



21. A Connection between Replication and Site-Specific Recombination Most wild strains of *S. cerevisiae* have multiple copies of the circular DNA plasmid 2μ (named for its contour length of about 2 μm), which has ~6,300 bp. For its replication, the plasmid uses the host replication system, under the same strict control as the host cell chromosomes, replicating only once per cell cycle. Replication of the plasmid is bidirectional, with both replication forks initiating at a single, well-defined origin. However, one replication cycle of a 2μ plasmid can result in more than two copies of the plasmid, allowing amplification of the plasmid copy number (number of plasmid copies per cell) whenever plasmid segregation at cell division leaves one daughter cell with fewer than the normal complement of plasmid copies. Amplification requires a site-specific recombination system encoded by the plasmid, which serves to invert one part of the plasmid relative to the other. Explain how a site-specific inversion event could result in amplification of the plasmid copy number. (Hint: Consider the situation when replication forks have duplicated one recombination site but not the other.)

DATA ANALYSIS PROBLEM

22. Mutagenesis in *Escherichia coli* Many mutagenic compounds act by alkylating the bases in DNA. The alkylating agent R7000 (7-methoxy-2-nitronaphtho[2,1-*b*]furan) is an extremely potent mutagen.

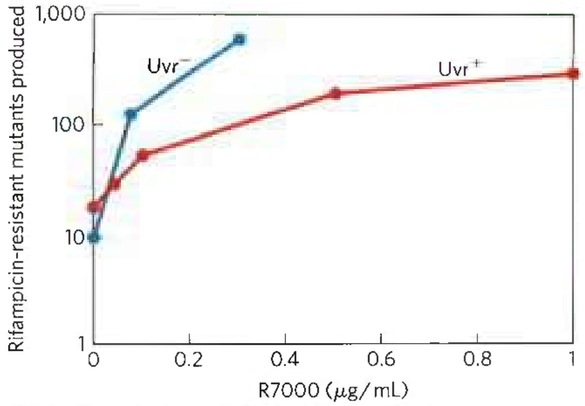


R7000

In vivo, R7000 is activated by the enzyme nitroreductase, and this more reactive form covalently attaches to DNA—primarily, but not exclusively, to G≡C base pairs.

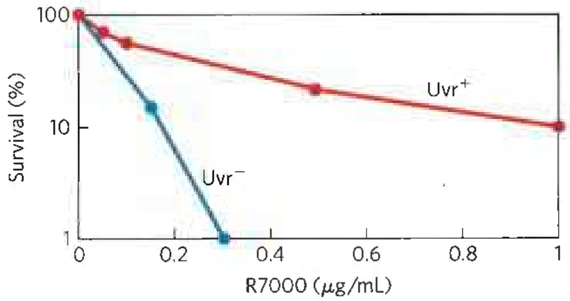
In a 1996 study, Quillardet, Touati, and Hofnung explored the mechanisms by which R7000 causes mutations in *E. coli*. They compared the genotoxic activity of R7000 in two strains of *E. coli*: the wild-type (Uvr$^+$) and mutants lacking *uvrA* activity (Uvr$^-$). They first measured rates of mutagenesis. Rifampicin is an inhibitor of RNA polymerase. In its presence, cells will not grow unless certain mutations occur in the gene encoding RNA polymerase; the appearance of rifampicin-resistant colonies thus provides a useful measure of mutagenesis rates.

The investigators determined the effects of different concentrations of R7000. Their results are shown in the following graph:



(a) Why are some mutants produced even when no R7000 is present?

Quillardet and colleagues also measured the survival rate of bacteria treated with different concentrations of R7000, with the following results:



(b) Explain how treatment with R7000 is lethal to cells.

(c) Explain the differences in the mutagenesis curves and in the survival curves for the two types of bacteria, Uvr$^+$ and Uvr$^-$, as shown in the graphs.

The researchers went on to measure the amount of R7000 covalently attached to the DNA in Uvr$^+$ and Uvr$^-$ *E. coli*. They incubated bacteria with [^{3}H]R7000 for 10 or 70 minutes, extracted the DNA, and measured its ^{3}H content in counts per minute (cpm) per microgram of DNA.

	^{3}H in DNA (cpm/μg)	
Time (min)	**Uvr$^+$**	**Uvr$^-$**
10	76	159
70	69	228

(d) Explain why the amount of ^{3}H drops over time in the Uvr$^+$ strain and rises over time in the Uvr$^-$ strain.

Quillardet and colleagues then examined the particular DNA sequence changes caused by R7000 in the Uvr$^+$ and Uvr$^-$ bacteria. For this, they used six different strains of *E. coli*, each with a different point mutation in the *lacZ* gene, which encodes β-galactosidase. Cells with any of these mutations have a nonfunctional β-galactosidase and are unable to metabolize lactose (i.e., a Lac$^-$ phenotype). Each type of point mutation required a specific reverse mutation to restore *lacZ* gene function and Lac$^+$ phenotype. By plating cells on a medium containing lactose as the sole carbon source, the researchers selected for these reverse-mutated, Lac$^+$ cells. And by counting the number of Lac$^+$ cells following mutagenesis of a particular strain, they could measure the frequency of each type of mutation.

First, they looked at the mutation spectrum in Uvr$^-$ cells. The following table shows the results for the six strains, CC101 through CC106 (with the point mutation required to produce Lac$^+$ cells indicated in parentheses).

Number of Lac$^+$ cells (average ± SD)

R7000 (μg/mL)	CC101 (A=T to C≡G)	CC102 (G≡C to A=T)	CC103 (G≡C to C≡G)	CC104 (G≡C to T=A)	CC105 (A=T to T=A)	CC106 (A=T to G≡C)
0	6 ± 3	11 ± 9	2 ± 1	5 ± 3	2 ± 1	1 ± 1
0.075	24 ± 19	34 ± 3	8 ± 4	82 ± 23	40 ± 14	4 ± 2
0.15	24 ± 4	26 ± 2	9 ± 5	180 ± 71	130 ± 50	3 ± 2

(e) Which types of mutation show significant increases above the background rate due to treatment with R7000? Provide a plausible explanation for why some have higher frequencies than others.

(f) Can all of the mutations you listed in (e) be explained as resulting from covalent attachment of R7000 to a G≡C base pair? Explain your reasoning.

(g) Figure 25-26b shows how methylation of guanine residues can lead to a G≡C to A=T mutation. Using a similar pathway, show how an R7000–G adduct could lead to the G≡C to A=T or T=A mutations shown above. Which base pairs with the R7000–G adduct?

The results for the Uvr$^+$ bacteria are shown in the following table.

Number of Lac$^+$ cells (average ± SD)

R7000 (μg/mL)	CC101 (A=T to C≡G)	CC102 (G≡C to A=T)	CC103 (G≡C to C≡G)	CC104 (G≡C to T=A)	CC105 (A=T to T=A)	CC106 (A=T to G≡C)
0	2 ± 2	10 ± 9	3 ± 3	4 ± 2	6 ± 1	0.5 ± 1
1	7 ± 6	21 ± 9	8 ± 3	23 ± 15	13 ± 1	1 ± 1
5	4 ± 3	15 ± 7	22 ± 2	68 ± 25	67 ± 14	1 ± 1

(h) Do these results show that all mutation types are repaired with equal fidelity? Provide a plausible explanation for your answer.

Reference

Quillardet, P., E. Touati, and M. Hofnung. 1996. Influence of the *uvr*-dependent nucleotide-excision repair on DNA adducts formation and mutagenic spectrum of a potent genotoxic agent: 7-methoxy-2-nitronaphtho[2,1-*b*]furan (R7000). *Mutat. Res.* 358:113–122.

Chapter 26

RNA METABOLISM

26.1 DNA-Dependent Synthesis of RNA *961*

26.2 RNA Processing *972*

26.3 RNA-Dependent Synthesis of RNA and DNA *988*

26.4 Catalytic RNAs and the RNA World Hypothesis *995*

Expression of the information in a gene generally involves production of an RNA molecule transcribed from a DNA template. Strands of RNA and DNA may seem similar at first glance, differing only in that RNA has a hydroxyl group at the 2′ position of the aldopentose, and uracil usually replaces thymine. However, unlike DNA, most RNAs carry out their functions as single strands, strands that fold back on themselves and have the potential for much greater structural diversity than DNA (Chapter 8). RNA is thus suited to a variety of cellular functions.

RNA is the only macromolecule known to have a role both in the storage and transmission of information and in catalysis, which has led to much speculation about its possible role as an essential chemical intermediate in the development of life on this planet. The discovery of catalytic RNAs, or **ribozymes**, has changed the very definition of an enzyme, extending it beyond the domain of proteins. Proteins nevertheless remain essential to RNA and its cellular functions. In the biosphere of today, all nucleic acids, including RNAs, are complexed with proteins. In the case of RNA, these complexes are called **ribonucleoproteins** or **RNPs**. Some of these RNPs are quite elaborate, and RNA can assume both structural and catalytic roles within complicated biochemical machines.

All RNA molecules except the RNA genomes of certain viruses are derived from information permanently stored in DNA. During **transcription**, an enzyme system converts the genetic information in a segment of double-stranded DNA into an RNA strand with a base sequence complementary to one of the DNA strands. Four major kinds of RNA are produced. **Messenger RNAs (mRNAs)** encode the amino acid sequence of one or more polypeptides specified by a gene or set of genes. **Transfer RNAs (tRNAs)** read the information encoded in the mRNA and transfer the appropriate amino acid to a growing polypeptide chain during protein synthesis. **Ribosomal RNAs (rRNAs)** are constituents of ribosomes, the intricate cellular machines that synthesize proteins. **Noncoding RNAs (ncRNAs)** have a variety of catalytic, structural, and regulatory functions.

During replication the entire chromosome is usually copied, but transcription is more selective. Only particular genes or groups of genes are transcribed at any one time, and some portions of the DNA genome are never transcribed. The cell restricts the expression of genetic information to the formation of gene products needed at any particular moment. The sum of all the RNA molecules produced in a cell under a given set of conditions is called the cellular **transcriptome**. Given the relatively small fraction of the human genome devoted to protein-coding genes (about 2%), we might expect that only a small part of the human genome is transcribed. This is not the case. Transcriptome analyses have revealed that approximately 76% of the human genome is transcribed into RNA. The products are predominantly not mRNAs but rather ncRNAs. Many ncRNAs are involved in regulating gene expression by interaction with other RNAs, genomic DNA, or proteins. However, the rapid pace of their discovery has forced us to realize that we do not yet know the function of the majority of our genomic ncRNA transcripts.

In this chapter we examine the synthesis of RNA on a DNA template and the postsynthetic processing, location, and turnover of RNA molecules. In doing so, we encounter many of the specialized functions of RNA, including catalytic functions. We also describe systems in which RNA is the template and DNA the product, rather than vice versa. The information pathways thus come full circle and reveal that template-dependent nucleic acid synthesis has standard rules, regardless of the nature of template or product (RNA or DNA). This examination of the biological interconversion of DNA and RNA as information carriers leads inevitably to a discussion of the evolutionary origin of biological information and processing. We will be guided by four principles.

P1 **RNA is synthesized by RNA polymerases using DNA templates and ribonucleoside 5′-triphosphates.** RNA is made in the 5′→3′ direction and complementary to the template DNA strand. Transcription is highly regulated and initiates by recruitment of the transcription machinery to gene promoters. Although bacterial and eukaryotic polymerases share many conserved features, the transcriptional machinery and its regulation are much more complex in eukaryotes.

P2 **Many RNAs must be modified and processed to become functional.** RNAs can be modified by nucleases, by excision of certain RNA segments, and/or by chemical modification of the RNA nucleotides. In humans, nearly all mRNAs are 5′ capped, spliced, and polyadenylated before being exported from the nucleus for translation in the cytoplasm.

P3 **RNA can be used as a template for synthesis of DNA by reverse transcriptases.** RNA carries genetic information that can be reverse transcribed into DNA. Retroviruses, such as HIV or those responsible for some cancers, must convert their RNA genomes into DNA by reverse transcription. Reverse transcription by telomerase is also responsible for producing the telomeres that protect the DNA found at the ends of eukaryotic chromosomes.

P4 **RNA can act as both a catalyst and carrier of genetic information.** Ribozymes can catalyze chemical transformations using many of the same strategies as protein-based enzymes. The dual capacity of RNA as both a carrier of genetic information and a catalyst is support for the RNA world hypothesis for the evolution of life on Earth.

26.1 DNA-Dependent Synthesis of RNA

Our discussion of RNA synthesis begins with a comparison between transcription and DNA replication (Chapter 25). Transcription resembles replication in its fundamental chemical mechanism, its polarity (direction of synthesis), and its use of a template. And like replication, transcription has initiation, elongation, and termination phases. Transcription differs from replication in that it does not require a primer and, generally, involves only limited segments of a DNA molecule. Additionally, only one DNA strand serves as a template for a particular RNA molecule.

RNA Is Synthesized by RNA Polymerases

The discovery of DNA polymerase and its dependence on a DNA template spurred a search for an enzyme that synthesizes RNA complementary to a DNA strand. By 1960, four research groups had independently detected an enzyme in cellular extracts that could form an RNA polymer from ribonucleoside 5′-triphosphates. Subsequent work on the purified *Escherichia coli* RNA polymerase helped to define the fundamental properties of transcription (**Fig. 26-1**). P1 **DNA-dependent RNA polymerase** requires, in addition to a DNA template, all four ribonucleoside 5′-triphosphates (ATP, GTP, UTP, and CTP) as precursors of the nucleotide units of RNA, as well as Mg^{2+}. The chemistry and mechanism of RNA synthesis closely resemble those used by DNA polymerases (see Fig. 25-3). RNA polymerase elongates an RNA strand by adding ribonucleotide units to the 3′-hydroxyl end, building RNA in the 5′→3′ direction. The 3′-hydroxyl group acts as a nucleophile, attacking the α phosphate of the incoming ribonucleoside triphosphate (Fig. 26-1a) and releasing pyrophosphate. The overall reaction is

$$\underset{\text{RNA}}{(\text{NMP})_n} + \text{NTP} \longrightarrow \underset{\text{Lengthened RNA}}{(\text{NMP})_{n+1}} + \text{PP}_\text{i}$$

RNA polymerase requires DNA for activity and is most active when bound to a double-stranded DNA. As noted above, only one of the two DNA strands serves as a template. The template DNA strand is copied in the 3′→5′ direction (antiparallel to the new RNA strand), just as in DNA replication. Each nucleotide in the newly formed RNA is selected by Watson-Crick base-pairing interactions: U residues are inserted in the RNA to pair with A residues in the DNA template, G residues are inserted to pair with C residues, and so on. Base-pair geometry (see Fig. 25-5) may also play a role in base selection.

Unlike DNA polymerase, RNA polymerase does not require a primer to initiate synthesis. Initiation occurs when RNA polymerase binds at specific DNA sequences called promoters (described below). The 5′-triphosphate group of the first residue in a nascent (newly formed) RNA molecule is not cleaved to release PP_i, but instead remains intact and functions in eukaryotes as a substrate for the RNA-capping machinery (see Fig. 26-13). During the elongation phase of transcription, the growing end of the new RNA strand base-pairs temporarily with the DNA template to form a short hybrid RNA-DNA double helix, about 8 bp long (Fig. 26-1b). The RNA in this hybrid duplex "peels off" shortly after its formation, and the DNA duplex re-forms.

To enable RNA polymerase to synthesize an RNA strand complementary to one of the DNA strands, the DNA duplex must unwind over a short distance, forming a transcription "bubble." During transcription, the *E. coli* RNA polymerase generally keeps about 17 bp unwound. The 8 bp RNA-DNA hybrid occurs in this unwound region. Elongation of a transcript by *E. coli* RNA polymerase proceeds at a rate of 50 to 90 nucleotides/s. Because DNA is a helix, movement of a transcription bubble requires considerable strand rotation of the nucleic acid molecules. DNA strand rotation is restricted in most DNAs by DNA-binding proteins and other structural barriers. As a result, a moving RNA polymerase generates

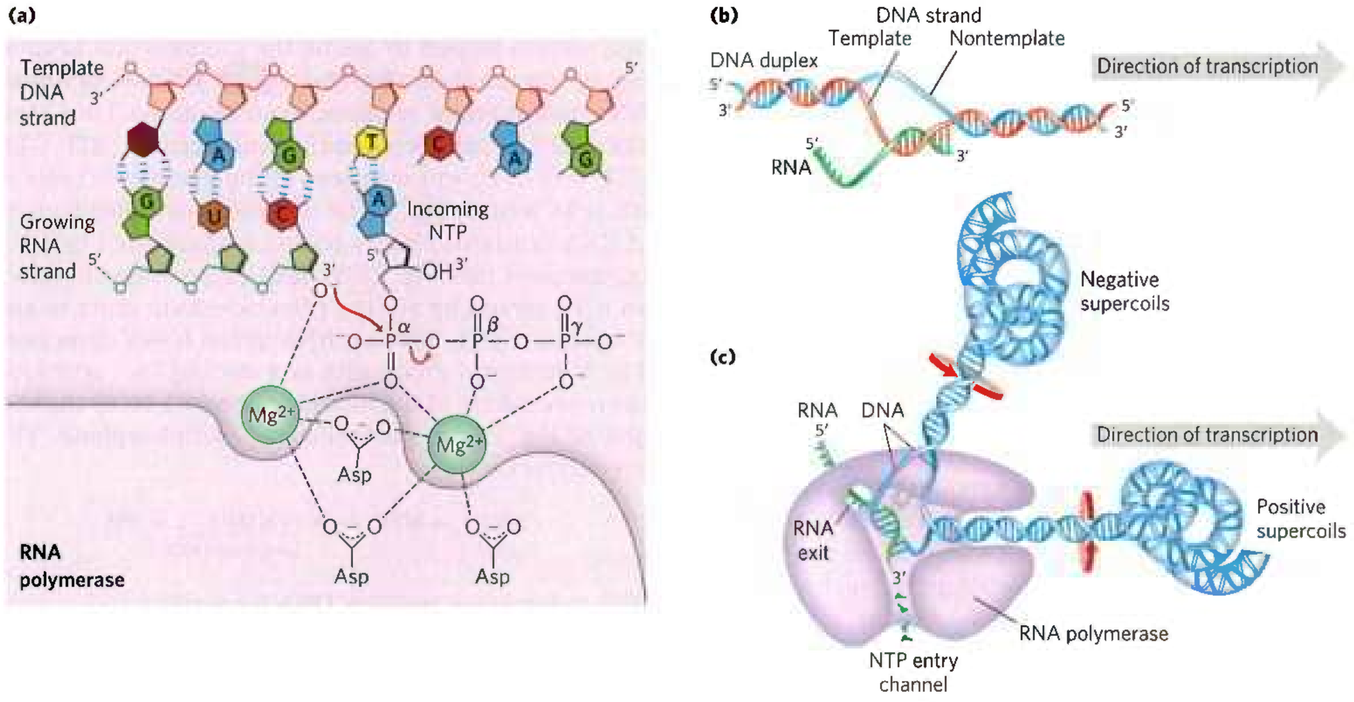


FIGURE 26-1 Transcription by RNA polymerase in *E. coli*. For synthesis of an RNA strand complementary to one of two DNA strands in a double helix, the DNA is transiently unwound. (a) Catalytic mechanism of RNA synthesis by RNA polymerase. Notice that this is essentially the same mechanism used by DNA polymerases. The reaction involves two Mg^{2+} ions coordinated to the phosphate groups of the incoming nucleoside triphosphates (NTPs) and to three Asp residues, which are highly conserved in the RNA polymerases of all species. One Mg^{2+} ion facilitates attack by the 3′-hydroxyl group on the α phosphate of the NTP; the other Mg^{2+} ion facilitates displacement of the pyrophosphate. Both metal ions stabilize the pentacovalent transition state. (b) About 17 bp of DNA are unwound at any given time. RNA polymerase and the transcription bubble move from left to right along the DNA as shown, facilitating RNA synthesis. The DNA is unwound ahead and rewound behind as RNA is transcribed. As the DNA is rewound, the RNA-DNA hybrid is displaced and the RNA strand is extruded. (c) Movement of an RNA polymerase along DNA tends to create positive supercoils (overwound DNA) ahead of the transcription bubble and negative supercoils (underwound DNA) behind it. The RNA polymerase is in close contact with the DNA ahead of the transcription bubble as well as with the separated DNA strands and the RNA within and immediately behind the bubble. A channel in the protein funnels new NTPs to the polymerase active site. The polymerase footprint encompasses about 35 bp of DNA during elongation.

waves of positive supercoils ahead of the transcription bubble and negative supercoils behind (Fig. 26-1c). This has been observed both in vitro and in vivo (in bacteria). In the cell, the topological problems caused by transcription are relieved through the action of topoisomerases (Chapter 24).

KEY CONVENTION The two complementary DNA strands have different roles in transcription. The strand that serves as template for RNA synthesis is called the **template strand.** The DNA strand complementary to the template, the **nontemplate strand,** or **coding strand,** is identical in base sequence to the RNA transcribed from the gene, with U in the RNA in place of T in the DNA (Fig. 26-2). The coding strand for a particular gene may be located in either strand of a given chromosome (as shown in Fig. 26-3 for a virus). By convention, the regulatory sequences that control transcription (described later in this chapter) are designated by the sequences in the coding strand. ■

The DNA-dependent RNA polymerase of *E. coli* is a large, complex enzyme with five core subunits ($\alpha_2\beta\beta'\omega$; M_r 390,000) and a sixth subunit, one of a group designated

(5′) C G C T A T A G C G T T T (3′)	DNA nontemplate (coding) strand
(3′) G C G A T A T C G C A A A (5′)	DNA template strand
(5′) C G C U A U A G C G U U U (3′)	RNA transcript

FIGURE 26-2 Template and nontemplate (coding) DNA strands. The two complementary strands of DNA are defined by their function in transcription. The RNA transcript is synthesized on the template strand and is identical in sequence (with U in place of T) to the nontemplate strand, or coding strand.

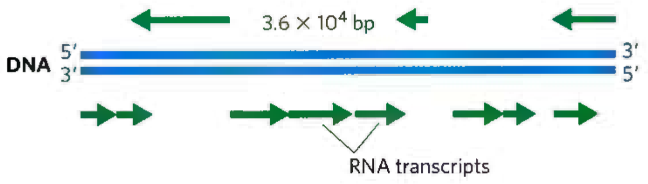


FIGURE 26-3 Organization of coding information in the adenovirus genome. The genetic information of the adenovirus genome is encoded by a double-stranded DNA molecule of 36,000 bp, both strands of which encode proteins. The information for most proteins is encoded by (that is, identical to) the top strand — by convention, the strand is oriented 5′ to 3′ from left to right. The bottom strand acts as template for these transcripts. However, a few proteins are encoded by the bottom strand, which is transcribed in the opposite direction (and uses the top strand as template).

σ, with variants designated by size (molecular weight). The σ subunit binds transiently to the core and directs the enzyme to specific binding sites on the DNA (described below). These six subunits constitute the RNA polymerase holoenzyme (**Fig. 26-4**). The RNA polymerase holoenzyme of *E. coli* thus exists in several forms, depending on the type of σ subunit. The most common subunit is σ^{70} (M_r 70,000), and the upcoming discussion focuses on the corresponding RNA polymerase holoenzyme.

RNA polymerases lack a separate proofreading $3' \rightarrow 5'$ exonuclease active site (such as that of many DNA polymerases), and the error rate for transcription is higher than that for chromosomal DNA replication—approximately one error for every 10^4 to 10^5 ribonucleotides incorporated into RNA. Because many copies of an RNA are generally produced from a single gene, and nearly all RNAs are eventually degraded and replaced, a mistake in an RNA molecule is of less consequence to the cell than a mistake in the permanent information stored in DNA. Many RNA polymerases, including bacterial RNA polymerase and the eukaryotic RNA polymerase II (discussed below), do pause when a mispaired base is added during transcription, and they can remove mismatched nucleotides from the 3′ end of a transcript by direct reversal of the polymerase reaction. But we do not yet know whether this activity is a true proofreading function and to what extent it may contribute to the fidelity of transcription.

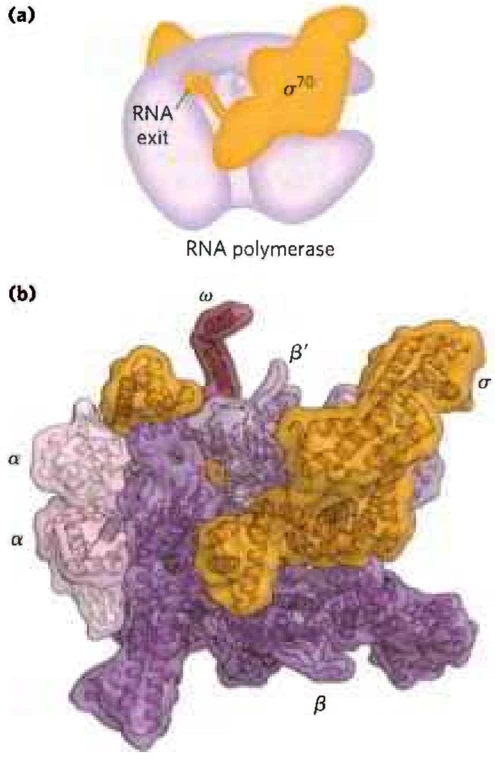


FIGURE 26-4 Structure of the σ^{70} RNA polymerase holoenzyme of *E. coli*. (a) The several subunits of the bacterial RNA polymerase give the enzyme the shape of a crab claw (purple). The σ^{70} subunit rests on top of the crab claw and threads through the RNA exit channel. (b) In this crystal structure of the RNA polymerase holoenzyme, each of the six subunits ($\alpha_2\beta\beta'\omega$ and σ^{70}) can be identified. The pincers of the crab claw are formed by the β and β' subunits. [Data from PDB ID 4MEY, D. Degen et al., *eLife* 3:e02451, 2014.]

RNA Synthesis Begins at Promoters

Initiation of RNA synthesis at random points in a DNA molecule would be an extraordinarily wasteful process. **P1** Instead, an RNA polymerase binds to specific sequences in the DNA called **promoters**, which direct the transcription of adjacent segments of DNA (genes). The sequences where RNA polymerases bind are variable, and much research has focused on identifying the particular sequences that are critical to promoter function.

In *E. coli*, RNA polymerase binding occurs within a region stretching from about 70 bp before the transcription start site to about 30 bp beyond it. By convention, the DNA base pairs that correspond to the beginning of an RNA molecule are given positive numbers, and those preceding the RNA start site are given negative numbers. The promoter region thus extends between positions −70 and +30. Analyses and comparisons of the most common class of bacterial promoters (those recognized by an RNA polymerase holoenzyme containing σ^{70}) have revealed consensus sequences centered about positions −10 and −35 (**Fig. 26-5a**). Although the sequences are not identical for all bacterial promoters in this class, certain nucleotides that are particularly common at each position form a **consensus sequence**. The consensus sequence at the −10 region is (5′)TATAAT(3′); at the −35 region it is (5′)TTGACA(3′). A third AT-rich recognition element, called the UP (upstream promoter) element, occurs between positions −40 and −60 in the promoters of certain highly expressed genes. The UP element is bound by the α subunit of RNA polymerase. The efficiency with which an RNA polymerase containing σ^{70} binds to a promoter and initiates transcription is determined in large measure by these sequences, the spacing between them, and their distance from the transcription start site. A change in only one base pair in the promoter can decrease the rate of binding by several orders of magnitude. The promoter sequence thus establishes a basal level of expression that can vary greatly from one *E. coli* gene to the next. The x-ray crystal structure of the σ^{70} RNA polymerase holoenzyme bound to its promoter shows how the σ factor recognizes both the RNA polymerase and the −10 and −35 regions by introducing a large bend in the DNA (Fig. 26-5b). Information about these interactions can also be obtained using the method illustrated in **Box 26-1**.

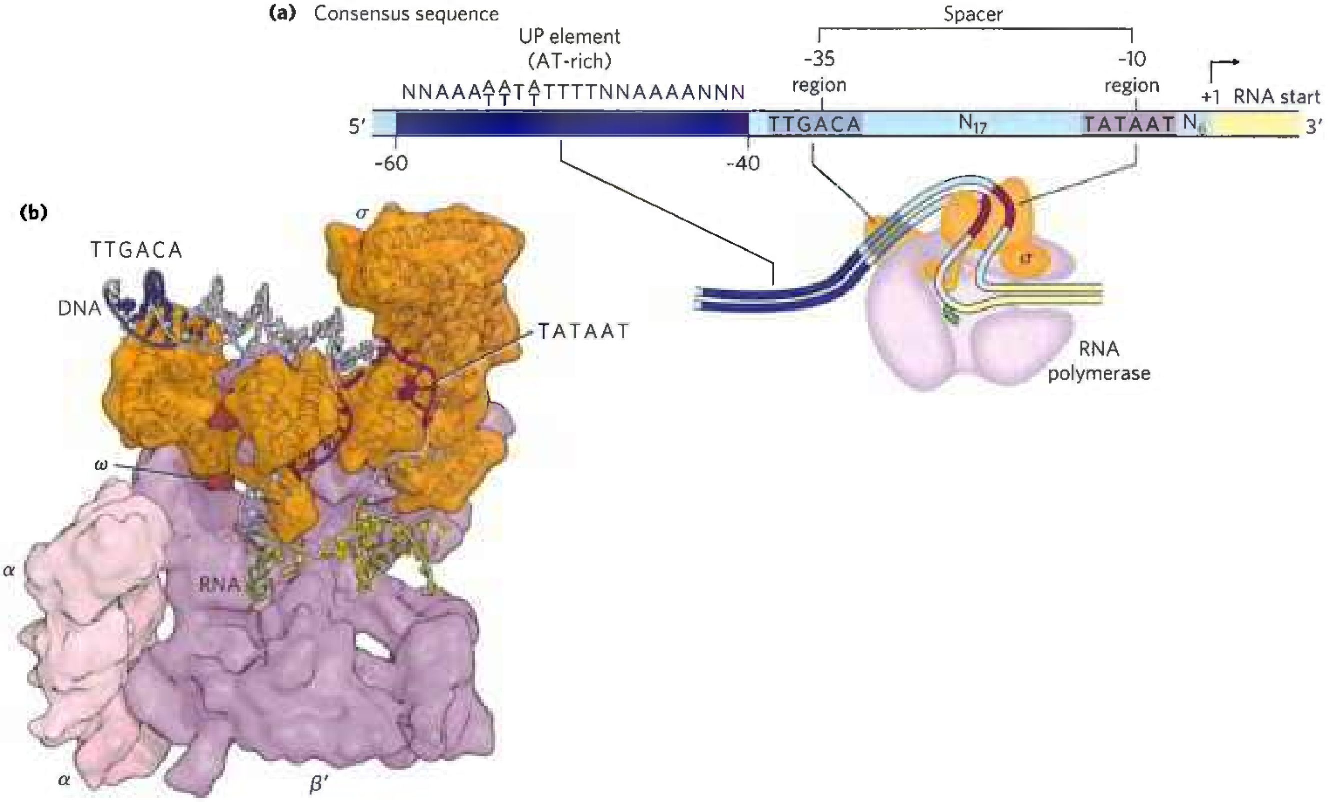


FIGURE 26-5 Promoter recognition by RNA polymerase holoenzymes containing σ^{70}. (a) The nontemplate strand of the consensus sequence for *E. coli* promoters recognized by σ^{70} is shown, read in the 5′→3′ direction, as is the convention for representations of this kind. The sequences differ from one promoter to the next, but comparisons of many promoters reveal similarities, particularly in the −10 and −35 regions. The sequence element UP, not present in all *E. coli* promoters, generally occurs in the region between −40 and −60 and strongly stimulates transcription at the promoters that contain them. Spacer regions contain slightly variable numbers of nucleotides (N). Only the first nucleotide coding the RNA transcript (at position +1) is shown. (b) This x-ray crystallographic structure of the *E. coli* holoenzyme bound to a promoter shows that the σ^{70} subunit introduces a sharp bend in the DNA template, allowing it to simultaneously contact the −35 and −10 regions as well as the RNA polymerase core. In this view the *β* subunit was omitted to allow the path of the DNA through the polymerase to be more easily seen. Due to the low resolution of the structure (5.5 Å) not all of the DNA backbone could be modeled, and these unmodeled regions are missing from the DNA shown in the figure. A cartoon schematic of the structure is also shown below (a), aligned with consensus promoter elements. [Data from PDB ID 4YLN, Y. Zuo and T. A. Steitz, *Mol. Cell* 58:534, 2015.]

The pathway of transcription initiation and the fate of the σ subunit are illustrated in **Figure 26-6**. The pathway consists of two major parts, binding and initiation, each with multiple steps. First, the polymerase, directed by its bound σ factor, binds to the promoter. A **closed complex** (in which the bound DNA remains double-stranded) and an **open complex** (in which the bound DNA is partially unwound near the −10 sequence) form in succession. Second, transcription is initiated within the complex, leading to a conformational change that converts the complex to the elongation form, followed by movement of the transcription complex away from the promoter (promoter clearance). Any of these steps can be affected by the specific makeup of the promoter sequences. The σ subunit dissociates at random as the polymerase enters the elongation phase of transcription. The protein NusA (M_r 54,430) binds to the elongating RNA polymerase, competitively with the σ subunit. Once transcription is complete, NusA dissociates from the enzyme, the RNA polymerase dissociates from the DNA, and a σ factor (σ^{70} or another) can again bind to the enzyme to initiate transcription.

E. coli has other classes of promoters bound by RNA polymerase holoenzymes with different σ subunits, such as the promoters of the heat shock genes. The products of this set of genes are made at higher levels when the cell is exposed to environmental stress, such as a sudden increase in temperature. RNA polymerase binds to the promoters of these genes only when σ^{70} is replaced with the σ^{32} (M_r 32,000) subunit, which is specific for the heat shock promoters (see Fig. 28-3). By using different σ subunits, the cell can coordinate the expression of sets of genes, permitting major changes in cell physiology. Which sets of genes are expressed is determined by the availability of the various σ subunits. This, in turn, is determined by several factors: regulated rates of synthesis and degradation, posttranslational modifications that switch individual σ subunits between active and inactive forms, and a specialized class of anti-σ proteins, each type binding to and sequestering a particular σ subunit to render it unavailable for transcription initiation.

BOX 26-1 METHODS

RNA Polymerase Leaves Its Footprint on a Promoter

Footprinting, a technique derived from principles used in DNA sequencing, identifies the DNA sequences bound by a particular protein. Researchers isolate a DNA fragment thought to contain sequences recognized by a DNA-binding protein, and then radiolabel one end of one strand (Fig. 1). They then use chemical or enzymatic reagents to introduce random breaks in the DNA fragment (averaging about one break per molecule). Separation of the labeled cleavage products (broken fragments of various lengths) by high-resolution electrophoresis produces a ladder of radioactive bands. In a separate tube, the cleavage procedure is repeated on copies of the same DNA fragment in the presence of the DNA-binding protein. The researchers then subject the two sets of cleavage products to electrophoresis and compare them side by side. A gap ("footprint") in the series of radioactive bands derived from the DNA-protein sample, attributable to protection of the DNA by the bound protein, identifies the sequences that the protein binds.

The precise location of the protein-binding site can be determined by directly sequencing (see Fig. 8-35) copies of the same DNA fragment and including the sequencing lanes (not shown here) on the same gel with the footprint. Figure 2 shows footprinting results for the binding of RNA polymerase to a DNA fragment containing a promoter. The polymerase covers 60 to 80 bp; protection by the bound enzyme includes the −10 and −35 regions.

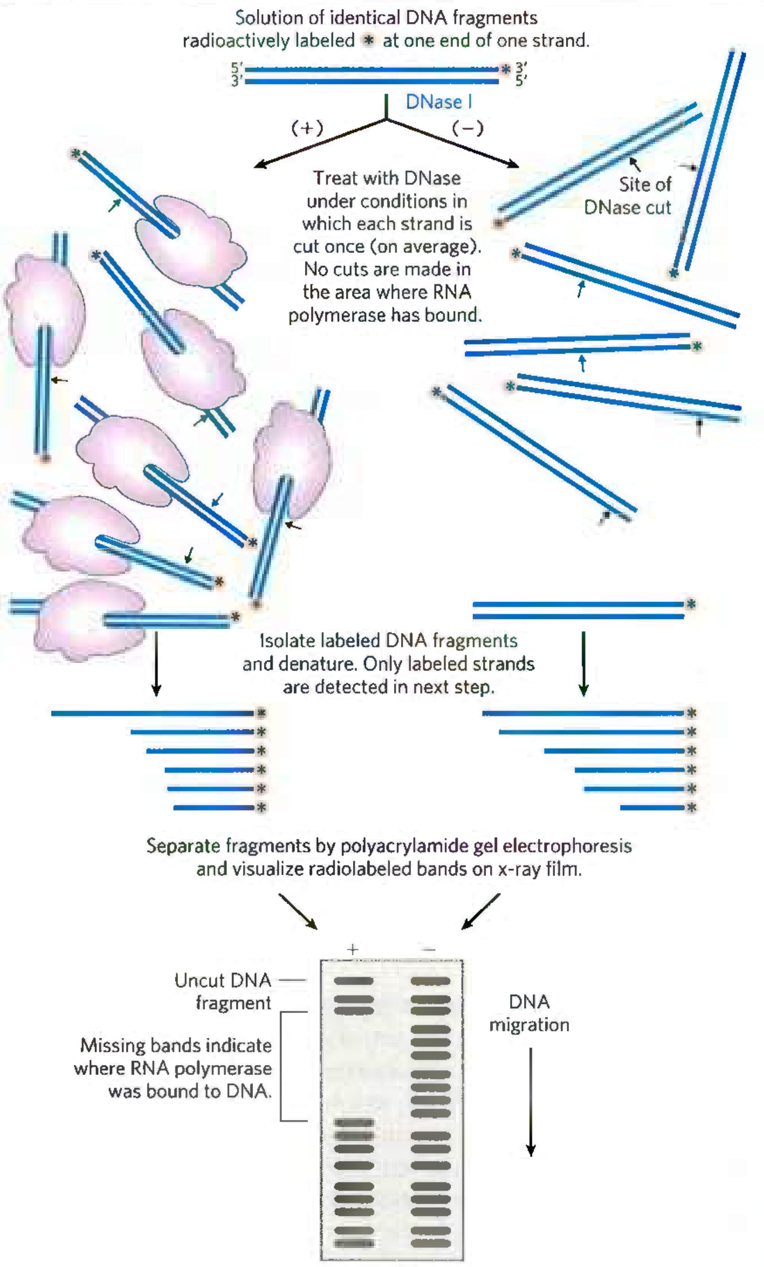

Solution of identical DNA fragments radioactively labeled ✱ at one end of one strand.
5′
3′
3′
5′
DNase I
(+)
(−)
Treat with DNase under conditions in which each strand is cut once (on average). No cuts are made in the area where RNA polymerase has bound.
Site of DNase cut
Isolate labeled DNA fragments and denature. Only labeled strands are detected in next step.
Separate fragments by polyacrylamide gel electrophoresis and visualize radiolabeled bands on x-ray film.
+
−
Uncut DNA fragment
Missing bands indicate where RNA polymerase was bound to DNA.
DNA migration

FIGURE 1 Footprint analysis of the RNA polymerase–binding site on a DNA fragment. Separate experiments are carried out in the presence (+) and absence (−) of the polymerase.

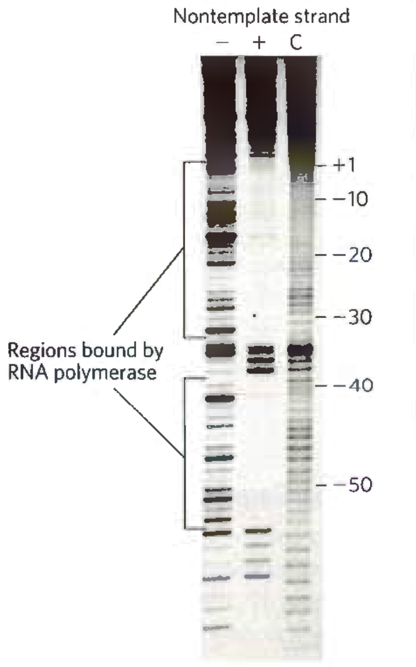
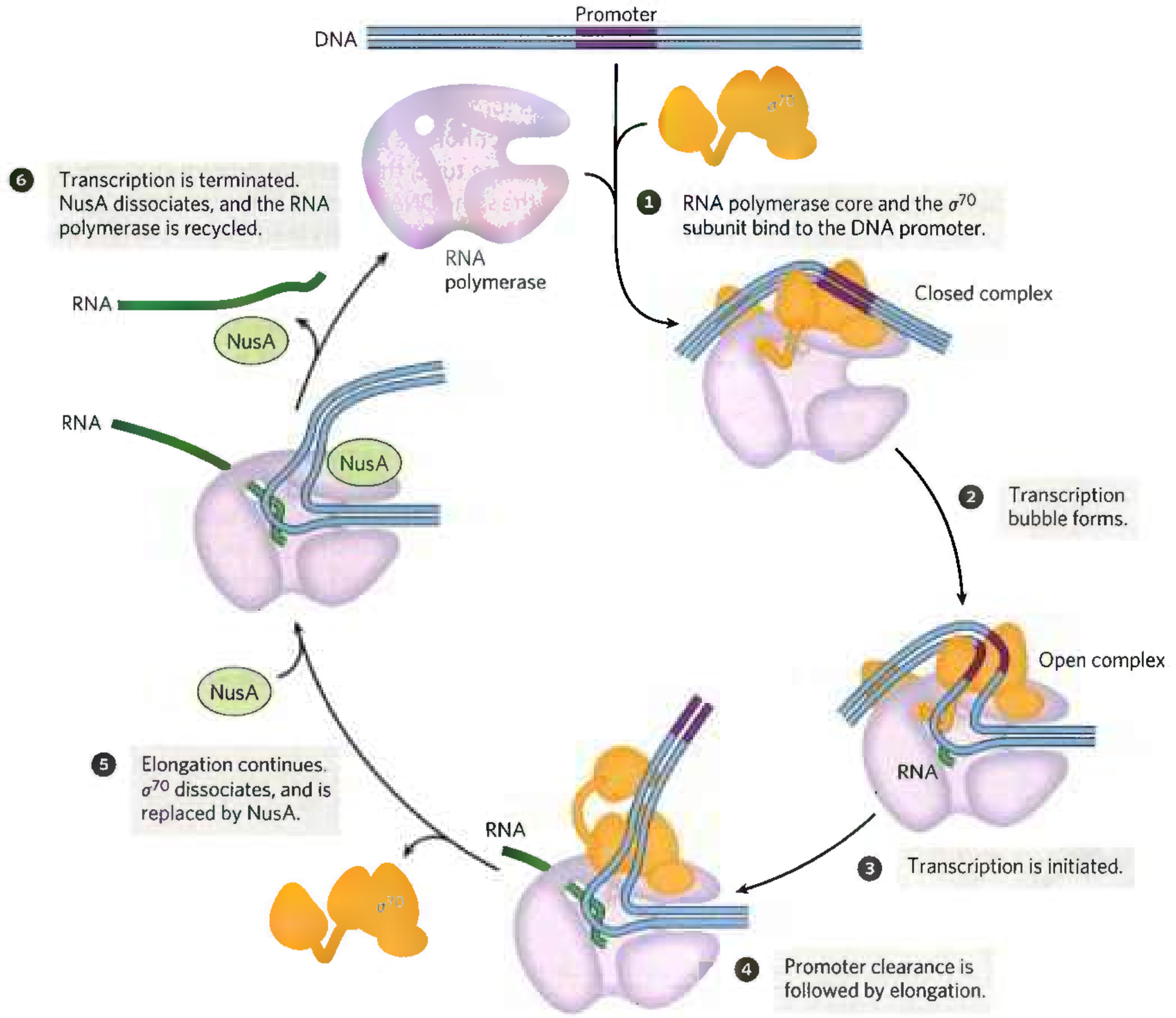

Nontemplate strand
−
+
C
+1
−10
−20
−30
−40
−50
Regions bound by RNA polymerase

FIGURE 2 Footprinting results of RNA polymerase binding to the *lac* promoter. In this experiment, the 5′ end of the nontemplate strand was radioactively labeled. Lane C is a control in which the labeled DNA fragments were cleaved with a chemical reagent that produces a more uniform banding pattern. [© Courtesy of Carol Gross Laboratory.]



① RNA polymerase core and the σ^{70} subunit bind to the DNA promoter.

② Transcription bubble forms.

③ Transcription is initiated.

④ Promoter clearance is followed by elongation.

⑤ Elongation continues. σ^{70} dissociates, and is replaced by NusA.

⑥ Transcription is terminated. NusA dissociates, and the RNA polymerase is recycled.

FIGURE 26-6 Transcription initiation and elongation by *E. coli* RNA polymerase. Initiation of transcription requires several steps generally divided into two phases: binding and initiation. In the binding phase, the initial interaction of the RNA polymerase with the promoter leads to formation of a closed complex, in which the promoter DNA is stably bound but not unwound. A 12 to 15 bp region of DNA—from within the −10 region to position +2 or +3—is then unwound to form an open complex. Additional intermediates (not shown) have been detected in the pathways leading to the closed and open complexes, along with several changes in protein conformation. The initiation phase encompasses promoter binding, transcription initiation, and promoter clearance (steps ① through ④ here). Once elongation commences, the σ subunit is released and is replaced by the protein NusA. The polymerase leaves the promoter and becomes committed to elongation of the RNA (step ⑤). When transcription is complete, the RNA is released, the NusA protein dissociates, and the RNA polymerase dissociates from the DNA (step ⑥). Another σ subunit binds to the RNA polymerase and the process begins again.

Transcription Is Regulated at Several Levels

Requirements for any gene product vary with cellular conditions or developmental stage, and transcription of each gene is carefully regulated to form gene products only in the proportions needed. P1 Regulation can occur at any step of transcription, including elongation and termination. However, much of the regulation is directed at the polymerase binding and transcription initiation steps outlined in Figure 26-6. Differences in promoter sequences are just one of several levels of control.

The binding of proteins to sequences both near to and distant from the promoter can also affect levels of gene expression. Protein binding can *activate* transcription by facilitating either RNA polymerase binding or steps farther along in the initiation process, or it can *repress* transcription by blocking the activity of the polymerase. In *E. coli*, one protein that activates transcription is the **cAMP receptor protein (CRP)**, which increases the transcription of genes coding for enzymes that metabolize sugars other than glucose when cells are grown in the absence of glucose. **Repressors** are proteins that block the synthesis of RNA at specific genes. In the case of the Lac repressor, transcription of the genes for the enzymes of lactose metabolism is blocked when lactose is unavailable.

As described further in Chapter 27, transcription of mRNAs and their translation are tightly coupled in bacteria. As a protein-coding gene is being transcribed, ribosomes rapidly bind to and begin to translate the mRNA before its synthesis is complete. Another protein, NusG, binds directly to both the ribosome and RNA polymerase, linking the two complexes. The rate of translation directly affects the rate of transcription. In contrast, eukaryotes carry out transcription in the nucleus and translation in the cytoplasm, making it impossible for these two steps to be physically coupled.

Specific Sequences Signal Termination of RNA Synthesis

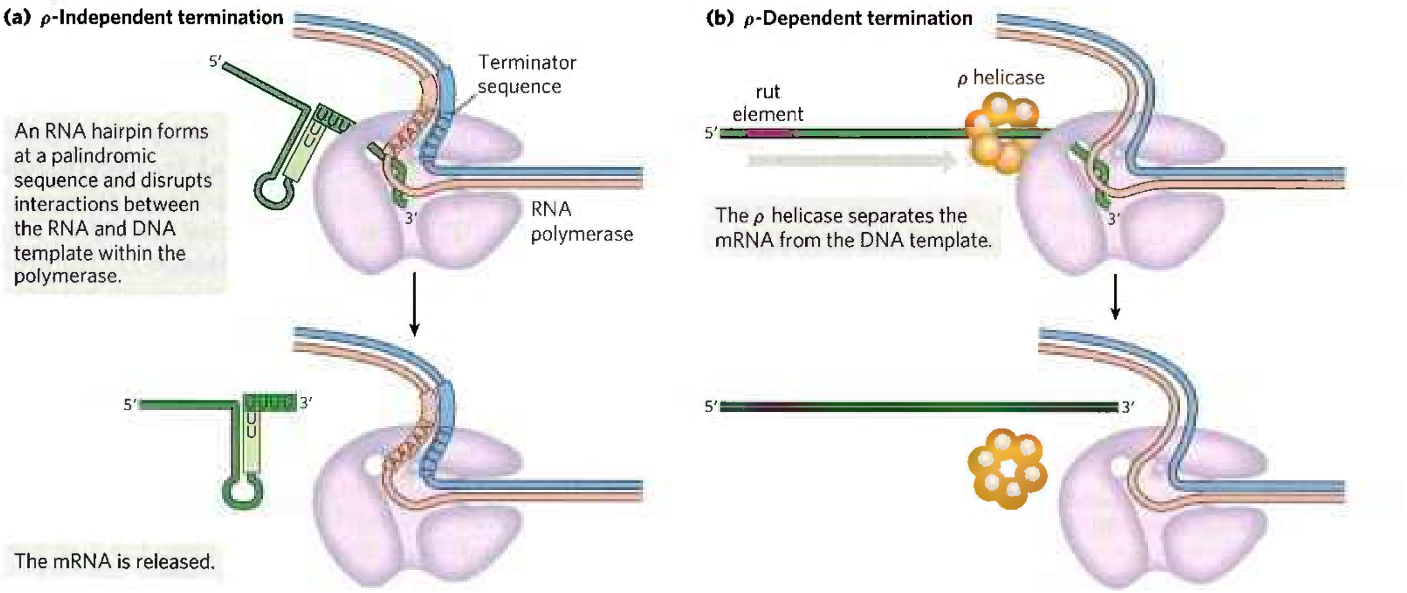
RNA synthesis is processive; that is, the RNA polymerase introduces a large number of nucleotides into a growing RNA molecule before dissociating (p. 917). This is necessary because, if the polymerase released an RNA transcript prematurely, it could not resume synthesis of the same RNA and would have to start again from the beginning of the gene. However, an encounter with certain DNA sequences results in a pause in RNA synthesis, and at some of these sequences transcription is terminated. Our focus here is again on the well-studied systems in bacteria. *E. coli* has at least two classes of termination signals: one class relies on a protein factor called ρ (rho), and the other is ρ-independent.

Most ρ-independent terminators have two distinguishing features. The first is a region that produces an RNA transcript with self-complementary sequences, permitting the formation of a hairpin structure (see Fig. 8-19a) centered 15 to 20 nucleotides before the projected end of the RNA strand. The second feature is a highly conserved string of three A residues in the template strand that are transcribed into U residues near the 3′ end of the hairpin. When a polymerase arrives at a termination site with this structure, it pauses (**Fig. 26-7a**). Formation of the hairpin structure in the RNA disrupts several A=U base pairs in the RNA-DNA hybrid segment and may disrupt important interactions between RNA and the RNA polymerase, facilitating dissociation of the transcript.

The ρ-dependent terminators lack the sequence of repeated A residues in the template strand but usually include a CA-rich sequence called a *rut* (*r*ho *ut*ilization) element. The ρ protein associates with the RNA at specific binding sites and migrates in the 5′→3′ direction until it reaches the transcription complex that is paused at a termination site (Fig. 26-7b). Here it promotes release of the RNA transcript. The ρ protein has an ATP-dependent RNA-DNA helicase activity that permits translocation of the protein along the RNA, and ATP is hydrolyzed by the ρ protein during the termination process. The detailed mechanism by which the protein promotes the release of the RNA transcript is not known.

Eukaryotic Cells Have Three Kinds of Nuclear RNA Polymerases

The transcriptional machinery in the nucleus of a eukaryotic cell is much more complex than that in bacteria. Eukaryotes have three nuclear RNA polymerases, designated I, II, and III, which are distinct complexes but have certain subunits in common. Each polymerase has a specific function (**Table 26-1**) and is recruited to


(a) ρ-Independent termination

An RNA hairpin forms at a palindromic sequence and disrupts interactions between the RNA and DNA template within the polymerase.

The mRNA is released.

(b) ρ-Dependent termination

The ρ helicase separates the mRNA from the DNA template.

FIGURE 26-7 Termination of transcription in *E. coli*. (a) ρ-Independent termination. RNA polymerase pauses at a variety of DNA sequences, some of which are terminators. One of two outcomes is then possible: either the polymerase bypasses the site and continues on its way, or the complex undergoes a conformational change (isomerization). During isomerization, intramolecular pairing of complementary sequences in the newly formed RNA transcript may form a hairpin that disrupts the RNA-DNA hybrid, the interactions between RNA and polymerase, or both. An A=U hybrid region at the 3′ end of the new transcript is relatively unstable, and the RNA dissociates from the complex completely, leading to termination. At nonterminating pause sites, the complex may escape after the isomerization step to continue RNA synthesis. (b) ρ-Dependent termination. RNAs that include a rut element recruit the ρ helicase. The ρ helicase migrates along the mRNA in the 5′→3′ direction and separates it from the polymerase.

TABLE 26-1 Eukaryotic Nuclear RNA Polymerases

RNA polymerase	Types of RNA synthesized
I	Pre-ribosomal RNA
II	mRNA ncRNA
III	tRNA 5S rRNA ncRNA

a specific promoter sequence. In addition, eukaryotic mitochondria and chloroplasts have their own RNA polymerases for transcription of genes encoded in their own DNA (see Fig. 19-40). The RNA polymerases in these organelles are similar to bacterial RNA polymerases and less elaborate than the nuclear transcription machinery discussed below.

RNA polymerase I (Pol I) is responsible for the synthesis of only one type of RNA, a transcript called pre-ribosomal RNA (or pre-rRNA), which contains the precursor for the 18S, 5.8S, and 28S rRNAs. The principal function of RNA polymerase II (Pol II) is the synthesis of mRNAs and many ncRNAs. This enzyme can recognize thousands of promoters that vary greatly in sequence. Some Pol II promoters have a few sequence features in common, including a TATA box (eukaryotic consensus sequence TATA(A/T)A(A/T)(A/G)) near base pair −30 and an Inr sequence (initiator) near the RNA start site at +1 (**Fig. 26-8**). However, such promoters are in the minority, and elaborate interactions with regulatory proteins guide Pol II function at many promoters that lack these features.

RNA polymerase III (Pol III) makes tRNAs, the 5S rRNA, and other small, specialized ncRNAs, including the U6 RNA component of the spliceosome, which we will discuss in Section 26.2. The promoters recognized by Pol III are well characterized. Some of the sequences required for the regulated initiation of transcription by Pol III are located within the gene itself, whereas others are in more conventional locations upstream of the RNA start site (Chapter 28).

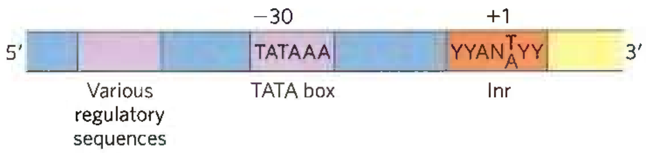


FIGURE 26-8 Some common features of TATA box promoters recognized by eukaryotic RNA polymerase II. The TATA box is the major assembly point for the proteins of the preinitiation complexes of Pol II. The DNA is unwound at the initiator sequence (Inr), and the transcription start site is usually within or very near this sequence. In the Inr consensus sequence shown here, N represents any nucleotide and Y represents a pyrimidine nucleotide. Additional sequences around the TATA box and downstream (to the right as shown here) of Inr may be recognized by one or more transcription factors. The sequence elements of Pol II promoters summarized here are much more variable and complex in comparison to *E. coli* promoters (see Fig. 26-5).

RNA Polymerase II Requires Many Other Protein Factors for Its Activity

RNA polymerase II is central to eukaryotic gene expression and has been studied extensively. P1 Although this polymerase is strikingly more complex than its bacterial counterpart, the complexity masks a remarkable conservation of structure, function, and mechanism. Pol II isolated from either yeast or human cells is a 12-subunit enzyme with an aggregate molecular weight of more than 510,000. The largest subunit (RBP1) exhibits a high degree of homology to the β' subunit of bacterial RNA polymerase. Another subunit (RBP2) is structurally similar to the bacterial β subunit, and two others (RBP3 and RBP11) show some structural homology to the two bacterial α subunits. Pol II must function with genomes that are more complex and with DNA molecules more elaborately packaged than in bacteria. The need for protein-protein contacts with the numerous other protein factors required to navigate this labyrinth accounts in large measure for the added complexity of the eukaryotic polymerase.

The largest subunit of Pol II (RBP1) also has an unusual feature, a long carboxyl-terminal tail consisting of many repeats of a consensus heptad amino acid sequence, —YSPTSPS—. There are 26 repeats in the yeast enzyme (19 exactly matching the consensus) and 52 (21 exact) in the mouse and human enzymes. This **carboxyl-terminal domain (CTD)** is separated from the main body of the enzyme by an intrinsically disordered linker sequence. The CTD has many important roles in Pol II function, as outlined below.

RNA polymerase II requires an array of other proteins, called **transcription factors**, to form the active transcription complex. The **general transcription factors** required at every Pol II promoter (factors usually designated TFII with an additional identifier) are highly conserved in all eukaryotes (**Table 26-2**). The process of transcription by Pol II can be described in terms of several phases—assembly, initiation, elongation, termination—each associated with characteristic proteins (**Fig. 26-9**). The step-by-step pathway described below leads to active transcription in vitro. In the cell, many of the proteins may be present in larger, preassembled complexes, simplifying the pathways for assembly on promoters. As you read about this process, consult Figure 26-9 and Table 26-2 to help keep track of the many participants.

Assembly of RNA Polymerase and Transcription Factors at a Promoter The formation of a closed complex begins when the TATA-binding protein (TBP) binds to the TATA box (Fig. 26-9a, step ①). At promoters lacking a TATA box, TBP arrives as part of a multisubunit complex called

TABLE 26-2 Proteins Required for Initiation of Transcription at the RNA Polymerase II (Pol II) Promoters of Eukaryotes

Transcription protein	Number of different subunits	Subunit(s) M_r[a]	Function(s)
Initiation			
Pol II	12	7,000–220,000	Catalyzes RNA synthesis
TBP (TATA-binding protein)	1	38,000	Specifically recognizes the TATA box
TFIIA	2	13,000, 42,000	Stabilizes binding of TFIIB and TBP to the promoter
TFIIB	1	35,000	Binds to TBP; recruits Pol II–TFIIF complex
TFIID[b]	13–14	14,000–213,000	Required for initiation at promoters lacking a TATA box
TFIIE	2	33,000, 50,000	Recruits TFIIH; has ATPase and helicase activities
TFIIF	2–3	29,000–58,000	Binds tightly to Pol II; binds to TFIIB and prevents binding of Pol II to nonspecific DNA sequences
TFIIH	10	35,000–89,000	Unwinds DNA at promoter (helicase activity); phosphorylates Pol II CTD; recruits nucleotide-excision repair proteins
Elongation[c]			
ELL[d]	1	80,000	
pTEFb	2	43,000, 124,000	Phosphorylates Pol II CTD
SII (TFIIS)	1	38,000	
Elongin (SIII)	3	15,000, 18,000, 110,000	

[a] M_r reflects the subunits present in the complexes of human cells.

[b] The presence of multiple copies of some TFIID subunits brings the total subunit composition of the complex to 21–22.

[c] The function of all elongation factors is to suppress the pausing or arrest of transcription by the Pol II–TFIIF complex.

[d] Name derived from eleven-nineteen *l*ysine-rich *l*eukemia. The gene for ELL is the site of chromosomal recombination events frequently associated with acute myeloid leukemia.

TFIID. TBP is bound, in turn, by the transcription factor TFIIB. TFIIA then binds and, along with TFIIB, helps to stabilize the TBP-DNA complex. The TFIIB-TBP complex is next bound by another complex consisting of TFIIF and Pol II. TFIIF helps target Pol II to its promoters, both by interacting with TFIIB and by reducing the binding of the polymerase to nonspecific sites on the DNA. Finally, TFIIE and TFIIH bind to create the closed, **preinitiation complex (PIC)**.

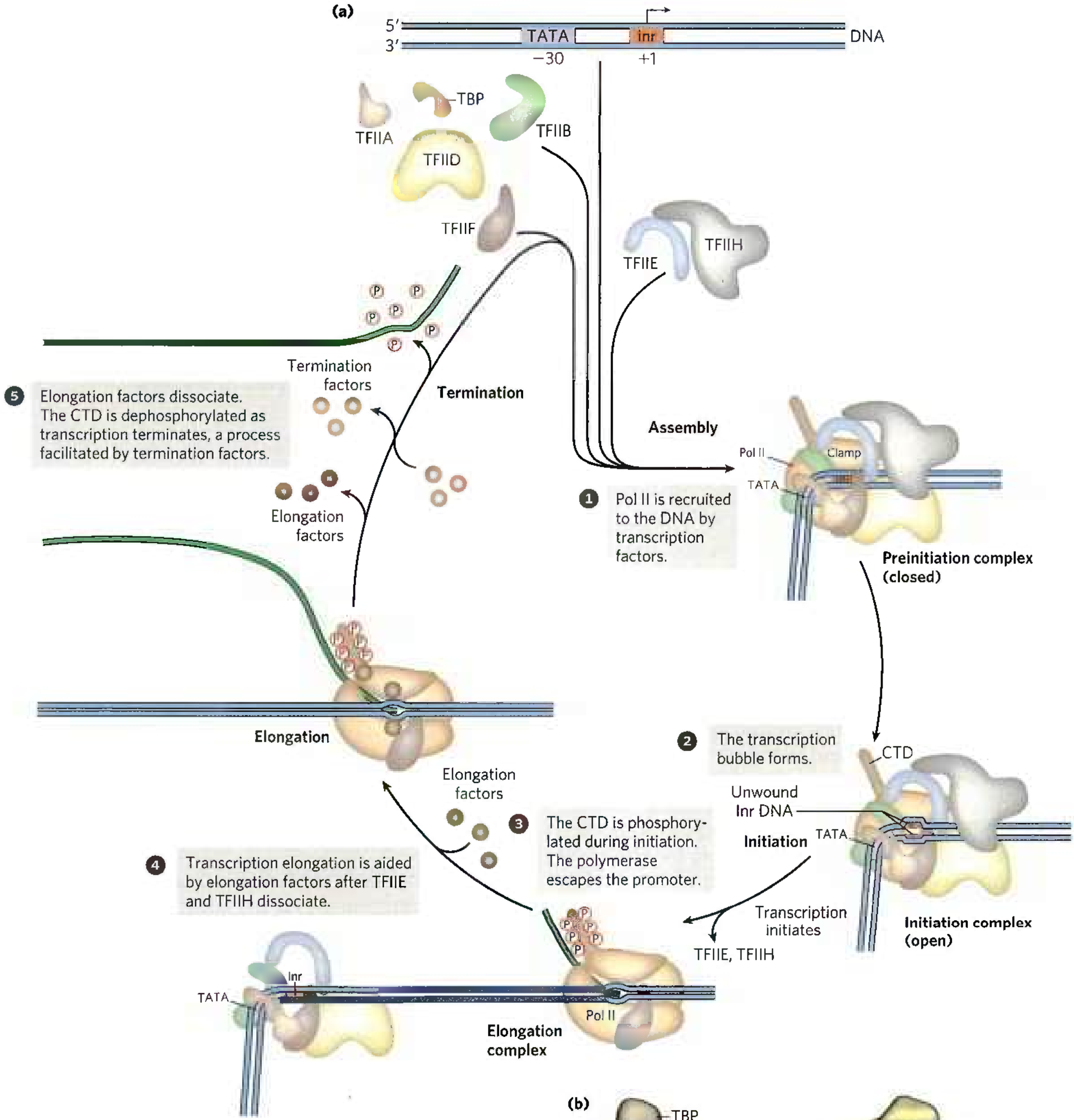
A key function of TFIID in the PIC is to position TBP on the promoter, which in turn dictates the location of Pol II loading and transcription initiation. Because most human promoters (~80%) lack a TATA box, how TFIID correctly positions TBP and Pol II relative to the transcription start site was poorly understood until their structures were determined by cryo-EM (Fig. 26-9b). These structures showed that TFIID binds the promoter DNA in an elongated complex that is anchored by TBP–DNA interactions on one end and extends linearly over 70 base pairs. The **Inr sequence** is positioned roughly in the middle, straddled on both ends by TFIID subunits. TFIID thus acts as a scaffold to direct binding of Pol II and other PIC components and uses its structure and interactions with TBP to help define the transcription start site.

TFIIH has multiple subunits and includes a DNA helicase activity that promotes the unwinding of DNA near the RNA start site (a process requiring the hydrolysis of ATP), thereby creating an open initiation complex (Fig. 26-9a, step ❷). Counting all the subunits of the various factors (including TFIIA and the subunits of TFIID), this active initiation complex can have more than 50 polypeptides.

RNA Strand Initiation and Promoter Clearance TFIIH has an additional function during the initiation phase. A kinase activity in one of its subunits phosphorylates Pol II at many places in the CTD (Fig. 26-9a, step ❸). Several other protein kinases, including CDK9 (cyclin-dependent kinase 9), which is part of the complex pTEFb (positive transcription elongation factor b), also phosphorylate the CTD, primarily on Ser residues of the CTD repeat sequence. CTD phosphorylation causes a conformational change in the overall complex, initiating transcription. During the subsequent elongation phase of transcription, the phosphorylation state of the CTD changes, affecting which RNA processing components are bound to the transcription complexes (**Fig. 26-10**).

During synthesis of the initial 60 to 70 nucleotides of RNA, first TFIIE, then TFIIH is released, and Pol II enters the elongation phase of transcription (Fig. 26-9a, step ❹).

Elongation, Termination, and Release TFIIF remains associated with Pol II throughout elongation. During this stage, polymerase activity is greatly enhanced by protein elongation factors (Table 26-2). The elongation factors,


(a)
5′
3′
TATA
Inr
DNA
−30
+1
TBP
TFIIA
TFIIB
TFIID
TFIIF
TFIIE
TFIIH
Assembly
Pol II
Clamp
TATA
① Pol II is recruited to the DNA by transcription factors.
Preinitiation complex (closed)
② The transcription bubble forms.
CTD
Unwound Inr DNA
Initiation
TATA
Initiation complex (open)
Transcription initiates
TFIIE, TFIIH
③ The CTD is phosphorylated during initiation. The polymerase escapes the promoter.
Elongation factors
P
Pol II
Elongation complex
Inr
TATA
④ Transcription elongation is aided by elongation factors after TFIIE and TFIIH dissociate.
Elongation
Elongation factors
Termination factors
Termination
⑤ Elongation factors dissociate. The CTD is dephosphorylated as transcription terminates, a process facilitated by termination factors.

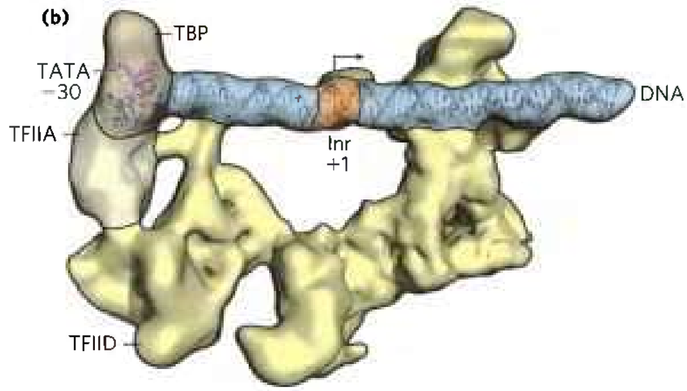

(b)
TBP
TATA
−30
TFIIA
DNA
Inr
+1
TFIID

FIGURE 26-9 Transcription at RNA polymerase II promoters. (a) TBP (often with TFIIA and sometimes with TFIID) and TFIIB bind sequentially to a promoter. TFIIF plus Pol II are then recruited to that complex. The further addition of TFIIE and TFIIH results in a closed complex. Within the complex, the DNA is unwound at the Inr region by the helicase activity of TFIIH and perhaps of TFIIE, creating an open complex that completes assembly. The carboxyl-terminal domain of the largest Pol II subunit is phosphorylated by TFIIH, and the polymerase then escapes the promoter and initiates transcription. Elongation is accompanied by the release of many transcription factors and is also enhanced by elongation factors (see Table 26-2). After termination, Pol II is released, dephosphorylated, and recycled. (b) Structure of the human TFIIA/TFIID/TBP complex bound to promoter DNA, determined by cryo-EM. The DNA is stretched linearly over 70 base pairs, with the Inr sequence positioned roughly in the middle of TFIID, anchored by TBP/TATA-box interactions on the end. [(a) Information from E. Nogales et al., *Curr. Opin. Struct. Biol.* 47:60, 2017, Fig. 4. (b) Data from PDB ID 5FUR, R. K. Louder et al., *Nature* 531:604, 2016.]

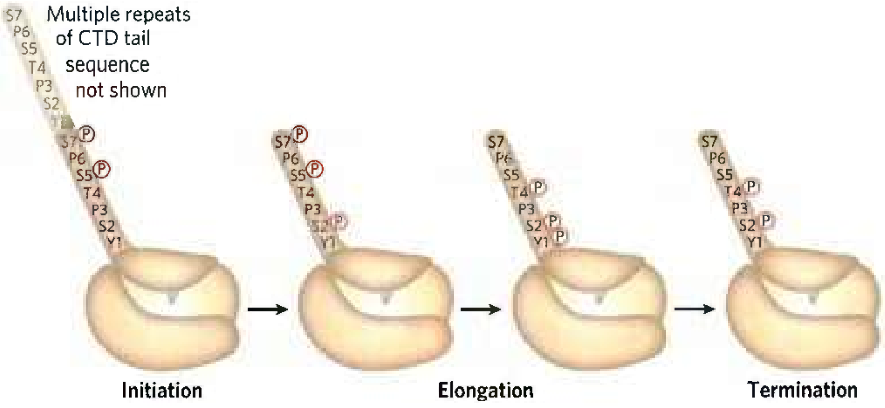


FIGURE 26-10 Phosphorylation of the carboxyl-terminal domain (CTD) of RNA polymerase II. The phosphorylation pattern of the CTD changes during different phases of transcription due to the action of kinases and phosphorylases associated with the transcription machinery. Multiple repeats of the CTD tail are phosphorylated with the patterns shown here during each stage; however, these are not shown for clarity. Understanding the patterns and heterogeneity of CTD tail phosphorylation at different stages of transcription and on different genes is an active area of transcription research.

some bound to the phosphorylated CTD, suppress pausing during transcription and also coordinate interactions between the supramolecular complexes involved in the posttranscriptional processing of mRNAs. Once the RNA transcript is completed, transcription is terminated (Fig. 26-9a, step ⑤). The Pol II CTD is dephosphorylated and the transcription machinery recycled, ready to initiate another transcript.

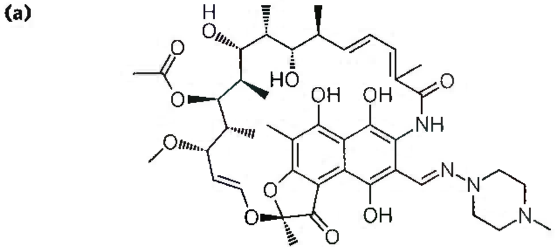
Regulation of transcription at Pol II promoters is an elaborate process. It involves the interaction of a wide variety of other proteins with the preinitiation complex. Some of these regulatory proteins interact with transcription factors, others with Pol II itself. The regulation of eukaryotic transcription is described in more detail in Chapter 28.

RNA Polymerases Are Drug Targets

Both bacterial and eukaryotic RNA polymerases are the targets of a large number of chemical inhibitors. Some of these molecules inhibit transcription of both types of RNA polymerases; others selectively inhibit only certain types of polymerase.

The elongation of RNA strands by RNA polymerase in both bacteria and eukaryotes is inhibited by the antibiotic **actinomycin D**. The planar portion of this molecule inserts (intercalates) into the double-helical DNA between successive G≡C base pairs, deforming the DNA duplex. This prevents movement of the polymerase along the DNA during transcription. Because actinomycin D inhibits RNA elongation in intact cells as well as in cell extracts, it can be used to identify cell processes that depend on RNA synthesis.

Rifampin (Fig. 26-11a) inhibits bacterial RNA synthesis by preventing the promoter clearance step of transcription. Rifampin is an important antibiotic for the treatment of tuberculosis (TB), which is caused by the bacterium *Mycobacterium tuberculosis* and kills approximately 1.8 million people each year. The antibiotic



Rifampin

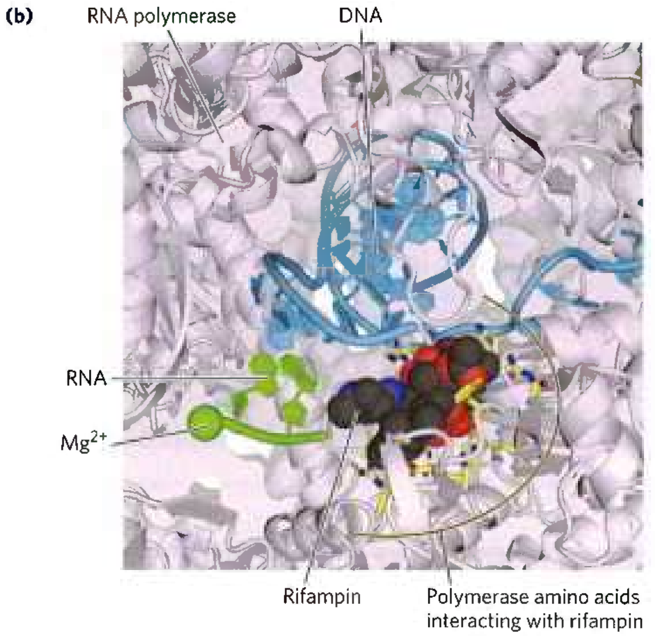


FIGURE 26-11 Inhibition of RNA polymerase by rifampin. (a) Chemical structure of rifampin. (b) X-ray crystallographic structure of rifampin bound to the active site of *M. tuberculosis* RNA polymerase. In this slab view, much of the surrounding polymerase has been removed so that active site details, including one of the essential Mg^{2+} ions, can be highlighted. Rifampin (shown in spacefill) binds within the active site and blocks extension of the RNA transcript. Many RNA polymerase amino acids make direct contact with rifampin, and mutation of these amino acids can result in rifampicin-resistant RNA polymerase and TB infection. [(b) Data from PDB ID 5UH6 and information from W. Lin et al., *Mol. Cell* 66:169, 2017.]

binds near the active site of RNA polymerase and prevents extension of the RNA product beyond 2 to 3 nucleotides. Unfortunately, *M. tuberculosis* can develop resistance to rifampin; more than 600,000 cases of rifampin-resistant TB are reported each year. In many cases, resistance is due to mutation in the rifampin binding site (Fig. 26-11b), particularly at Asp^{516}, His^{526}, and Ser^{531} of the β subunit. New drugs that inhibit *M. tuberculosis* RNA polymerase are desperately needed for treatment of drug-resistant TB.

The death cap mushroom *Amanita phalloides* has a very effective defense mechanism against predators. It produces **α-amanitin**, which disrupts transcription in animal cells by blocking Pol II and, at higher concentrations, Pol III. Neither Pol I nor bacterial RNA polymerase is sensitive to α-amanitin—nor is the RNA polymerase II of *A. phalloides* itself. Because α-amanitin is selective for inhibiting the function of only certain RNA polymerases, it has proven useful for identifying the functions of different polymerases in the cell. Mitochondrial and bacterial RNA polymerases share significant similarities to one another, including α-amanitin resistance. By exposing eukaryotic cells to α-amanitin, it is possible to detect newly synthesized mRNAs that arise only from mitochondrial and not nuclear transcription. Researchers using α-amanitin need to exercise abundant caution because it is highly toxic to humans. An amount of α-amanitin the size of a grain of rice contains a lethal dose. ■

Amanita phalloides, the death cap mushroom [Wolstenholme Images/Alamy Stock Photo.]

SUMMARY 26.1 *DNA-Dependent Synthesis of RNA*

- Transcription is catalyzed by DNA-dependent RNA polymerases, which use ribonucleoside 5′-triphosphates to synthesize RNA in the 5′→3′ direction, complementary to the template strand of duplex DNA. Transcription occurs in several phases: binding of RNA polymerase to a DNA site called a promoter, initiation of transcript synthesis, elongation, and termination.
- RNA polymerases bind regions of DNA called promoters to initiate transcription of nearby genes. Promoter sequences help to establish the level of gene expression and in *E. coli* are recognized by variable RNA polymerase subunits called σ factors. Transcription initiation involves formation of the closed and open complexes. DNA is unwound in the open complex to allow it to serve as the transcription template.
- As the first committed steps in transcription, binding of RNA polymerase to the promoter and initiation of transcription are closely regulated.
- Bacterial transcription stops at sequences called terminators. *E. coli* commonly uses two types of termination signals: ρ-dependent and ρ-independent.
- Eukaryotic cells have three types of nuclear RNA polymerases. The vast majority of cellular mRNAs and ncRNAs are synthesized by Pol II.
- Binding of Pol II to its promoters requires an array of proteins called transcription factors. Ultimately, a large molecular complex called the preinitiation complex, PIC, forms at the promoter. Elongation factors participate in the elongation phase of transcription. The phosphorylation state of the long carboxyl-terminal domain of the largest Pol II subunit changes in the initiation and elongation phases and determines what components are part of the initiation and elongation complexes.
- RNA polymerases can be inhibited by a number of drugs, some of which are specific to either bacterial or eukaryotic polymerases. Drugs that inhibit bacterial RNA polymerase are commonly used to treat infections such as tuberculosis.

26.2 RNA Processing

P2 Many of the RNA molecules in bacteria and virtually all RNA molecules in eukaryotes are processed to some degree after synthesis. Processing can include addition or deletion of nucleotide sequences as well as chemical modification of RNA nucleotides. All of these events can be used to control the posttranscriptional fate of the RNA in the cell. As a result, many mature RNAs are not exact copies of the DNA genes from which they were transcribed. Some of the most interesting molecular events in RNA metabolism occur during posttranscriptional processing. Intriguingly, several of the enzymes that catalyze these reactions have active sites composed of RNA rather than protein. The discovery of these catalytic RNAs, or **ribozymes**, has brought a revolution in thinking about RNA function and about the origin of life, as we will discuss in Section 26.4.

A newly synthesized RNA molecule is called a **primary** or **precursor transcript**. Perhaps the most extensive processing of primary transcripts occurs in eukaryotic precursor mRNAs (pre-mRNAs) and in the tRNAs of both bacteria and eukaryotes. However, many ncRNAs are also processed.

The precursor transcript for a eukaryotic mRNA typically contains sequences encompassing one gene, although the sequences encoding the polypeptide may not be contiguous. Noncoding tracts that break up the coding region of the transcript are called **introns**, and the coding segments are called **exons** (see the discussion of introns and exons in DNA in Chapter 24). **P2** In a process called **RNA splicing**, the introns are removed from the pre-mRNA, and the exons are spliced together to form a continuous sequence that specifies a functional polypeptide. Virtually all human genes contain introns, the average being eight introns per gene. Eukaryotic mRNAs are also modified at each end. A modified nucleotide structure called a 5′ cap is added at the 5′ end. The 3′ end is cleaved, and 80 to 250 A residues are added to create a poly(A) "tail." The sometimes elaborate protein complexes that carry out 5′ capping, splicing, and 3′ polyadenylation mRNA-processing reactions do not operate independently. They are organized in association with each other and with the phosphorylated CTD of Pol II; each complex affects the function of the others, as outlined in **Figure 26-12**.

Proteins involved in mRNA transport to the cytoplasm are also associated with the mRNA in the nucleus, and the processing of the transcript is coupled to its transport. In effect, a eukaryotic mRNA, as it is synthesized, is ensconced in an elaborate and dynamic supramolecular **messenger ribonucleoprotein (mRNP) complex** comprising dozens of proteins. The composition of the mRNP changes as the transcript is processed, transported to the cytoplasm, and delivered to the ribosome for translation. Associated proteins can dramatically modulate the cellular destination, function, and fate of an mRNA.

In addition to splicing and 5′ and 3′ end modification, individual purine and pyrimidine nucleotides within primary transcripts can undergo chemical modification. Many eukaryotic mRNAs contain modified nucleotides that affect their interactions with RNA-binding proteins and regulate gene expression; however, RNA modification has been best characterized in primary tRNA transcript processing. Many bases and sugars in tRNAs are modified in both bacteria and eukaryotes, including with unusual bases not found in other nucleic acids (see

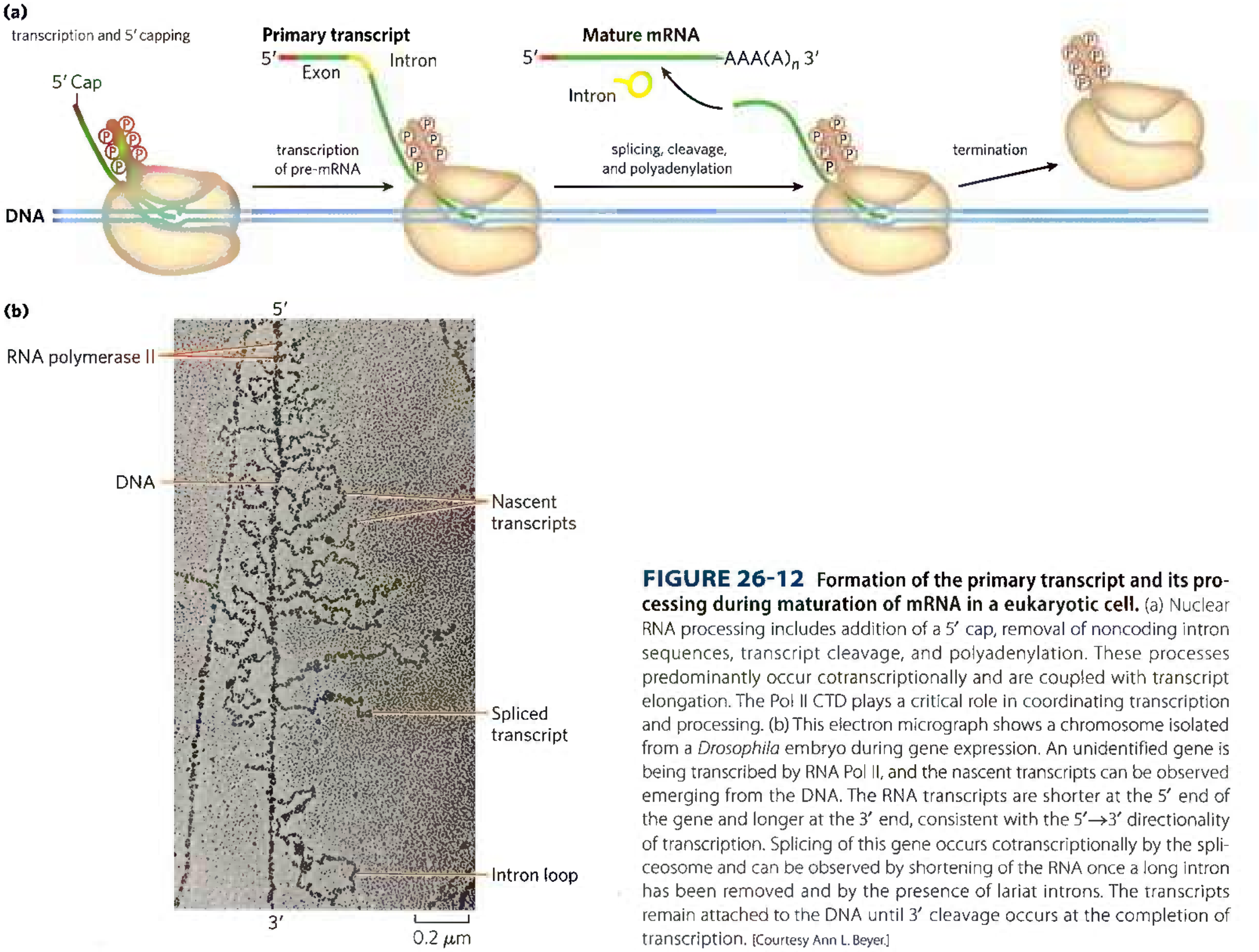


FIGURE 26-12 Formation of the primary transcript and its processing during maturation of mRNA in a eukaryotic cell. (a) Nuclear RNA processing includes addition of a 5′ cap, removal of noncoding intron sequences, transcript cleavage, and polyadenylation. These processes predominantly occur cotranscriptionally and are coupled with transcript elongation. The Pol II CTD plays a critical role in coordinating transcription and processing. (b) This electron micrograph shows a chromosome isolated from a *Drosophila* embryo during gene expression. An unidentified gene is being transcribed by RNA Pol II, and the nascent transcripts can be observed emerging from the DNA. The RNA transcripts are shorter at the 5′ end of the gene and longer at the 3′ end, consistent with the 5′→3′ directionality of transcription. Splicing of this gene occurs cotranscriptionally by the spliceosome and can be observed by shortening of the RNA once a long intron has been removed and by the presence of lariat introns. The transcripts remain attached to the DNA until 3′ cleavage occurs at the completion of transcription. [Courtesy Ann L. Beyer.]

Fig. 26-22). Many ncRNAs also undergo elaborate processing, often involving the removal of segments from one or both ends.

The ultimate fate of any RNA is its complete and regulated degradation. The rate of turnover of RNAs plays a critical role in determining their steady-state levels and the rate at which cells can shut down expression of a gene when its product is no longer needed. During the development of multicellular organisms, for example, certain proteins must be expressed at one stage only, and the mRNA encoding such a protein must be made and destroyed at the appropriate times.

Eukaryotic mRNAs Are Capped at the 5′ End

P2 Most eukaryotic mRNAs have a **5′ cap**, a residue of 7-methylguanosine linked to the 5′-terminal residue of the mRNA through an unusual 5′,5′-triphosphate linkage (**Fig. 26-13**). The 5′ cap helps protect mRNA from ribonucleases. It also binds to specific cap-binding complexes of proteins and participates in binding of the mRNA to the ribosome to initiate translation (Chapter 27).

The 5′ cap is formed by condensation of a molecule of GTP with the triphosphate at the 5′ end of the transcript. The guanine is subsequently methylated at N-7, and additional methyl groups are often added at the 2′ hydroxyls of the first and second nucleotides adjacent to the cap (Fig. 26-13a). The methyl groups are derived from *S*-adenosylmethionine. All these reactions (Fig. 26-12b) occur very early in transcription, after the first 20 to 30 nucleotides of the transcript have been added. All four of the enzymes in the cap-synthesizing complex, and through them the 5′ end of the transcript itself, are associated with the RNA polymerase II CTD (Fig. 26-13c) until the cap is synthesized. The capped 5′ end is then released from the cap-synthesizing complex and bound by the nuclear cap-binding complex, which facilitates both splicing and nuclear export of the RNA.

The 5′ cap does not provide permanent protection of the transcript. Eukaryotes also contain cellular decapping enzymes, which are important for RNA regulation. Cap removal allows RNAs to be degraded by exonucleases that hydrolyze the RNA in the 5′→3′ direction. Some viruses have also evolved elaborate mechanisms for removing the 5′ cap from host mRNAs. The influenza virus needs no specialized enzymes for the synthesis of 5′ caps on its viral RNAs; instead, it borrows these

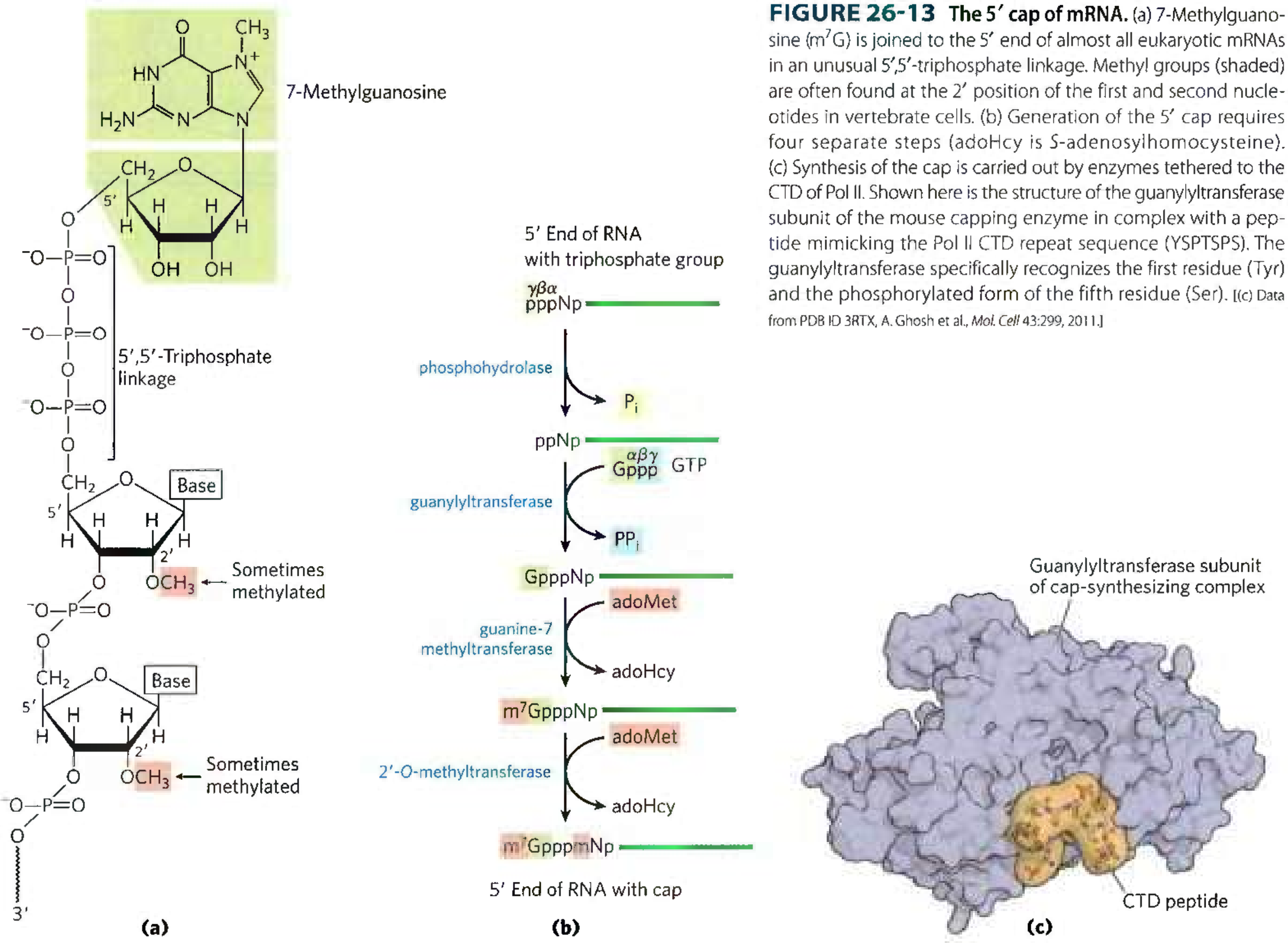

7-Methylguanosine
5′,5′-Triphosphate linkage
Base
Base
Sometimes methylated
Sometimes methylated
OCH3
OCH3
5′
2′
3′
(a)
5′ End of RNA with triphosphate group
γβα pppNp
phosphohydrolase
Pi
ppNp
αβγ Gppp GTP
guanylyltransferase
PPi
GpppNp
adoMet
guanine-7 methyltransferase
adoHcy
m7GpppNp
adoMet
2′-O-methyltransferase
adoHcy
m7GpppmNp
5′ End of RNA with cap
(b)
Guanylyltransferase subunit of cap-synthesizing complex
CTD peptide
(c)

FIGURE 26-13 The 5′ cap of mRNA. (a) 7-Methylguanosine (m^7G) is joined to the 5′ end of almost all eukaryotic mRNAs in an unusual 5′,5′-triphosphate linkage. Methyl groups (shaded) are often found at the 2′ position of the first and second nucleotides in vertebrate cells. (b) Generation of the 5′ cap requires four separate steps (adoHcy is *S*-adenosylhomocysteine). (c) Synthesis of the cap is carried out by enzymes tethered to the CTD of Pol II. Shown here is the structure of the guanylyltransferase subunit of the mouse capping enzyme in complex with a peptide mimicking the Pol II CTD repeat sequence (YSPTSPS). The guanylyltransferase specifically recognizes the first residue (Tyr) and the phosphorylated form of the fifth residue (Ser). [(c) Data from PDB ID 3RTX, A. Ghosh et al., *Mol. Cell* 43:299, 2011.]

structures from host-cell transcripts in a process termed "cap-snatching." A capped host transcript is bound by the viral RNA polymerase and cleaved by an endonuclease. The influenza RNA polymerase can then use the resulting capped oligonucleotide to prime viral RNA synthesis.

Both Introns and Exons Are Transcribed from DNA into RNA

In bacteria, the mRNA used for translation is generally a direct copy of the DNA gene sequence, continuing along the DNA template without interruption until the information needed to specify the polypeptide is complete. However, the notion that *all* genes are continuous was disproved in 1977 when Phillip Sharp and Richard Roberts independently discovered that many genes for polypeptides in eukaryotes are interrupted by noncoding sequences (introns).

P2 The vast majority of genes in vertebrates contain introns; among the few exceptions are those that encode histones. The occurrence of introns in other eukaryotes varies. Many genes of the yeast *Saccharomyces cerevisiae* lack introns, but introns are more common in some other yeast species. Introns are also found in a few bacterial and archaeal genes. Introns in DNA are transcribed along with the rest of the gene by RNA polymerases. The introns in the primary RNA transcript are then spliced, and the exons are joined to form a mature, functional RNA. In eukaryotic mRNAs, most exons are less than 1,000 nucleotides long, with many in the 100 to 200 nucleotide size range, encoding stretches of 30 to 60 amino acids within a longer polypeptide. Introns vary in size from 50 to more than 700,000 nucleotides, with a median length of about 1,800. Genes of higher eukaryotes, including humans, typically have much more DNA devoted to introns than to exons. For example, the human dystrophin gene encodes a pre-mRNA more than 2 million nucleotides long. However, the final mRNA is 14,000 nucleotides long, indicating that more than 99% of the transcribed RNA is found within introns and removed by splicing. Deficiencies in dystrophin expression can lead to muscular dystrophies. The ~20,000 genes of the human genome include more than 200,000 introns.

RNA Catalyzes the Splicing of Introns

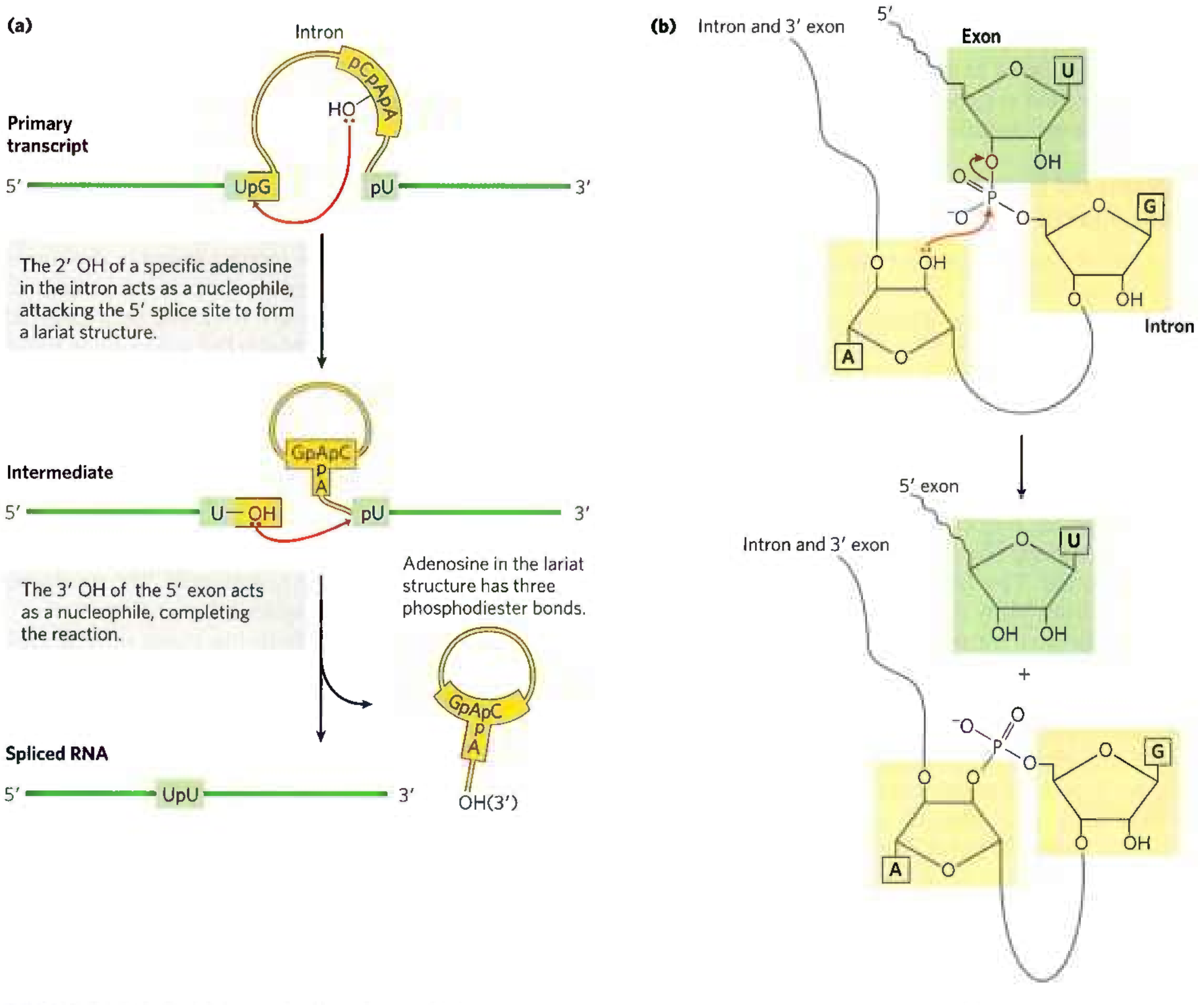
There are four classes of introns (**Table 26-3**). The first two, the group I and group II introns, differ in the details of their splicing mechanisms but share one surprising characteristic: they are *self-splicing*—no proteins are needed to carry out catalysis. The introns found in the nuclear-encoded genes of eukaryotes comprise the third class. These pre-mRNA introns are removed by a large RNP called the spliceosome. Although the spliceosome requires dozens of proteins for its function, its active site includes RNA. The final class of introns requires protein enzymes for their removal. These introns are found in some tRNAs as well as certain mRNAs, such as that encoding the Xbox binding protein 1, Xbp1. Protein-mediated splicing of the XBP1 transcript regulates the cellular response to unfolded proteins that occurs under conditions of endoplasmic reticulum stress in human cells. The mechanisms of tRNA and XBP1 mRNA splicing are similar.

Group I introns are found in some nuclear, mitochondrial, and chloroplast genes that code for rRNAs, mRNAs, and tRNAs. Group II introns are generally found in the primary transcripts of mitochondrial or chloroplast mRNAs in fungi, algae, and plants. Group I and group II introns are also among the rare examples of introns in bacteria. The splicing mechanisms in both groups involve two transesterification reaction steps (**Fig. 26-14**), in which a ribose 2′- or 3′-hydroxyl group makes a nucleophilic attack on a phosphorus, and a new phosphodiester bond is formed at the expense of the old, maintaining the balance of energy. These reactions are very similar to the DNA breaking and rejoining reactions promoted by topoisomerases (see Fig. 24-18) and site-specific recombinases (see Fig. 25-37).

The group I splicing reaction requires a guanine nucleoside or nucleotide cofactor, but the cofactor is not used as a source of energy; instead, the 3′-hydroxyl group of guanosine is used as a nucleophile in the first step of the

TABLE 26-3 Mechanisms of RNA Splicing

Mechanism	Components	Features	Cellular locations
Group I Intron	Catalytic RNA	Self-splicing using a guanine-derived cofactor	Found in nuclear, mitochondrial, and chloroplast genes that encode mRNAs, rRNAs, or tRNAs. Can be found in bacteria.
Group II Intron	Catalytic RNA; maturase and reverse transcriptase proteins	Self-splicing using a nucleophile within the intron to form a lariat	Primarily found in mitochondrial and chloroplast genes of fungi, algae, and plants. Can be found in bacteria.
Spliceosome	Catalytic snRNAs; dozens of protein splicing factors	Requires a large RNP for processing using a nucleophile within the intron to form a lariat	Found in nuclear genes of eukaryotes. Capable of alternative splicing to create multiple products from a given transcript.
Protein-catalyzed	Protein enzymes	Uses a splicing endonuclease and ligase	Found in tRNAs and a few mRNAs.



The 2′ OH of a specific adenosine in the intron acts as a nucleophile, attacking the 5′ splice site to form a lariat structure.

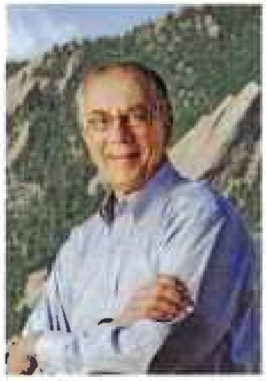
The 3′ OH of the 5′ exon acts as a nucleophile, completing the reaction.

Adenosine in the lariat structure has three phosphodiester bonds.

FIGURE 26-14 Splicing mechanism of group II introns. (a) In the first step, the 2′ OH of an internal A residue (called the branch point) attacks the phosphodiester bond at the 5′ splice site, resulting in 5′ splice site cleavage and lariat formation. In the second step, the free 3′ OH of the 5′ exon attacks the phosphodiester bond at the 3′ splice site, resulting in exon ligation and intron-lariat release. The spliceosome uses the same chemistry for intron removal, although different RNA sequences mark the intron boundaries and location of the branch point. (b) In the transesterification reaction that occurs during lariat formation, one phosphodiester bond is broken as a second one is created. This forms a lariatlike structure in which one branch is a 2′,5′-phosphodiester bond (the linkage between the intron branchpoint A and intron G nucleotides).

splicing pathway. In group II splicing reactions, the nucleophile is the 2′-hydroxyl group of an A residue *within* the intron (Fig. 26-14a). A branched lariat structure is formed as an intermediate (Fig. 26-14b). In both group I introns and group II introns, the 3′ hydroxyl of the exon that is displaced in the first step then acts as a nucleophile in a similar reaction at the 3′ end of the intron. The result is precise excision of the intron and ligation of the exons.

Self-splicing of introns was first revealed in 1982 in studies of the splicing mechanism of the group I rRNA intron from the ciliated protozoan *Tetrahymena thermophila*, conducted by Thomas Cech and colleagues. These workers transcribed isolated *Tetrahymena* DNA (including the intron) in vitro, using purified bacterial RNA polymerase. The resulting RNA spliced itself accurately without any protein enzymes from *Tetrahymena*. The discovery that RNAs could have catalytic functions was a milestone in our understanding of biological systems and a major step forward in the understanding of how life probably evolved. Catalytic RNAs like group I and group II introns share many features with protein-based enzymes, including folding into well-defined secondary and tertiary structures (**Fig. 26-15**). Catalytic RNAs and their significance in evolution are described in greater detail in Section 26.4.


Thomas Cech
[Photo by Glenn Asakawa/ University of Colorado.]

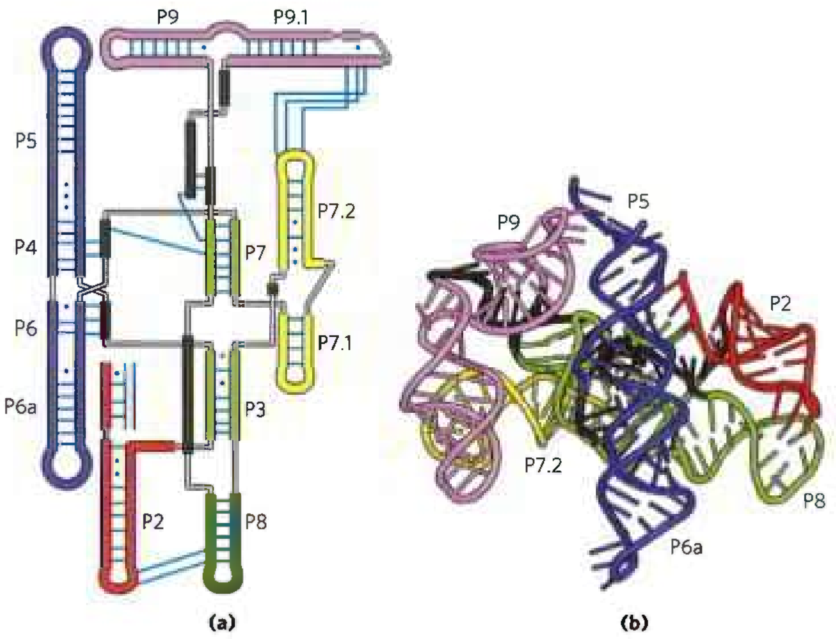


FIGURE 26-15 Structure of a group I intron. (a) The secondary structure of the group I intron ribozyme from phage Twort, a mycobacterium phage named for Frederick Twort, the physician who discovered phage in 1915. Like most catalytic RNAs, this intron adopts well-defined secondary structure. It is composed of multiple RNA duplexes (P2–P9, each differently colored) capped by hairpin structures. (b) The tertiary structure of the intron bound to a spliced RNA product, obtained by x-ray crystallography, shows that the RNA duplexes pack closely with one another to yield a compact ribozyme. The RNA duplexes are colored and named as in (a). [Data from PDB ID 1Y0Q, B. Golden et al., *Nat. Struct. Biol.* 12:82, 2005.]

In Eukaryotes the Spliceosome Carries out Nuclear pre-mRNA Splicing

In eukaryotes, most introns undergo splicing by the same lariat-forming mechanism as the group II introns. P2 However, the intron splicing takes place within a **spliceosome**, a large complex made up of multiple specialized RNP complexes called *s*mall *n*uclear *r*ibo*n*ucleo*p*roteins (snRNPs, pronounced *snurps*) and dozens of non-snRNP proteins. Each snRNP contains one of a class of eukaryotic RNAs, 100 to 200 nucleotides long, known as **small nuclear RNAs (snRNAs)**. Five snRNAs (U1, U2, U4, U5, U6) involved in splicing reactions are generally found in abundance in eukaryotic nuclei. The U3 snRNP is also found in the nucleus but is involved in ribosome assembly and is not part of the spliceosome.

The role of snRNPs in the splicing reaction was discovered by Joan Steitz in a remarkable example of "bedside-to-bench" science. Using antibodies isolated from patients with autoimmune diseases, members of the Steitz laboratory were able to purify the spliceosome's snRNP components and identify the associated snRNAs. Based on complementarity between the 5′ end of the U1 snRNA and the 5′ splice site of nuclear pre-mRNA introns (**Fig. 26-16**), Steitz proposed that snRNPs participate in the splicing reaction. Subsequently, it was discovered that patients suffering from the autoimmune disease lupus can generate antibodies against protein components of their own spliceosomes.

Joan Steitz [Photo by Robert A. Lisak, courtesy Yale School of Medicine.]

In yeast, the various snRNPs include about 80 different proteins, most of which have close homologs in all other eukaryotes. In humans, these conserved protein components are augmented by more than 200 additional proteins, which mostly participate in regulating the splicing reaction. Spliceosomes are thus among the most complex supramolecular machines in any eukaryotic cell. The RNA components of a spliceosome are the catalysts of the various splicing steps. The overall complex can be considered a highly flexible ribonucleoprotein enzyme that can adapt to the great diversity in size and sequence of nuclear pre-mRNAs.

Spliceosomal introns generally have the dinucleotide sequence GU at the 5′ end and AG at the 3′ end, marking the sites where splicing occurs. The U1 snRNA contains a sequence complementary to the 5′ splice site (Fig. 26-16b), and the U1 snRNP binds to this region by forming an RNA duplex with the pre-mRNA. A U2 snRNP binds to the 3′ end, also by base-pairing, and identifies the A residue that becomes the nucleophile used during the first transesterification reaction (Fig. 26-14). Addition of a complex of the U4, U5, and U6 snRNPs, called the tri-snRNP, leads to formation of the spliceosome (Fig. 26-16c).

Key parts of the splicing active site found in the U6 snRNA are initially sequestered by base-pairing to parts of U4 snRNA to prevent aberrant cleavage of nontarget phosphodiester bonds. In a process called activation, the U6 and U4 snRNAs must be unwound and separated to expose the active site needed for the first step in splicing. Unwinding of U4 and U6 as well as many other steps in splicing require ATP hydrolysis by a set of eight different ATPases that are part of the splicing machinery.

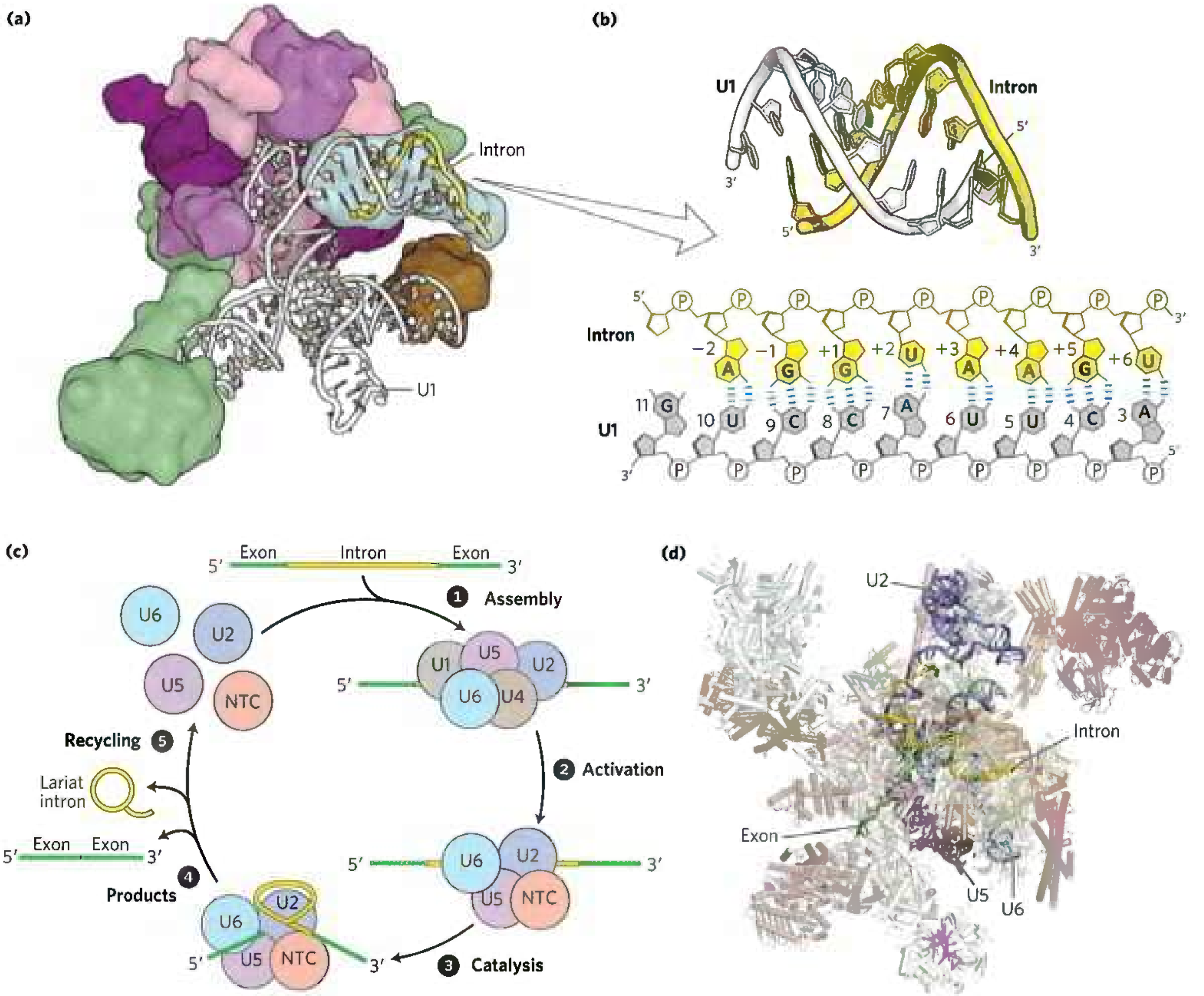


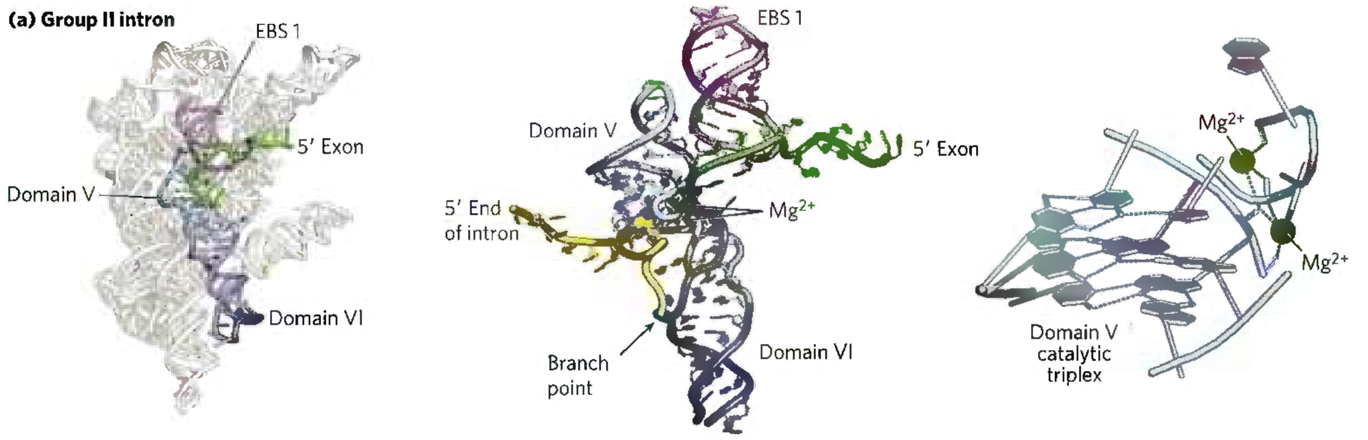
FIGURE 26-16 Processing of pre-mRNA primary transcripts by the spliceosome. (a) Small nuclear RNPs, such as the human U1 snRNP shown here, contain snRNAs associated with a number of proteins. The human U1 snRNP contains a single copy of the U1 snRNA and 10 associated polypeptides. (b) The U1 snRNP recognizes the 5′ splice site by base-pairing between the U1 snRNA and conserved RNA sequences within the intron that mark the 5′ exon/intron boundary. The sequences and structure shown were obtained from x-ray crystal structure shown in (a). The human U1 snRNA normally contains pseudouridines at positions 5 and 6; however, an unmodified RNA was used to determine this structure. (c) Spliceosomes are assembled on introns from snRNPs and proceed through stages of assembly, activation, catalysis of intron removal, release of the RNA products, and recycling of the splicing factors. In addition to the U snRNPs discussed in the text, a large protein-only supramolecular complex called the Prp19-containing complex (also known as NineTeen Complex, or NTC) is required for splicing and joins the spliceosome during the activation step. (d) Structure of the human spliceosome, determined by cryo-EM. Nuclear pre-mRNA splicing requires this large molecular machine composed of dozens of proteins and five snRNAs to remove many different introns. By comparison, using similar chemistry, group II introns can catalyze their own removal. In order to highlight the RNA catalytic core of the spliceosome and the U2, U5, and U6 snRNAs, some protein splicing factors are not shown in this view. The RNA appears discontinuous where the structure could not be resolved. [Data from (a, b) PDB ID 4PJO, Y. Kondo et al., *eLife* 4:e04986, 2015; (d) PDB ID 6QDV, S. Fica et al., *Science* 363:710, 2019.]

Spliceosomes are single turnover enzymes, meaning that each spliceosome can remove only one intron from a single transcript. As a result, spliceosomes undergo a complex cycle of assembly, activation, catalysis, product release, and recycling of the snRNP components each time an intron is removed (Fig. 26-16c, steps ❶ through ❺).

The chemical events of splicing—5′ splice site cleavage by formation of an intron lariat followed by exon ligation—are identical in mechanism to that of group II introns, despite the former requiring dozens of proteins for activity and the latter being a self-splicing ribozyme. The similarities in chemistry as well as conservation between essential RNA components of each enzyme suggests that group II introns and spliceosomes are evolutionarily related to one another. Comparison of x-ray crystal structures of group II introns and cryo-EM structures of spliceosomes provide strong support for this hypothesis. Despite being surrounded by a large protein scaffold, the catalytic center of the spliceosome is composed of RNA and arranged in a nearly identical manner to that of group II introns (**Fig. 26-17**). Thus, the spliceosome uses a ribozyme core to carry out pre-mRNA splicing. As we shall see, some group II introns also contain domains that are themselves translated as mRNAs and encode proteins that bear striking similarity to those in the spliceosome, strengthening this evolutionary connection.

About 1% of human introns are spliced by a less common type of spliceosome, called the minor spliceosome, in which the U1, U2, U4, and U6 snRNPs are replaced by the U11, U12, U4atac, and U6atac snRNPs. Whereas U1- and U2-containing spliceosomes remove introns with (5′)GU and AG(3′) terminal sequences, the minor spliceosomes remove a rare class of introns that have (5′)AU and AC(3′) terminal sequences to mark the splice sites. Introns removed by either the major or the minor spliceosome most often remain in the nucleus and are degraded.

(a) Group II intron



(b) Spliceosome

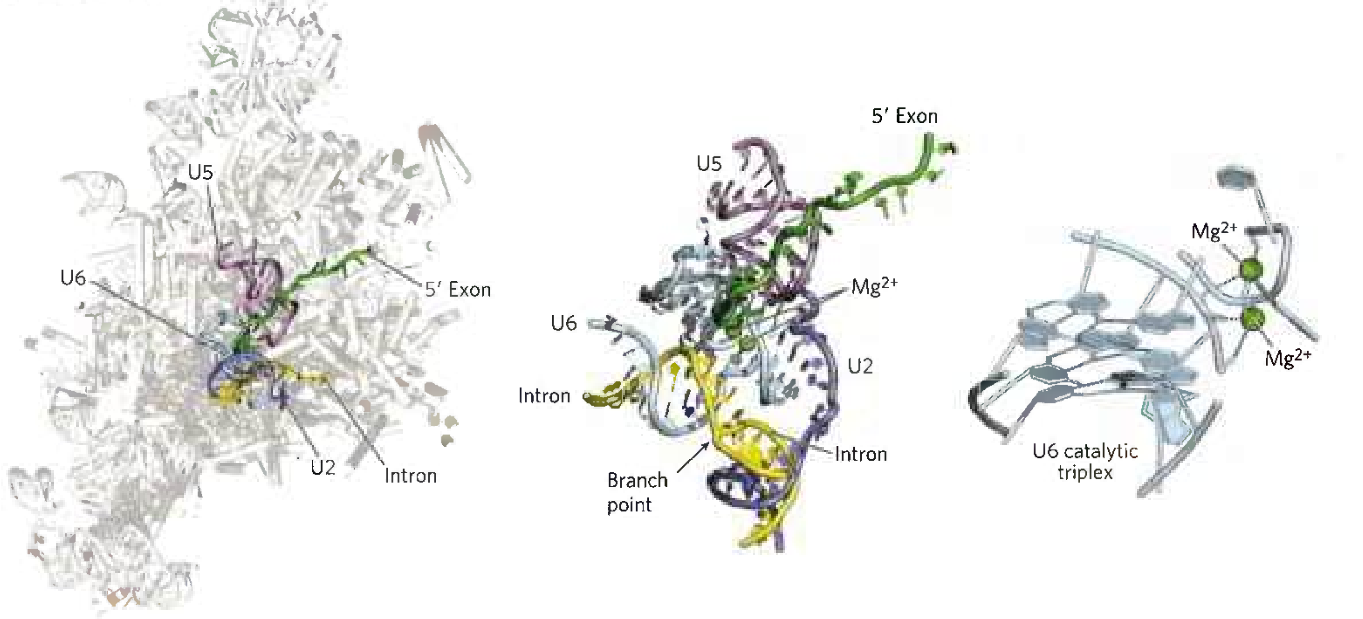


FIGURE 26-17 RNA active site conservation between group II introns and the spliceosome. A close-up examination of the active sites of (a) the *Pylaiella littoralis* group II intron and (b) the *S. cerevisiae* spliceosome reveal a similar arrangement of catalytic RNAs. In both cases, a catalytic RNA (domain V of the group II intron or U6 snRNA of the spliceosome) promotes a transesterification reaction by orienting the phosphodiester bond located at the 5′ splice site/intron junction for nucleophilic attack and by coordinating essential Mg^{2+} ions. In addition to similarities between domain V of group II introns and the U6 snRNA, group II intron domain VI and exon binding site 1 (EBS 1) play functional roles that are similar to those of the U2 and U5 snRNAs in the spliceosome. Close examination shows that the active sites of the group II intron and the spliceosome are nearly identical, formed by a complex arrangement of nucleotides called the catalytic triplex. The triplex is responsible for binding Mg^{2+} ions essential for catalysis as well as orienting the substrates for the splicing reaction. Conservation of sequence, chemistry, and three-dimensional structure suggests that the spliceosome evolved from a group II intron–like ribozyme. [Data from (a) left and center PDB ID 4R0D, A. R. Robart et al., *Nature* 514:193, 2014; right PDB ID 6QDV, S. M. Fica et al., *Science* 363:710, 2019; (b) left PDB ID 5LJ3, W. P. Galej et al., *Nature* 537:197, 2016; center PDB ID 5MQ0, S. M. Fica et al., *Nature* 542:377, 2017; right PDB ID 6ME0, D. B. Haack et al., *Cell* 178:612, 2019. Information from W. Galej et al., *Chem. Rev.* 118:4156, 2018.]

Some components of the splicing apparatus are tethered to the CTD of RNA polymerase II, indicating that splicing, like other RNA processing reactions, is tightly coordinated with transcription. Most splicing in humans occurs cotranscriptionally, meaning that splicing occurs while Pol II is still transcribing the gene. For this to occur correctly, the rates of transcription, capping, splicing, and 3′ end formation must be carefully regulated. Splicing of a pre-mRNA in the nucleus can also have profound effects on the function of the mRNA in the cytoplasm. Lynne Maquat and Melissa Moore discovered that the human spliceosome leaves behind a set of proteins on each spliced mRNA near the junction between two exons. This exon junction complex is retained on the mRNA as it is exported to the cytoplasm, where it can regulate the extent to which an mRNA can be translated over its lifetime before degradation.

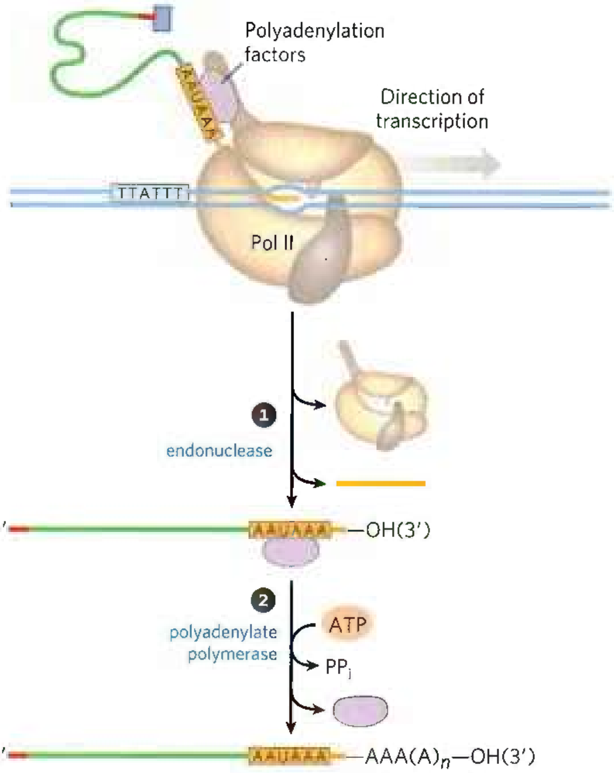


FIGURE 26-18 Addition of the poly(A) tail to the primary RNA transcript of eukaryotes. Pol II synthesizes RNA beyond the segment of the transcript containing the cleavage signal sequences, including the highly conserved upstream sequence (5′)AAUAAA. This cleavage signal sequence is bound by an enzyme complex that includes an endonuclease, a polyadenylate polymerase, and several other multisubunit proteins involved in sequence recognition, stimulation of cleavage, and regulation of the length of the poly(A) tail; all of these proteins are tethered to the CTD. ❶ The RNA is cleaved by the endonuclease at a point 10 to 30 nucleotides 3′ to (downstream of) the sequence AAUAAA. ❷ The polyadenylate polymerase synthesizes a poly(A) tail 80 to 250 nucleotides long, beginning at the cleavage site.

Proteins Catalyze Splicing of tRNAs

A fourth and final class of introns, found in certain tRNAs and a few mRNAs such as XBP1, is distinguished from other intron types in that the splicing reaction requires endonucleases and ligases made of protein and does not involve catalytic RNAs. The splicing endonuclease cleaves the phosphodiester bonds at both ends of the intron, and the two exons are joined by a mechanism similar to the DNA ligase reaction (see Fig. 25-15).

Eukaryotic mRNAs Have a Distinctive 3′ End Structure

P2 At their 3′ end, most eukaryotic mRNAs undergoing translation in the cell cytoplasm have a string of A residues, about 30 residues in yeast and 50 to 100 in animals, called the **poly(A) tail**. This tail serves as a binding site for one or more specific proteins. The poly(A) tail and its associated proteins have a variety of roles in coordinating transcription and translation, and may help protect mRNA from enzymatic destruction. Many bacterial mRNAs also acquire poly(A) tails, but these tails stimulate decay of mRNA rather than protecting it from degradation.

The poly(A) tail is added in a multistep process. The transcript is extended beyond the site where the poly(A) tail is to be added, then is cleaved at the poly(A) addition site by an endonuclease component of a large enzyme complex, again associated with the CTD of RNA polymerase II (Fig. 26-18). The mRNA site where cleavage occurs is marked by two sequence elements: the highly conserved sequence (5′)AAUAAA(3′), 10 to 30 nucleotides on the 5′ side (upstream) of the cleavage site, and a less well-defined sequence rich in G and U residues, 20 to 40 nucleotides downstream of the cleavage site. Cleavage generates the free 3′-hydroxyl group that defines the end of the mRNA, to which A residues are immediately added by **polyadenylate polymerase**, which catalyzes the reaction

$$\text{RNA} + n\text{ATP} \longrightarrow \text{RNA—(AMP)}n + n\text{PP}_i$$

where $n = 80$ to 250. This enzyme does not require a template but does require the cleaved mRNA as a primer. These longer poly(A) tails are added in the nucleus, and then shortened significantly after the mRNA is transported to the cytoplasm.

The overall processing of a typical eukaryotic mRNA is summarized in Figure 26-19. In some cases, the polypeptide-coding region of the mRNA is also modified by RNA "editing" (see Section 27.1 for details). This editing includes processes that add or delete bases in the coding regions of primary transcripts or that change the sequence (such as by enzymatic deamination of a C residue to create a U residue). A particularly dramatic example occurs in trypanosomes, which are parasitic protists: large regions of an mRNA are synthesized without any uridylate, and the U residues are inserted later by RNA editing.

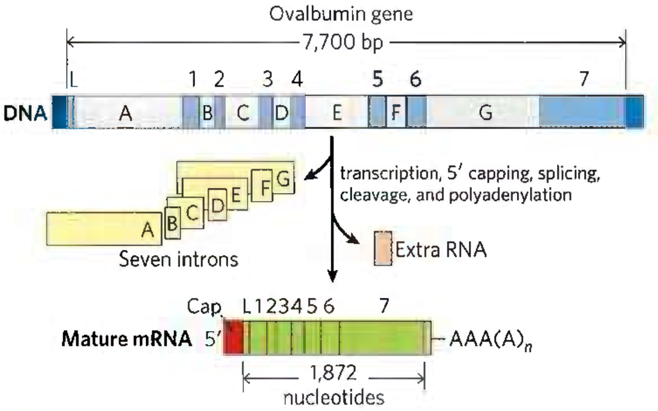


FIGURE 26-19 Overview of the processing of a eukaryotic mRNA. The ovalbumin gene, shown here, has introns A to G and exons 1 to 7 and L (L encodes a signal peptide sequence that targets the protein for export from the cell; see Fig. 27-38). About three-quarters of the RNA is removed during processing. Pol II extends the primary transcript well beyond the cleavage and polyadenylation site ("extra RNA") before terminating transcription.

A Gene Can Give Rise to Multiple Products by Differential RNA Processing

One of the paradoxes of modern genomics is that the apparent complexity of organisms does not correlate with the number of protein-coding genes, or even the amount of genomic DNA. Some eukaryotic mRNA transcripts can be processed in more than one way to produce *different* mRNAs and thus different polypeptides. Much of the variability in processing is the result of **alternative splicing**, in which a particular exon may or may not be incorporated into the mature mRNA transcript. Alternative splicing occurs in a relatively small number of transcripts in yeast, but in more than 95% of human genes. Changes in alternative splicing can have profound impact on the development of an organism (**Box 26-2**). Alternative splicing of a single transcription factor in the staple grain quinoa differentiates palatable sweet varieties from those too bitter to ingest without processing. In *Drosophila*, sex is determined by alternative splicing of the *sex lethal* (*Sxl*) transcript based on the number of X chromosomes present in the cell.

Figure 26-20a illustrates how alternative splicing patterns can produce more than one protein from a common pre-mRNA. The pre-mRNA contains molecular signals for all the alternative processing pathways, and the pathway favored in a given cell or metabolic situation is determined by processing factors, RNA-binding proteins that promote one particular path. For example, splicing regulatory proteins or heterogeneous ribonuclear proteins (hnRNPs) may bind these signals and promote or inhibit spliceosome assembly at that site. There are many additional patterns of alternative splicing.

Complex transcripts can also have more than one site where poly(A) tails can form (Fig. 26-20b). If there are two or more sites for cleavage and polyadenylation, use of the one closest to the 5′ end will remove more of the primary transcript sequence. This mechanism,

BOX 26-2 MEDICINE

Alternative Splicing and Spinal Muscular Atrophy

Alternative splicing is one of the least understood steps in human gene regulation, in part because a gene product can be spliced together in many ways: entire exons can be left out of the mRNA (skipped) or spliced in (retained). More subtle changes can also occur in which alternative 5′ or 3′ splice sites are used that differ from their canonical positions by just a few nucleotides. The spliced isoform that is generated is determined by interactions among the spliceosome, a large number of regulatory factors that associate with the pre-mRNA transcript, and other cellular machinery, including the transcription complex.

Despite this complexity, scientists are learning how to control alternative splicing to treat genetic diseases such as spinal muscular atrophy (SMA). SMA is a progressive neurodegenerative disease and in its most severe form is always fatal. It is the most common genetic cause of death in infants. SMA is caused by a defect in the *SMN1* (survival of motor neuron 1) gene. *SMN1* encodes a protein that is essential for assembly of cellular snRNPs, including those that make up the spliceosome.

Humans have two *SMN* genes: *SMN1* and *SMN2*. However, only *SMN1* is capable of being correctly spliced to produce a functional protein (Fig. 1). *SMN2* encodes an RNA sequence called a silencer, which causes exon 7 to be excluded from the mRNA. As a result, *SMN2* cannot produce functional protein. Healthy individuals are able to get all of the SMN protein they require for snRNP assembly from the *SMN1* gene. But those with a mutation in *SMN1* do not produce enough SMN protein to assemble an adequate number of snRNPs. This leads to neuromuscular degeneration that is often fatal.

One possible solution for treating SMA would be to find a way to change the alternative splicing pattern of the SMN2 gene so that exon 7 would be included rather than skipped. This would produce functional SMN protein. This is exactly the strategy that

(Continued on next page)

BOX 26-2 MEDICINE

Alternative Splicing and Spinal Muscular Atrophy (*Continued*)

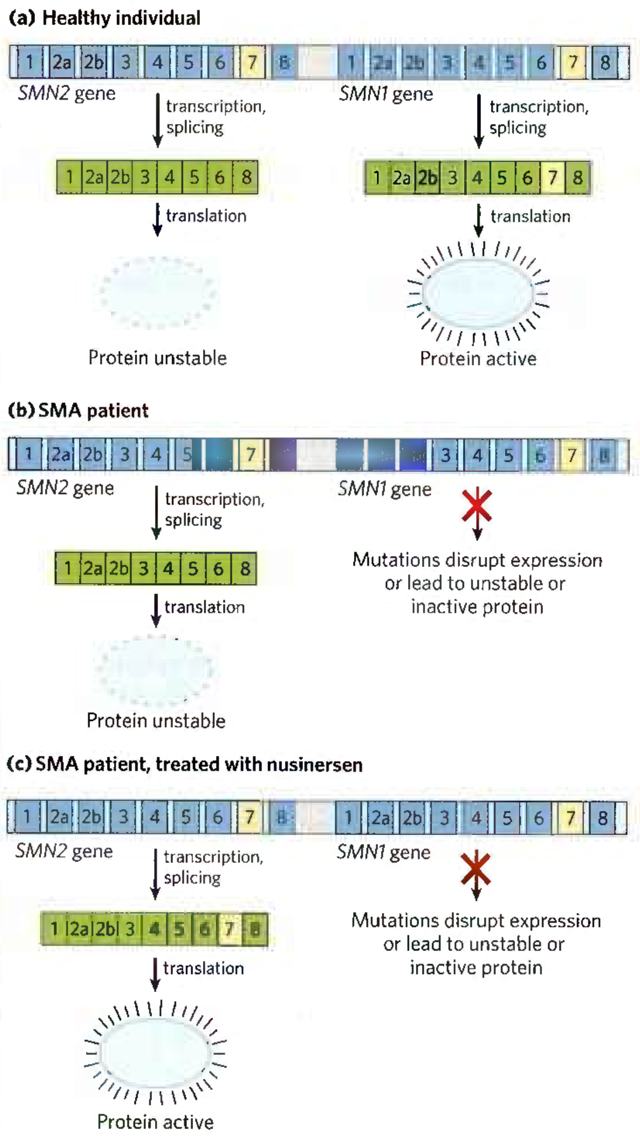
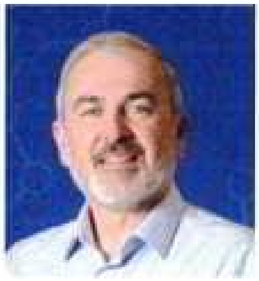

(a) Healthy individual
1 2a 2b 3 4 5 6 7 8
SMN2 gene
transcription, splicing
1 2a 2b 3 4 5 6 8
translation
Protein unstable
1 2a 2b 3 4 5 6 7 8
SMN1 gene
transcription, splicing
1 2a 2b 3 4 5 6 7 8
translation
Protein active
(b) SMA patient
1 2a 2b 3 4 5 7
SMN2 gene
transcription, splicing
1 2a 2b 3 4 5 6 8
translation
Protein unstable
3 4 5 6 7 8
SMN1 gene
Mutations disrupt expression or lead to unstable or inactive protein
(c) SMA patient, treated with nusinersen
1 2a 2b 3 4 5 6 7 8
SMN2 gene
transcription, splicing
1 2a 2b 3 4 5 6 7 8
translation
Protein active
1 2a 2b 3 4 5 6 7 8
SMN1 gene
Mutations disrupt expression or lead to unstable or inactive protein

FIGURE 1 Alternative splicing of the *SMN1* and *SMN2* gene transcripts in healthy individuals and in those with SMA. (a) In healthy individuals, active protein is produced by translation of mRNAs that include exon 7 of the *SMN1* gene. Alternative splicing of the *SMN2* transcript skips exon 7, resulting in an mRNA that cannot produce functional protein. (b) In SMA patients, a mutation in the *SMN1* gene results in no functional protein being produced from either *SMN1* or *SMN2*. (c) Upon treatment with nusinersen, alternative splicing of *SMN2* results in an mRNA that includes exon 7 and produces functional protein. This can prevent neuromuscular degeneration. [Information from D. R. Corey, *Nat. Neurosci.* 20:497, 2017, Fig. 1.]



Adrian Krainer [Courtesy Adrian Krainer.]

Adrian Krainer used to correct SMA phenotypes in mice. Scientists in the Krainer lab discovered that if they injected mice with a synthetic oligonucleotide complementary to the silencer sequence of *SMN2* exon 7 (also called an *antisense* oligonucleotide, or ASO), the sequence would be hidden from the splicing machinery. This changes the splicing of the SMN2 gene so that exon 7 is included and the SMN2 gene is able to produce a functional SMN protein and prevent neurodegeneration (Fig. 1).

A drug called nusinersen has since been developed based on Krainer's research and became the first approved treatment for SMA. In SMA patients, injection of nusinersen into the central nervous system can correct splicing of the human *SMN2* gene product, restore SMN protein production, and halt neurodegeneration. Most pharmaceuticals are small, organic molecules, but nusinersen is an 18-mer oligonucleotide. To turn an oligonucleotide into a drug, researchers had to incorporate chemical modifications into the phosphate backbone and ribose sugar (Fig. 2). These modifications prevent the oligonucleotide from being destroyed by cellular nucleases and also improve binding to RNA targets. Currently very few nucleic acid–based drugs have been approved for medical use, but their importance in medicine will likely continue to grow.

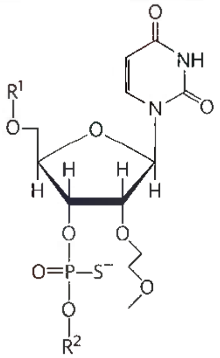

O
NH
N
O
R1
O
O
H
H
H
H
O
O
O=P—S⁻
O
O
R2

FIGURE 2 The modified nucleotides in nusinersen use a phosphorthioate backbone rather than phosphodiesters, and the 2′ hydroxyl groups are replaced by 2′-*O*-methoxyethyl groups.

called **poly(A) site choice**, generates diversity in the variable domains of immunoglobulin heavy chains (see Fig. 25-42).

Both alternative splicing and poly(A) site choice come into play in the expression of many genes. For example, a single RNA transcript is processed using both mechanisms to produce two different hormones: the calcium-regulating hormone calcitonin in rat thyroid and calcitonin-gene-related peptide (CGRP) in rat brain (**Fig. 26-21**). **P2** Together, alternative splicing and poly(A) site choice greatly increase the variety of proteins generated from the genomes of higher eukaryotes.

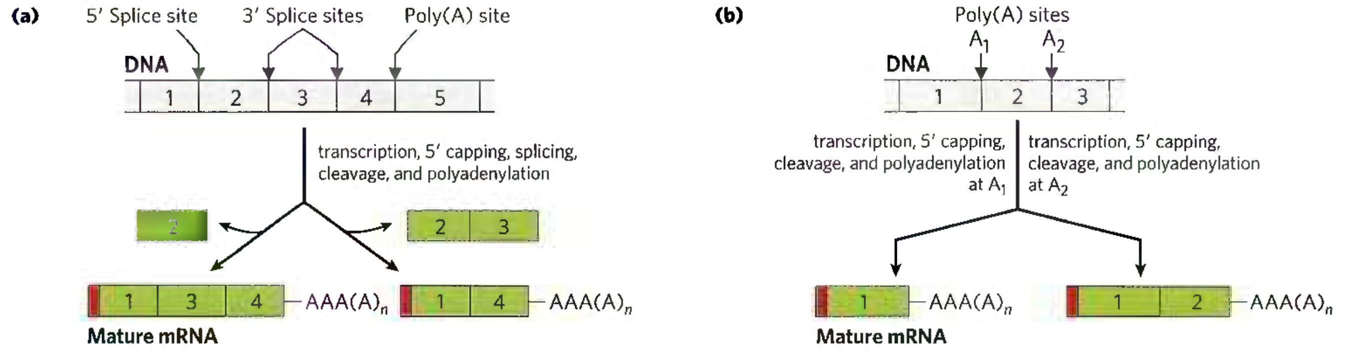

(a)
5′ Splice site
3′ Splice sites
Poly(A) site
DNA
1
2
3
4
5
transcription, 5′ capping, splicing, cleavage, and polyadenylation
2
2
3
1
3
4
AAA(A)$_n$
1
4
AAA(A)$_n$
Mature mRNA
(b)
Poly(A) sites
A_1
A_2
DNA
1
2
3
transcription, 5′ capping, cleavage, and polyadenylation at A_1
transcription, 5′ capping, cleavage, and polyadenylation at A_2
1
AAA(A)$_n$
1
2
AAA(A)$_n$
Mature mRNA

FIGURE 26-20 Alternative transcript production in eukaryotes. (a) Alternative splicing patterns. Two 3′ splice sites are shown. Different mature mRNAs are produced from the same primary transcript. (b) Two alternative cleavage and polyadenylation sites, A_1 and A_2.

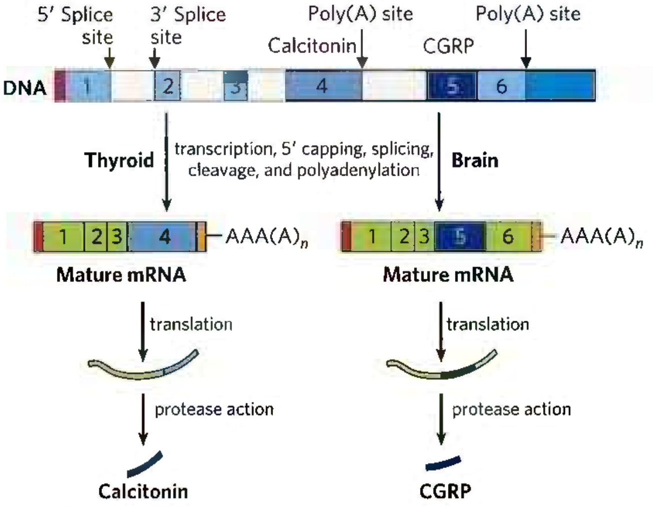

5′ Splice site
3′ Splice site
Poly(A) site
Calcitonin
Poly(A) site
CGRP
DNA
1
2
3
4
5
6
Thyroid
transcription, 5′ capping, splicing, cleavage, and polyadenylation
Brain
1
2
3
4
AAA(A)$_n$
1
2
3
5
6
AAA(A)$_n$
Mature mRNA
Mature mRNA
translation
translation
protease action
protease action
Calcitonin
CGRP

FIGURE 26-21 Alternative processing of the calcitonin gene transcript in rats. The calcitonin gene encodes a primary transcript with two poly(A) sites; one predominates in the brain, the other in the thyroid. In the brain, splicing eliminates the calcitonin exon (exon 4); in the thyroid, this exon is retained. The resulting peptides are processed further to yield the final hormone products: calcitonin in the thyroid and calcitonin-gene-related peptide (CGRP) in the brain.

Ribosomal RNAs and tRNAs Also Undergo Processing

Posttranscriptional processing is not limited to mRNA. Ribosomal RNAs of bacterial, archaeal, and eukaryotic cells are made from longer precursors called **pre-ribosomal RNAs**, or pre-rRNAs. Transfer RNAs are similarly derived from longer precursors. These RNAs may also contain a variety of modified nucleosides; some examples are shown in **Figure 26-22**.

Ribosomal RNAs In bacteria, 16S, 23S, and 5S rRNAs (and some tRNAs, although most tRNAs are encoded elsewhere) arise from a single 30S RNA precursor of about 6,500 nucleotides. RNA at both ends of the 30S precursor and segments between the rRNAs are removed during processing (**Fig. 26-23**). The 16S and 23S rRNAs contain modified nucleosides. In *E. coli*, the 11 modifications in the 16S rRNA include a pseudouridine and 10 nucleosides methylated on the base or the 2′-hydroxyl group or both. The 23S rRNA has 10 pseudouridines, 1 dihydrouridine, and 12 methylated nucleosides. In bacteria, each modification is generally catalyzed by a distinct enzyme. Methylation reactions use *S*-adenosylmethionine as cofactor. No cofactor is required for pseudouridine formation.

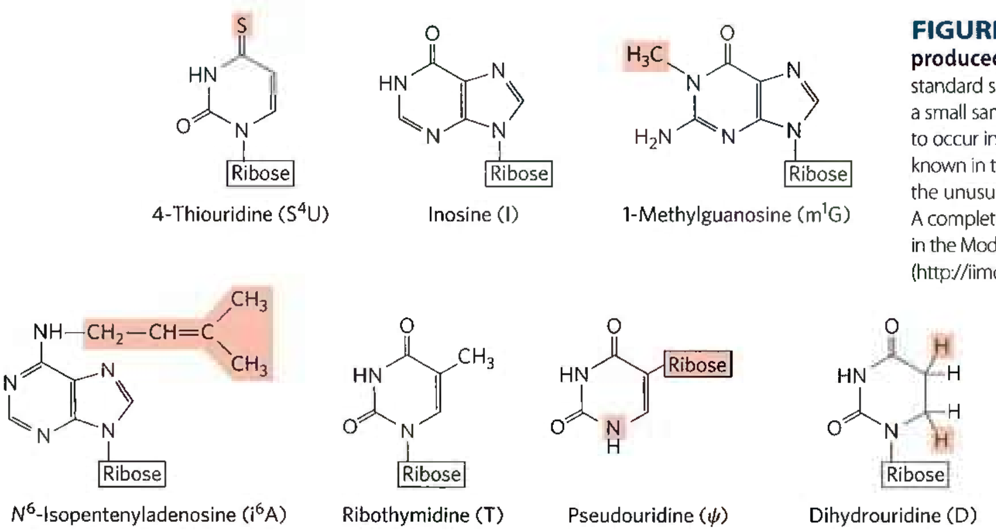

S
HN
O
N
Ribose
4-Thiouridine (S^4U)
O
HN
N
N
N
Ribose
Inosine (I)
H_3C
O
N
N
H_2N
N
N
Ribose
1-Methylguanosine (m^1G)
CH_3
NH—CH_2—CH=C
CH_3
N
N
N
N
Ribose
N^6-Isopentenyladenosine (i^6A)
O
HN
CH_3
O
N
Ribose
Ribothymidine (T)
O
HN
Ribose
O
N
H
Pseudouridine (ψ)
O
H
HN
H
H
O
N
H
Ribose
Dihydrouridine (D)

FIGURE 26-22 Some modified bases of RNA produced in posttranscriptional reactions. The standard symbols are shown in parentheses. This is just a small sampling of the 96 modified nucleosides known to occur in different RNA species, with 81 different types known in tRNAs and 30 observed to date in rRNAs. Notice the unusual ribose attachment point in pseudouridine. A complete listing of these modified bases can be found in the Modomics database of RNA modification pathways (http://iimcb.genesilico.pl/modomics/).

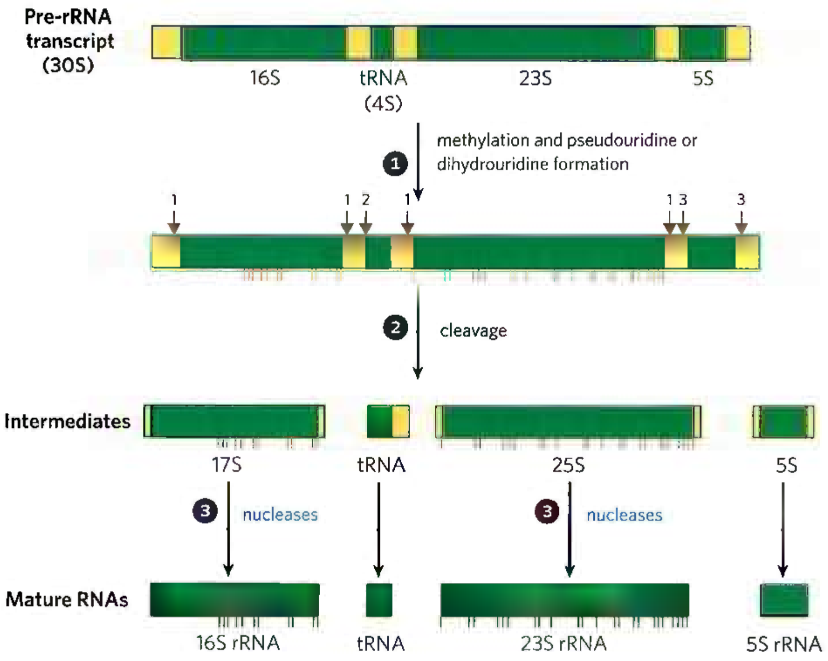


FIGURE 26-23 Processing of pre-rRNA transcripts in bacteria. ❶ Before cleavage, the 30S RNA precursor is methylated at specific bases (red tick marks), and some uridine residues are converted to pseudouridine (blue tick) or dihydrouridine (black tick) residues. The methylation reactions are of multiple types, some occurring on bases and some on 2′-hydroxyl groups. ❷ Cleavage liberates precursors of rRNAs and tRNA(s). Cleavage at the points labeled 1, 2, and 3 is carried out by the enzymes RNase III, RNase P, and RNase E, respectively. As discussed later in the text, RNase P is a ribozyme. ❸ The final 16S, 23S, and 5S rRNA products result from the action of a variety of specific nucleases. The seven copies of pre-rRNA gene in the *E. coli* chromosome differ in the number, location, and identity of tRNAs included in the primary transcript. Some copies of the gene have additional tRNA gene segments between the 16S and 23S rRNA segments and at the far 3′ end of the primary transcript.

The genome of *E. coli* encodes seven pre-rRNA molecules. All of these genes have essentially identical rRNA-coding regions, but they differ in the segments between these regions. The segment between the 16S and 23S rRNA genes generally encodes one or two tRNAs, with different tRNAs produced from different pre-rRNA transcripts. Coding sequences for tRNAs are also found on the 3′ side of the 5S rRNA in some precursor transcripts.

The situation in eukaryotes is even more complicated (see Fig. 27-17). The entire process is initiated in the nucleolus, in large complexes that assemble on the rRNA precursor as it is synthesized by Pol I. There is a tight coupling between rRNA transcription, rRNA maturation, and ribosome assembly in the nucleolus. Each complex includes the ribonucleases that cleave the rRNA precursor, the enzymes that modify particular bases, large numbers of ncRNAs called **small nucleolar RNAs**, or **snoRNAs**, that guide nucleoside modification and some cleavage reactions, and ribosomal proteins. In yeast, the entire process involves the pre-rRNA, more than 170 nonribosomal proteins, snoRNAs for each nucleoside modification (about 70, because some snoRNAs guide two types of modification), and the 78 ribosomal proteins. Humans have an even greater number of modified nucleosides, about 200, and a greater number of associated snoRNAs. The composition of the complexes may change as the ribosomes are assembled, and many of the intermediate complexes rival the ribosome itself in complexity. The 5S rRNA of most eukaryotes is made as a completely separate transcript by a different polymerase (Pol III).

The most common nucleoside modifications in eukaryotic rRNAs are conversion of uridine to pseudouridine and adoMet-dependent nucleoside methylation (often at 2′-hydroxyl groups). These reactions often rely on snoRNA-protein complexes, or **snoRNPs**, each consisting of a snoRNA and four or five proteins, including the enzyme that carries out the modification. There are two classes of snoRNPs, both defined by key conserved sequence elements referred to as lettered boxes. The box H/ACA snoRNPs function in pseudouridylylation, and box C/D snoRNPs in 2′-*O*-methylations. The snoRNAs are 60 to 300 nucleotides long. Each snoRNA includes a 10 to 21 nucleotide sequence that is perfectly complementary to some site on an rRNA and serves to identify the modification site (Fig. 26-24). The conserved sequence elements in the remainder of the snoRNA fold into structures that are bound by the snoRNP proteins.

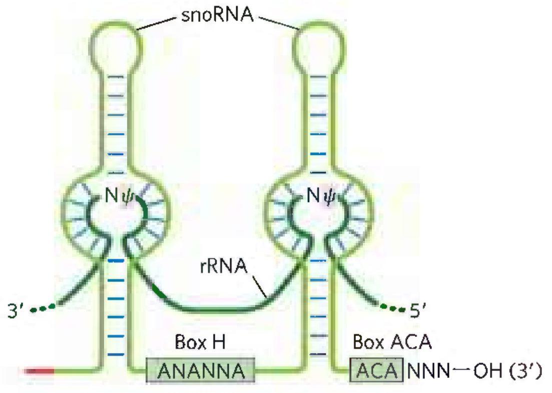


FIGURE 26-24 RNA pairing with box H/ACA snoRNAs to guide pseudouridylations. The pseudouridine conversion sites in the target rRNA are in the regions paired with the snoRNA, and the conserved H/ACA box sequences are protein-binding sites. [Information from T. Kiss, *Cell* 109:145, 2002.]

Transfer RNAs Most cells have 40 to 50 distinct tRNAs, and eukaryotic cells have multiple copies of many of the tRNA genes. Transfer RNAs are derived from longer RNA precursors by enzymatic removal of nucleotides from the 5′ and 3′ ends (**Fig. 26-25**). In eukaryotes, introns are present in a few tRNA transcripts and must be excised. Where two or more different tRNAs are contained in a single primary transcript, they are separated by enzymatic cleavage. The endonuclease RNase P, found in all organisms, removes RNA at the 5′ end of tRNAs. This enzyme contains both protein and RNA. The RNA component is essential for activity, and in bacterial cells it can carry out its processing function with precision even without the protein component. RNase P is another example of a catalytic RNA, as described in more detail below. The 3′ end of tRNAs is processed by one or more nucleases, including the exonuclease RNase D.

Transfer RNA precursors may undergo further post-transcriptional processing. The 3′-terminal trinucleotide CCA(3′) to which an amino acid is attached during protein synthesis (Chapter 27) is absent from some bacterial tRNA precursors and all eukaryotic tRNA precursors and is added during processing (Fig. 26-25). This addition is carried out by tRNA nucleotidyltransferase, an unusual enzyme that binds the three ribonucleoside triphosphate precursors in separate active sites and catalyzes formation of the phosphodiester bonds to produce the CCA(3′) sequence. The creation of this defined sequence of nucleotides is therefore not dependent on a DNA or RNA template—the template is the binding site of the enzyme.

The final type of tRNA processing is the modification of some bases by methylation, deamination, or reduction (Fig. 26-22). These modifications can change how the tRNA interacts with cellular proteins and even how the tRNA is used by the ribosome during translation. In the case of pseudouridine, the base (uracil) is removed and reattached to the sugar through C-5. Some of these modified bases occur at characteristic positions in all tRNAs (Fig. 26-25).

Special-Function RNAs Undergo Several Types of Processing

The number of known classes of special-function noncoding RNAs (ncRNAs) is expanding rapidly, as is the variety of functions known to be associated with them. Many of these ncRNAs also undergo processing.

The snRNAs and snoRNAs not only facilitate RNA processing reactions but also are themselves synthesized as larger precursors and then processed. Many snoRNAs are encoded within the introns of other genes. As the introns are spliced from the pre-mRNA, proteins bind to the snoRNA sequences and ribonucleases remove the extra RNA at the 5′ and 3′ ends to form the snoRNP. The snRNAs destined for spliceosomes are synthesized as pre-snRNAs, and ribonucleases remove the extra RNA at each end. Particular nucleosides in snRNAs are also subject to 11 types of modification, with 2′-*O*-methylation and conversion of uridine to pseudouridine predominating.

MicroRNAs (miRNAs) are a special class of noncoding RNAs involved in gene regulation. The miRNAs are about 22 nucleotides long, complementary in sequence to particular regions of mRNAs. Found in plants and in animals, from worms to mammals, they promote mRNA

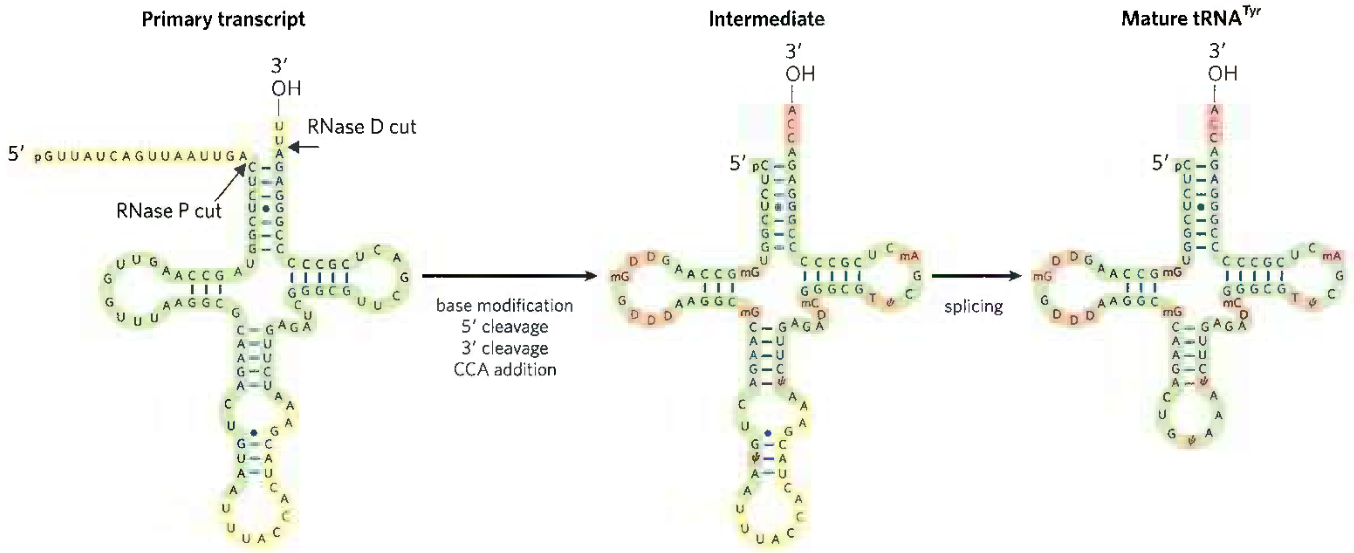


FIGURE 26-25 Processing of tRNAs in bacteria and eukaryotes. The yeast tRNA^{Tyr} (the tRNA specific for tyrosine binding; see Chapter 27) is used to illustrate the important steps. Short blue lines represent normal base pairing; blue dots indicate G–U base pairs. The nucleotide sequences shown in yellow are removed from the primary transcript. The ends are processed first, the 5′ end before the 3′ end. CCA is then added to the 3′ end, a necessary step in processing all eukaryotic tRNAs and for those bacterial tRNAs that lack this sequence in the primary transcript. While the ends are being processed, specific bases in the rest of the transcript are modified (see Fig. 26-22). For the eukaryotic tRNA shown here, the final step is splicing of the 14 nucleotide intron by a protein enzyme. Introns are found in some eukaryotic tRNAs but not in bacterial tRNAs.

degradation and suppress translation to fine-tune gene expression. About 1,500 human genes encode miRNAs, and one or more of these miRNAs affect the expression of most protein-coding genes.

The miRNAs are synthesized from much larger precursors, in several steps (**Fig. 26-26**). The primary transcripts for miRNAs (pri-miRNAs) vary greatly in size; some are encoded in the introns of other genes and are coexpressed with these host genes. Processing of pri-miRNA is mediated by two endoribonucleases in the RNase III family, Drosha and Dicer. First, in the nucleus, the pri-miRNA is reduced to a 70 to 80 nucleotide precursor miRNA (pre-miRNA) by a protein complex including Drosha and another protein, DGCR8. The pre-miRNA is then exported to the cytoplasm in a complex with two proteins, exportin-5 and the Ran GTPase (see Fig. 27-42). In the cytoplasm, Ran hydrolyzes the GTP, then exportin-5 and the pre-miRNA are released. The pre-miRNA is then acted on by Dicer to produce the nearly mature miRNA paired with a short RNA complement. The complement is removed by an RNA helicase, and the mature miRNA is incorporated into protein complexes, such as the RNA-induced silencing complex (RISC), which then bind a target mRNA. If the complementarity between miRNA and its target is nearly perfect, the target mRNA is cleaved. If the complementarity is only partial, the complex blocks translation of the target mRNA. The roles of miRNAs and RISC in gene regulation are detailed in Chapter 28.

Cellular mRNAs Are Degraded at Different Rates

The expression of genes is regulated at many levels. A crucial factor governing a gene's expression is the cellular concentration of its associated mRNA. The concentration of any molecule depends on two factors: its rate of synthesis and its rate of degradation. When synthesis and degradation of an mRNA are balanced, the concentration of the mRNA remains in a steady state. A change in either rate will lead to net accumulation or depletion of the mRNA. **P2** Degradative pathways ensure that mRNAs do not build up in the cell and direct the synthesis of unnecessary proteins.

The rates of degradation vary greatly for mRNAs from different eukaryotic genes. For a gene product that is needed only briefly, the half-life of its mRNA may be only minutes or even seconds. Gene products needed

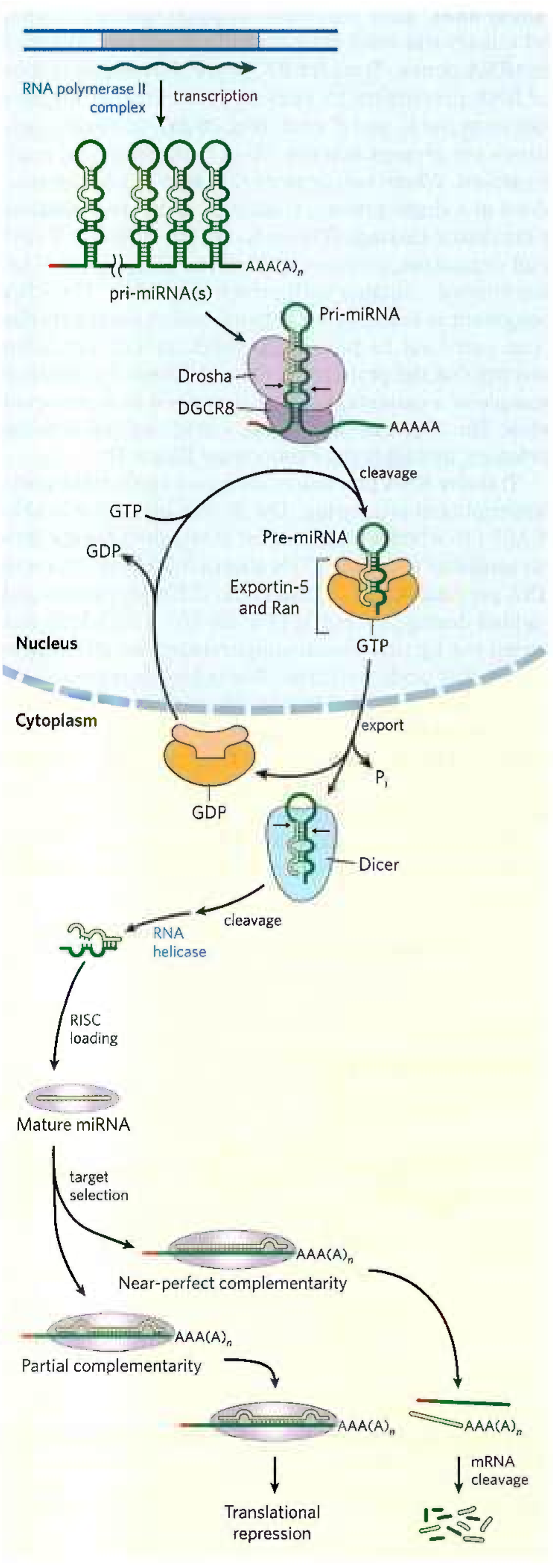

RNA polymerase II complex
transcription
AAA(A)$_n$
pri-miRNA(s)
Pri-miRNA
Drosha
DGCR8
AAAAA
cleavage
GTP
GDP
Pre-miRNA
Exportin-5 and Ran
Nucleus
GTP
Cytoplasm
export
P_i
GDP
Dicer
cleavage
RNA helicase
RISC loading
Mature miRNA
target selection
AAA(A)$_n$
Near-perfect complementarity
AAA(A)$_n$
Partial complementarity
AAA(A)$_n$
AAA(A)$_n$
mRNA cleavage
Translational repression

FIGURE 26-26 **Synthesis and processing of miRNAs.** The primary transcript of miRNAs is a larger RNA of variable length, called pri-miRNA. The pri-miRNA undergoes a number of processing events both in the nucleus and in the cytoplasm to make a mature miRNA. Once the miRNA has been loaded into a protein complex called RISC, it can then hybridize to mRNAs and repress their translation or trigger their cleavage and destruction. [Information from E. Wienholds and R. H. A. Plasterk, *FEBS Lett.* 579:5911, 2005; V. N. Kim et al., *Nat. Rev. Mol. Cell Biol.* 10:126, 2009, Figs 2–4.]

constantly by the cell may have mRNAs that are stable over many cell generations. The average half-life of the mRNAs of a vertebrate cell is about 3 hours, with the pool of each type of mRNA turning over about 10 times per cell generation. The half-life of bacterial mRNAs is much shorter—only about 1.5 min—perhaps because of regulatory requirements.

Messenger RNA is degraded by ribonucleases present in all cells. In *E. coli*, mRNAs typically contain 5′ triphosphates remaining from the initiation of transcription. These groups protect the mRNA from 5′ degradation. As a result, mRNA decay begins with one or several cuts by an endoribonuclease, followed by 3′→5′ degradation by exoribonucleases (**Fig. 26-27**). The initial cut by the endonuclease generates an RNA fragment with a 5′ monophosphate end, which serves to tether the endonuclease to the transcript and ensure its rapid destruction. Some bacteria (*Bacillus subtilis*, for example), have exonucleases that also recognize the 5′ monophosphate end and can degrade RNA fragments in the 5′→3′ direction.

Polynucleotide phosphorylase (PNPase) is a common 3′→5′ exoribonuclease responsible for the degradation of many mRNAs in bacteria, chloroplasts, and mitochondria. It catalyzes the reversible phosphorolysis (rather than hydrolysis) of the mRNA chain using orthophosphate as the nucleophile. The PNPase reaction is readily reversible and the enzyme can also add nucleotides to the 3′ ends of bacterial mRNAs. Decay of mRNAs containing complex 3′ end structures, such as the hairpins responsible for ρ-independent transcription termination (see Fig. 26-7), can involve multiple rounds of lengthening and shortening of the mRNA by PNPase until it is finally consumed. This unusual nontemplated RNA polymerization activity of PNPase proved to be critical for production of mRNA polymers used for deciphering the genetic code (Chapter 27).

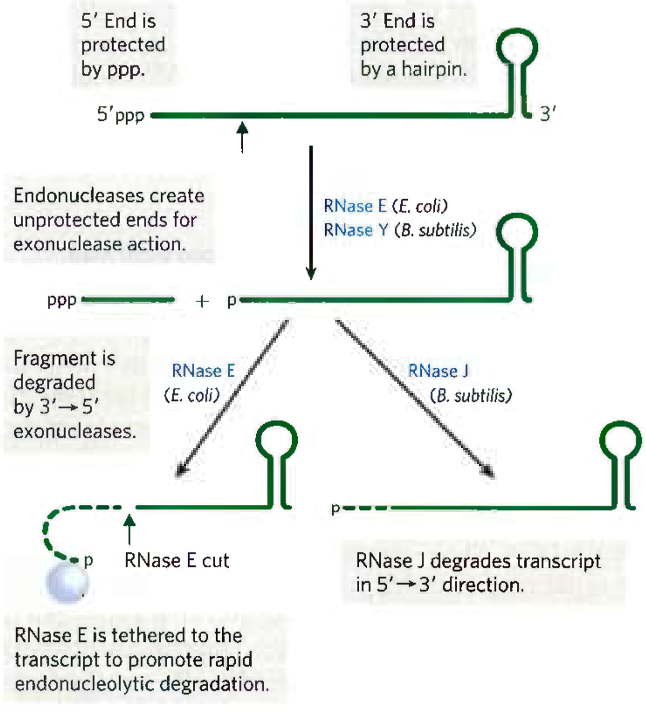

5′ End is protected by ppp.
3′ End is protected by a hairpin.
5′ppp
3′
Endonucleases create unprotected ends for exonuclease action.
RNase E (*E. coli*)
RNase Y (*B. subtilis*)
ppp
+ p
Fragment is degraded by 3′→5′ exonucleases.
RNase E (*E. coli*)
RNase J (*B. subtilis*)
p
p
RNase E cut
RNase J degrades transcript in 5′→3′ direction.
RNase E is tethered to the transcript to promote rapid endonucleolytic degradation.

FIGURE 26-27 Degradation of RNA in bacteria. In bacteria, mRNA degradation usually begins by endonucleolytic cleavage because the 5′ and 3′ ends of the mRNA are often protected by a triphosphate and hairpin structure, respectively. In *E. coli*, the RNase E endonuclease carries out this cleavage, whereas in *B. subtilis* the cleavage is carried out by the RNase Y endonuclease. The endonuclease activity produces RNA fragments that serve as substrates for 3′→5′ or 5′→3′ exonucleases. All bacteria contain 3′→5′ exonucleases such as PNPase, RNase R, or RNase II. Some species, like *B. subtilis*, also contain a 5′→3′ exonuclease called RNase J. The 5′ phosphate produced by the endonuclease after the first cleavage can also serve as a tether to link the RNase E endonuclease directly to the mRNA, ensuring its rapid destruction. [Information from M. Hui et al. *Annu. Rev. Genet.* 48:537, 2014.]

As we have previously seen with transcription and RNA processing, the analogous processes for RNA degradation in eukaryotes are much more complex than their bacterial counterparts. Eukaryotes have multiple pathways for mRNA decay, and the pathway used can depend on the mRNA location, its structure, its association with ribosomes, and other factors. However, in most cases, decapping the 5′ end and shortening the 3′ poly(A) tail are critical steps for allowing exonucleases to access the mRNA.

All eukaryotes also have large 3′→5′ exoribonucleases called **exosomes**, which are responsible for the degradation for nearly all types of RNA. Exosomes are multisubunit complexes containing about 10 proteins. Specialized exosomes exist in the nucleus, cytoplasm, and nucleolus. The core of the exosome is a barrel-like structure through which RNA is threaded (**Fig. 26-28**). Even though this core

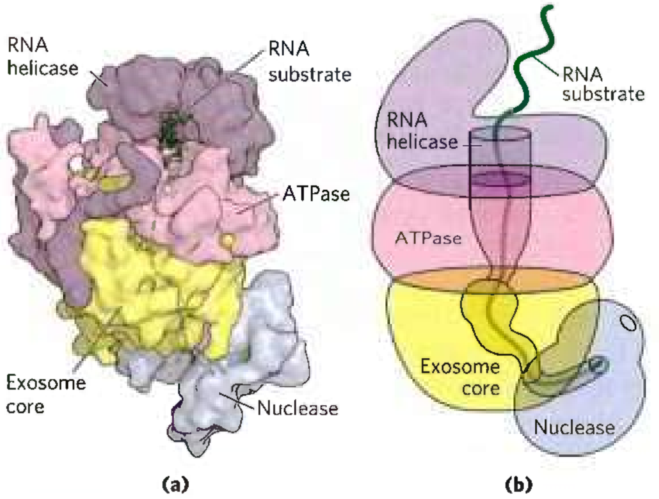

RNA helicase
RNA substrate
ATPase
Exosome core
Nuclease
RNA substrate
RNA helicase
ATPase
Exosome core
Nuclease
(a)
(b)

FIGURE 26-28 Essential role of the exosome in eukaryotic RNA degradation. (a) Exosomes are multisubunit enzymes in which RNA is threaded through a central barrel and fed into a nuclease. In this structure, the exosome core is topped by ATPase and RNA helicase modules that help unwind RNA secondary structures so that single-stranded RNA can pass into the core. Below the core is a nuclease responsible for cleaving the RNA. (b) In this cartoon schematic, the passage of the substrate RNA through the barrel-like exosome and to the nuclease is highlighted. [Data from PDB ID 4IFD, D. L. Makino et al., *Nature* 495:70, 2013; PDB ID 4OO1, E. V. Wasmuth et al., *Nature* 511:435, 2014. Information from K. Januszyk and C. D. Lima, *Curr. Opin. Struct. Biol.* 24:132, 2014.]

is structurally similar to bacterial PNPase, RNA is not degraded within the barrel. Instead, the barrel serves as an adapter that efficiently channels the RNA to associated enzymes with 3′→5′ exonuclease and endonuclease activity.

SUMMARY 26.2 ***RNA Processing***

- Many primary transcripts produced in bacteria and eukaryotes must be processed into a mature form to be functional. Processing can include modifications to the 5′ and 3′ ends of the RNA, removal of internal RNA sequences by splicing, and modifications of the RNA nucleotides.
- Eukaryotic mRNAs have an inverted 7-methylguanosine residue cap at their 5′ end. The cap helps to protect the RNA from degradation and interacts with proteins important for cellular transport and translation.
- Many organisms contain genes in which the coding information is interrupted by introns. Splicing removes these introns and joins the flanking exons. Nearly every human gene contains multiple introns, which can vary dramatically in size.
- There are four classes of introns: group I, group II, spliceosome-processed introns, and protein-processed introns. Group I and II introns are self-splicing with RNAs capable of carrying out catalysis independent of protein enzymes. Catalytic RNAs share features in common with protein-based enzymes.
- Nuclear-encoded introns in eukaryotes are removed by a large RNP machine called a spliceosome. A spliceosome is a single-turnover enzyme containing snRNA and protein that recognizes introns by base-pairing with the snRNAs. Even though a spliceosome contains dozens of proteins, it uses an RNA active site and mechanism similar to that of group II introns.
- Some tRNAs and a few mRNAs contain introns that must be removed by protein-based endonuclease and ligase enzymes.
- In eukaryotes, transcription terminates when an endonuclease cleaves the nascent RNA, freeing it from Pol II. A poly(A) tail is then added to the 3′ end of the RNA by a polyadenylate polymerase.
- Alternative splicing and alternative poly(A) site choice in eukaryotes allow for many different transcripts to be produced from a single gene.
- The primary transcripts of tRNAs, rRNAs, and miRNAs also undergo extensive processing, including endonucleolytic cleavage and chemical modification. Correct placement of these modifications is often guided by snoRNAs that base-pair with the target RNA.
- The cellular lifetime of RNAs can be highly variable, and RNA degradation is tightly regulated. In bacteria, endonucleases generate mRNA fragments for destruction by exonucleases. In eukaryotes, mRNAs typically must be decapped and the poly(A) tail shortened before degradation. The exosome is a supramolecular complex of 3′→5′ exo- and endonucleases involved in many steps of eukaryotic RNA decay.

26.3 RNA-Dependent Synthesis of RNA and DNA

In our discussion of DNA and RNA synthesis up to this point, the role of the template strand has been reserved for DNA. However, some enzymes use an RNA template for nucleic acid synthesis. With the important exception of viruses with an RNA genome, these enzymes play only supporting roles in information pathways. RNA viruses are the source of most characterized RNA-dependent polymerases, although some eukaryotes also use these enzymes to amplify double-stranded RNAs used in RNA interference.

The existence of RNA replication requires an elaboration of the central dogma—the notion that genetic information flows only from DNA to RNA to proteins. RNA-dependent polymerases allow the genetic information stored in RNA to be replicated and reverse transcribed into DNA. The enzymes of the RNA replication process have profound implications for investigations into the nature of self-replicating molecules that may have existed in prebiotic times.

Reverse Transcriptase Produces DNA from Viral RNA

P3 Certain RNA viruses that infect animal cells carry within the viral particle an RNA-dependent DNA polymerase called **reverse transcriptase**. On infection, the single-stranded RNA viral genome (~10,000 nucleotides) and the enzyme enter the host cell. The reverse transcriptase first catalyzes the synthesis of a DNA strand complementary to the viral RNA (**Fig. 26-29**), then degrades the RNA strand of the viral RNA-DNA hybrid and replaces it with DNA. The resulting duplex DNA often becomes incorporated into the genome of the eukaryotic host cell. These integrated (and dormant) viral genes can be activated and transcribed, and the gene products—viral proteins and the viral RNA genome itself—are packaged as new viruses. The RNA viruses that contain reverse transcriptases are known as **retroviruses** (*retro* is the Latin prefix for "backward").

The existence of reverse transcriptases in RNA viruses was predicted by Howard Temin in 1962, and the enzymes were ultimately detected by Temin and, independently, by David Baltimore in 1970. P3 Their discovery aroused much attention as dogma-shaking proof that genetic information can flow "backward" from RNA to DNA.

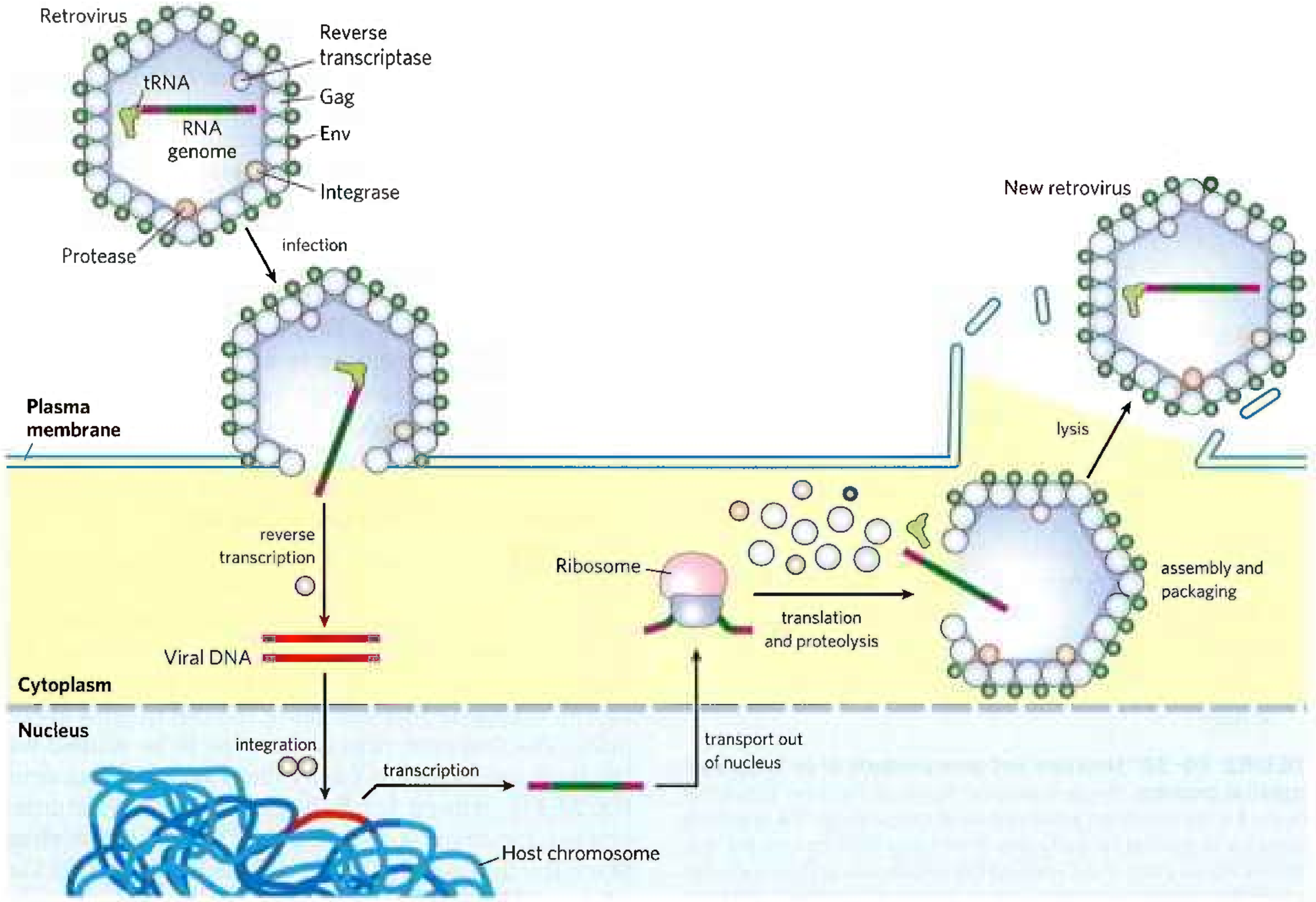
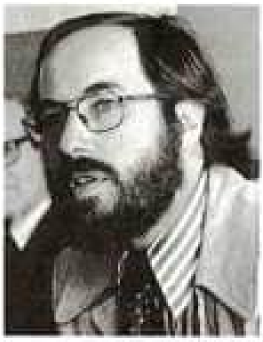

Retrovirus
Reverse transcriptase
tRNA
Gag
RNA genome
Env
Integrase
Protease
infection
New retrovirus
Plasma membrane
lysis
reverse transcription
Ribosome
assembly and packaging
translation and proteolysis
Viral DNA
Cytoplasm
Nucleus
integration
transcription
transport out of nucleus
Host chromosome

FIGURE 26-29 Retroviral infection of a mammalian cell and integration of the retrovirus into the host chromosome. Viral particles entering the host cell carry viral reverse transcriptase and a cellular tRNA (picked up from a former host cell) already base-paired to the viral RNA. The purple segments represent the long terminal repeats on the viral RNA. The tRNA facilitates immediate conversion of viral RNA to double-stranded DNA by the action of reverse transcriptase. The double-stranded DNA enters the nucleus and is integrated into the host genome. The integration is catalyzed by a virally encoded integrase. Integration of viral DNA into host DNA is mechanistically similar to the insertion of transposons in bacterial chromosomes (see Fig. 25-41). For example, a few base pairs of host DNA become duplicated at the site of integration, forming short repeats of 4 to 6 bp at each end of the inserted retroviral DNA (not shown). On transcription and translation of the integrated viral DNA, new viruses are formed and released by cell lysis (right). In the viruses, the viral RNA is enclosed by capsid proteins called Gag and outer envelope proteins called Env. Additional viral proteins (reverse transcriptase, integrase, and a viral protease needed for posttranslational processing of viral proteins) are packaged within the virus particle with the RNA.

Howard Temin, 1934–1994
[Bettmann/Getty Images]


David Baltimore [AP Images]

Retroviruses typically have three genes: *gag* (a name derived from the historical designation *g*roup *a*ssociated anti*g*en), *pol*, and *env* (**Fig. 26-30**). The transcript that contains *gag* and *pol* is translated into a long "polyprotein," a single large polypeptide that is cleaved into six proteins with distinct functions. The proteins derived from the *gag* gene make up the interior core of the viral particle. The *pol* gene encodes the protease that cleaves the long polypeptide, an integrase that inserts the viral DNA into the host chromosomes, and reverse transcriptase. Many reverse transcriptases have two subunits, α and β. The *pol* gene specifies the β subunit (M_r 90,000), and the α subunit (M_r 65,000) is simply a proteolytic fragment of the β subunit. The *env* gene encodes the proteins of the viral envelope. At each end of the linear RNA genome are long terminal repeat (LTR) sequences of a few hundred nucleotides. Transcribed into the duplex DNA, these sequences facilitate integration of the viral chromosome into the host DNA and contain promoters for viral gene expression.

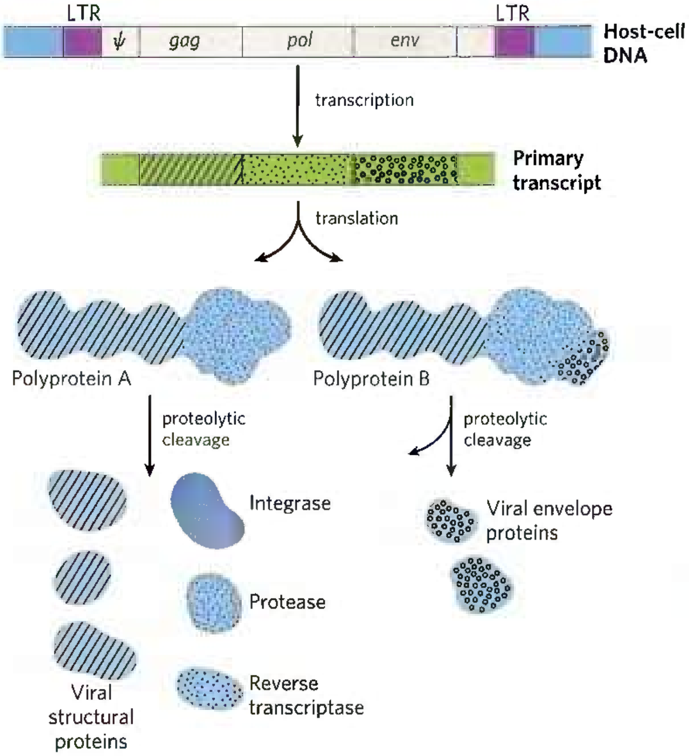


FIGURE 26-30 Structure and gene products of an integrated retroviral genome. The long terminal repeats (LTRs) have sequences needed for the regulation and initiation of transcription. The sequence denoted ψ is required for packaging of retroviral RNAs into mature viral particles. Transcription of the retroviral DNA produces a primary transcript encompassing the *gag*, *pol*, and *env* genes. Translation (Chapter 27) produces a polyprotein, a single long polypeptide derived from the *gag* and *pol* genes, which is cleaved into six distinct proteins. Splicing of the primary transcript yields an mRNA derived largely from the *env* gene, which is also translated into a polyprotein, then cleaved to generate viral envelope proteins.

Reverse transcriptases catalyze three different reactions: (1) RNA-dependent DNA synthesis, (2) RNA degradation, and (3) DNA-dependent DNA synthesis. Each transcriptase is most active with the RNA of its own virus, but each can be used experimentally to make DNA complementary to a variety of RNAs. The DNA and RNA synthesis and RNA degradation activities use separate active sites on the protein. For DNA synthesis to begin, the reverse transcriptase requires a primer, a cellular tRNA obtained during an earlier infection and carried in the viral particle. This tRNA is base-paired at its 3′ end with a complementary sequence in the viral RNA. The new DNA strand is synthesized in the 5′→3′ direction, as in all RNA and DNA polymerase reactions. Reverse transcriptases, like RNA polymerases, do not have 3′→5′ proofreading exonucleases. They generally have error rates of about 1 per 20,000 nucleotides added. An error rate this high is extremely unusual in DNA replication and seems to be a characteristic of most enzymes that replicate the genomes of RNA viruses. A consequence is a higher mutation rate and a faster rate of viral evolution, which is a factor in the frequent appearance of new strains of disease-causing retroviruses.

Reverse transcriptases have become important reagents in the study of DNA-RNA relationships and in DNA cloning techniques. They make possible the synthesis of DNA complementary to an mRNA template, and synthetic DNA prepared in this manner, called **complementary DNA (cDNA)**, can be used to clone cellular genes (see Fig. 9-13).

Some Retroviruses Cause Cancer and AIDS

P3 Retroviruses have featured prominently in the molecular understanding of cancer. Most retroviruses do not kill their host cells but remain integrated in the cellular DNA, replicating when the cell divides. Some retroviruses, classified as RNA tumor viruses, contain an oncogene that can cause the cell to grow abnormally. The first retrovirus of this type to be studied was the Rous sarcoma virus (also called avian sarcoma virus; **Fig. 26-31**), named for F. Peyton Rous, who studied chicken tumors now known to be caused by this virus. Since the initial discovery of oncogenes by Harold Varmus and Michael Bishop, many dozens of such genes have been found in retroviruses.

P3 The human immunodeficiency virus (HIV), which causes acquired immune deficiency syndrome (AIDS), is a retrovirus. Identified in 1983, HIV has an RNA genome with standard retroviral genes along with several other unusual genes (**Fig. 26-32**). Unlike many other retroviruses, HIV kills many of the cells it infects (principally T lymphocytes) rather than causing tumor formation. This gradually leads to suppression of the immune system in the host organism. The reverse transcriptase of HIV is even more error-prone than other known reverse transcriptases — 10 times more so — resulting in high mutation rates in this virus. One or more errors are generally made every time the viral genome is replicated, so any two viral RNA molecules are likely to differ.

Many modern vaccines for viral infections consist of one or more coat proteins of the virus, produced by methods described in Chapter 9. These proteins are not infectious on their own but stimulate the immune

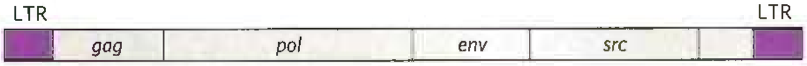


FIGURE 26-31 Rous sarcoma virus genome. The *src* gene encodes a tyrosine kinase, one of a class of enzymes that function in systems affecting cell division, cell-cell interactions, and intercellular communication (see Section 12.4). The same gene is found in chicken DNA (the usual host for this virus) and in the genomes of many other eukaryotes, including humans. When associated with the Rous sarcoma virus, this oncogene is often expressed at abnormally high levels, contributing to unregulated cell division and cancer.

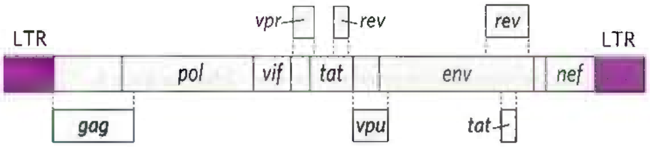


FIGURE 26-32 The genome of HIV, the virus that causes AIDS. In addition to the typical retroviral genes, HIV contains several small genes with a variety of functions (not identified here and not all known). Some of these genes overlap. Alternative splicing mechanisms produce many different proteins from this small (9.7×10^3 nucleotides) genome.

system to recognize and resist subsequent viral invasions (Chapter 5). Because of the high error rate of the HIV reverse transcriptase, the *env* gene in this virus (along with the rest of the genome) undergoes very rapid mutation, complicating the development of an effective vaccine. However, repeated cycles of cell invasion and replication are needed to propagate an HIV infection, so inhibition of viral enzymes offers the most effective therapy currently available. The HIV protease is targeted by a class of drugs called protease inhibitors (see Fig. 6-29). Reverse transcriptase is the target of some additional drugs widely used to treat HIV-infected individuals (**Box 26-3**). ■

Many Transposons, Retroviruses, and Introns May Have a Common Evolutionary Origin

Some well-characterized eukaryotic DNA transposons from sources as diverse as yeast and fruit flies have a structure very similar to that of retroviruses; these are sometimes called retrotransposons (**Fig. 26-33**). Retrotransposons

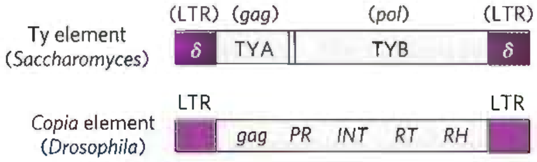


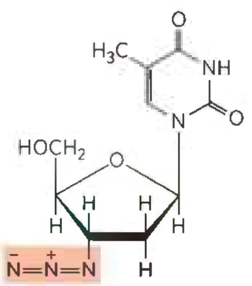
FIGURE 26-33 Eukaryotic transposons. The Ty element of the yeast *Saccharomyces* and the *copia* element of the fruit fly *Drosophila* are examples of eukaryotic retrotransposons, which often have a structure similar to retroviruses but lack the *env* gene. The δ sequences of the Ty element are functionally equivalent to retroviral LTRs. In the *copia* element, *INT* and *RT* are homologous to the integrase and reverse transcriptase segments, respectively, of the *pol* gene.

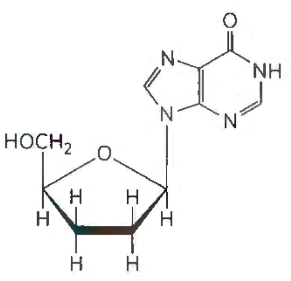
BOX 26-3 MEDICINE

Fighting AIDS with Inhibitors of HIV Reverse Transcriptase

Research into the chemistry of template-dependent nucleic acid biosynthesis, combined with modern techniques of molecular biology, has elucidated the life cycle and structure of the human immunodeficiency virus, the retrovirus that causes AIDS. A few years after the isolation of HIV, this research resulted in the development of drugs capable of prolonging the lives of people infected by HIV.

The first drug to be approved for clinical use was azidothymidine (AZT), a structural analog of deoxythymidine. AZT was first synthesized in 1964 by Jerome P. Horwitz. It failed as an anticancer drug (the purpose for which it was made), but in 1985 it was found to be a useful treatment for AIDS. AZT is taken up by T lymphocytes, immune system cells that are particularly vulnerable to HIV infection, and it is converted to AZT triphosphate. (AZT triphosphate taken directly would be ineffective because it cannot cross the plasma membrane.) HIV's reverse transcriptase has a higher affinity for AZT triphosphate than for deoxythymidine triphosphate (dTTP), and binding of AZT triphosphate to this enzyme competitively inhibits dTTP binding. When AZT is added to the 3′ end of the growing DNA strand, lack of a 3′ hydroxyl means that the DNA strand is terminated prematurely and viral DNA synthesis grinds to a halt.


3′-Azido-2′,3′-dideoxythymidine (AZT)


2′,3′-Dideoxyinosine (DDI)

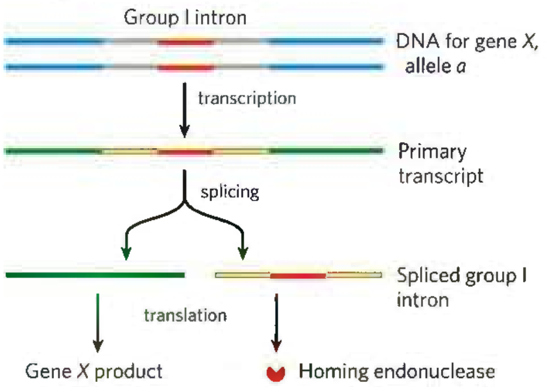
AZT triphosphate is not as toxic to the T lymphocytes themselves because *cellular* DNA polymerases have a lower affinity for this compound than for dTTP. At concentrations of 1 to 5 μM, AZT affects HIV reverse transcription but not most cellular DNA replication. Unfortunately, AZT seems to be toxic to the bone marrow cells that are the progenitors of erythrocytes, and many individuals taking AZT develop anemia. AZT can increase the survival time of people with advanced AIDS by about a year, and it delays the onset of AIDS in those who are still in the early stages of HIV infection. Some other AIDS drugs, such as dideoxyinosine (DDI), have a similar mechanism of action. Newer drugs target and inactivate the HIV protease. Because of the high error rate of HIV reverse transcriptase and the resulting rapid evolution of HIV, the most effective treatments of HIV infection use a combination of drugs directed at both the protease and the reverse transcriptase.

encode an enzyme homologous to the retroviral reverse transcriptase, and their coding regions are flanked by LTR sequences. They transpose from one position to another in the cellular genome by means of an RNA intermediate, using reverse transcriptase to make a DNA copy of the RNA, followed by integration of the DNA at a new site. Most transposons in eukaryotes use this mechanism for transposition, distinguishing them from bacterial transposons, which move as DNA directly from one chromosomal location to another (see Fig. 25-41).

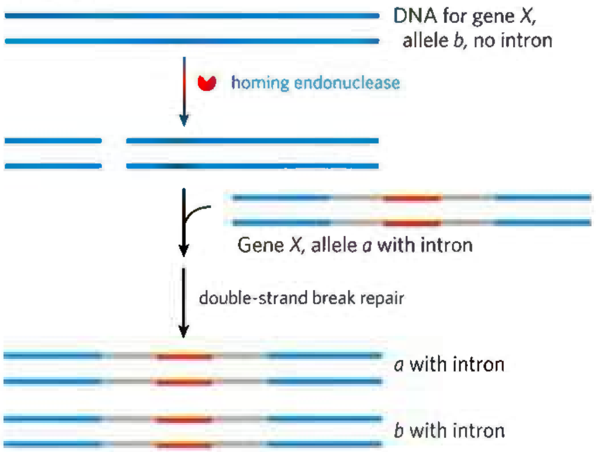
Retrotransposons lack an *env* gene and so cannot form viral particles. They can be thought of as defective viruses, trapped in cells. Comparisons between retroviruses and eukaryotic transposons suggest that reverse transcriptase is an ancient enzyme that predates the evolution of multicellular organisms.

Many group I and group II introns are also mobile genetic elements. In addition to their self-splicing activities, they encode DNA endonucleases that promote their movement. During genetic exchanges between cells of the same species, or when DNA is introduced into a cell by parasites or by other means, these endonucleases promote insertion of the intron into an identical site in another DNA copy of a homologous gene that does not contain the intron, in a process termed **homing** (**Fig. 26-34**). Whereas group I intron homing is DNA-based, group II intron homing occurs through an RNA intermediate. The endonucleases of the group II introns have associated reverse transcriptase activity. The proteins can form complexes with the intron RNAs themselves, after the introns are spliced from the primary transcripts. Because the homing process involves insertion of the RNA intron into DNA and reverse transcription of the intron, the movement of these introns has been called retrohoming. Over time, every copy of a particular gene in a population may acquire the intron.

(a) Production of homing endonuclease


Group I intron
DNA for gene *X*, allele *a*
transcription
Primary transcript
splicing
Spliced group I intron
translation
Gene *X* product
Homing endonuclease

(b) Homing


DNA for gene *X*, allele *b*, no intron
homing endonuclease
Gene *X*, allele *a* with intron
double-strand break repair
a with intron
b with intron

(c) Retrohoming

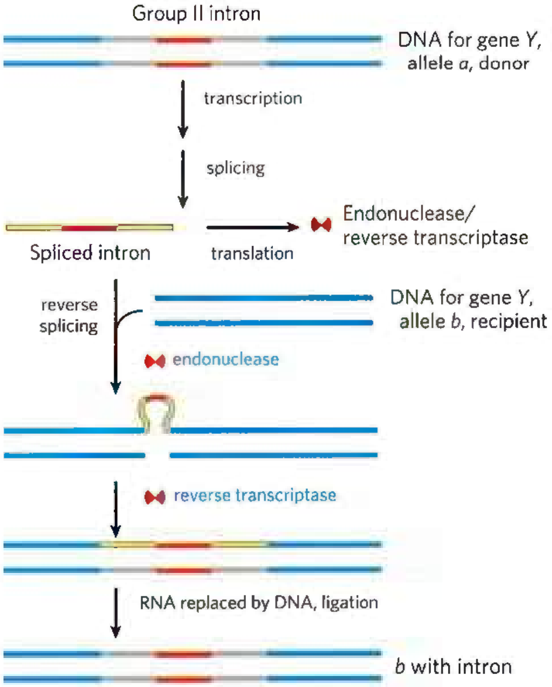

Group II intron
DNA for gene *Y*, allele *a*, donor
transcription
splicing
Spliced intron
translation
Endonuclease/ reverse transcriptase
reverse splicing
DNA for gene *Y*, allele *b*, recipient
endonuclease
reverse transcriptase
RNA replaced by DNA, ligation
b with intron

FIGURE 26-34 Introns that move: homing and retrohoming. Certain introns include a gene (shown in red) for enzymes that promote homing (some group I introns) or retrohoming (some group II introns). (a) The gene in the spliced intron is bound by a ribosome and translated. Group I homing introns specify a site-specific endonuclease, called a homing endonuclease. Group II retrohoming introns specify a protein with both endonuclease and reverse transcriptase activities (not shown here). (b) Homing. Allele *a* of a gene *X* containing a group I homing intron is present in a cell containing allele *b* of the same gene, which lacks the intron. The homing endonuclease produced by *a* cleaves *b* at the position corresponding to the intron in *a*, and double-strand break repair (recombination with allele *a*; see Fig. 25-34) then creates a new copy of the intron in *b*. (c) Retrohoming. Allele *a* of gene *Y* contains a retrohoming group II intron; allele *b* lacks the intron. The spliced intron inserts itself into the coding strand of *b* in a reaction that is the reverse of the splicing that excised the intron from the primary transcript (see Fig. 26-14), except that here the insertion is into DNA rather than RNA. The noncoding DNA strand of *b* is then cleaved by the intron-encoded endonuclease/reverse transcriptase. This same enzyme uses the inserted RNA as a template to synthesize a complementary DNA strand. The RNA is then degraded by cellular ribonucleases and replaced with DNA.

Much more rarely, the intron may insert itself into a new location in an unrelated gene. If this event does not kill the host cell, it can lead to the evolution and distribution of an intron in a new location. The structures and mechanisms used by mobile introns support the idea that at least some introns originated as molecular parasites whose evolutionary past can be traced to retroviruses and transposons.

Telomerase Is a Specialized Reverse Transcriptase

Telomeres, the structures at the ends of linear eukaryotic chromosomes (see Fig. 24-7), generally consist of many tandem copies of a short oligonucleotide sequence. This sequence usually has the form T_xG_y in one strand and C_yA_x in the complementary strand, where x and y are typically in the range of 1 to 4 (p. 890). Telomeres vary in length from a few dozen base pairs in some ciliated protozoans to tens of thousands of base pairs in mammals. The TG strand is longer than its complement, leaving a region of single-stranded DNA of up to a few hundred nucleotides at the 3′ end.

The ends of a linear chromosome are not readily replicated by cellular DNA polymerases. DNA replication requires a template and primer, and beyond the end of a linear DNA molecule no template is available for the pairing of an RNA primer. Without a special mechanism for replicating the ends, chromosomes would be shortened somewhat in each cell generation. The enzyme **telomerase**, discovered by Carol Greider and Elizabeth Blackburn, solves this problem by adding telomeres to chromosome ends.

Carol Greider [Courtesy Carol Greider.]

Elizabeth Blackburn [Micheline Pelletier/Getty Images.]

The discovery and purification of this enzyme provided insight into a reaction mechanism that is remarkable and unprecedented. Telomerase, like some other enzymes described in this chapter, is an RNP that contains both RNA and protein components. The RNA component in humans is about 150 nucleotides long and contains about 1.5 copies of the appropriate C_yA_x telomere repeat. This region of the RNA acts as a template for synthesis of the T_xG_y strand of the telomere. P3 Telomerase thereby acts as a cellular reverse transcriptase that provides the active site for RNA-dependent DNA synthesis. Unlike retroviral reverse transcriptases, telomerase copies only a small segment of RNA that it carries within itself. Telomere synthesis requires the 3′ end of a chromosome as primer and proceeds in the usual 5′→3′ direction. Having synthesized one copy of the repeat, the enzyme repositions to resume extension of the telomere (**Fig. 26-35a**).

After extension of the T_xG_y strand by telomerase, the complementary C_yA_x strand is synthesized by cellular DNA polymerases, starting with an RNA primer (see Fig. 25-11). The single-stranded region is protected by specific binding proteins in many lower eukaryotes, especially those species with telomeres of less than a few hundred base pairs. In higher eukaryotes (including mammals) with telomeres many thousands of base pairs long, the single-stranded end is sequestered in a specialized structure called a **T loop** (Fig. 26-35b). The single-stranded end is folded back and paired with its complement in the double-stranded portion of the telomere. The formation of a T loop involves invasion of the 3′ end of the telomere's single strand into the duplex DNA, perhaps by a mechanism similar to the initiation of homologous genetic recombination (see Fig. 25-34). In mammals, the looped DNA is bound by two proteins, TRF1 and TRF2, with the latter protein involved in formation of the T loop. T loops protect the 3′ ends of chromosomes, making them inaccessible to nucleases and the enzymes that repair double-strand breaks.

In protozoans (such as *Tetrahymena*), loss of telomerase activity results in a gradual shortening of telomeres with each cell division, ultimately leading to the death of the cell line. A similar link between telomere length and cell senescence (cessation of cell division) has been observed in humans. In germ-line cells, which contain telomerase activity, telomere lengths are maintained; in somatic cells, which lack telomerase, they are not. There is a linear, inverse relationship between the length of telomeres in cultured fibroblasts and the age of the individual from whom the fibroblasts were taken: telomeres in human somatic cells gradually shorten as an individual ages. If the telomerase reverse transcriptase is introduced into human somatic cells in vitro, telomerase activity is restored and the cellular life span increases markedly.

Is the gradual shortening of telomeres a key to the aging process? Is our natural life span determined by the length of the telomeres we are born with? Further research in this area should yield some fascinating insights.

Some RNAs Are Replicated by RNA-Dependent RNA Polymerase

Apart from the retroviruses, the RNA viruses include some *E. coli* bacteriophages as well as eukaryotic viruses such as the influenza virus and coronaviruses that cause SARS or COVID-19. The single-stranded RNA

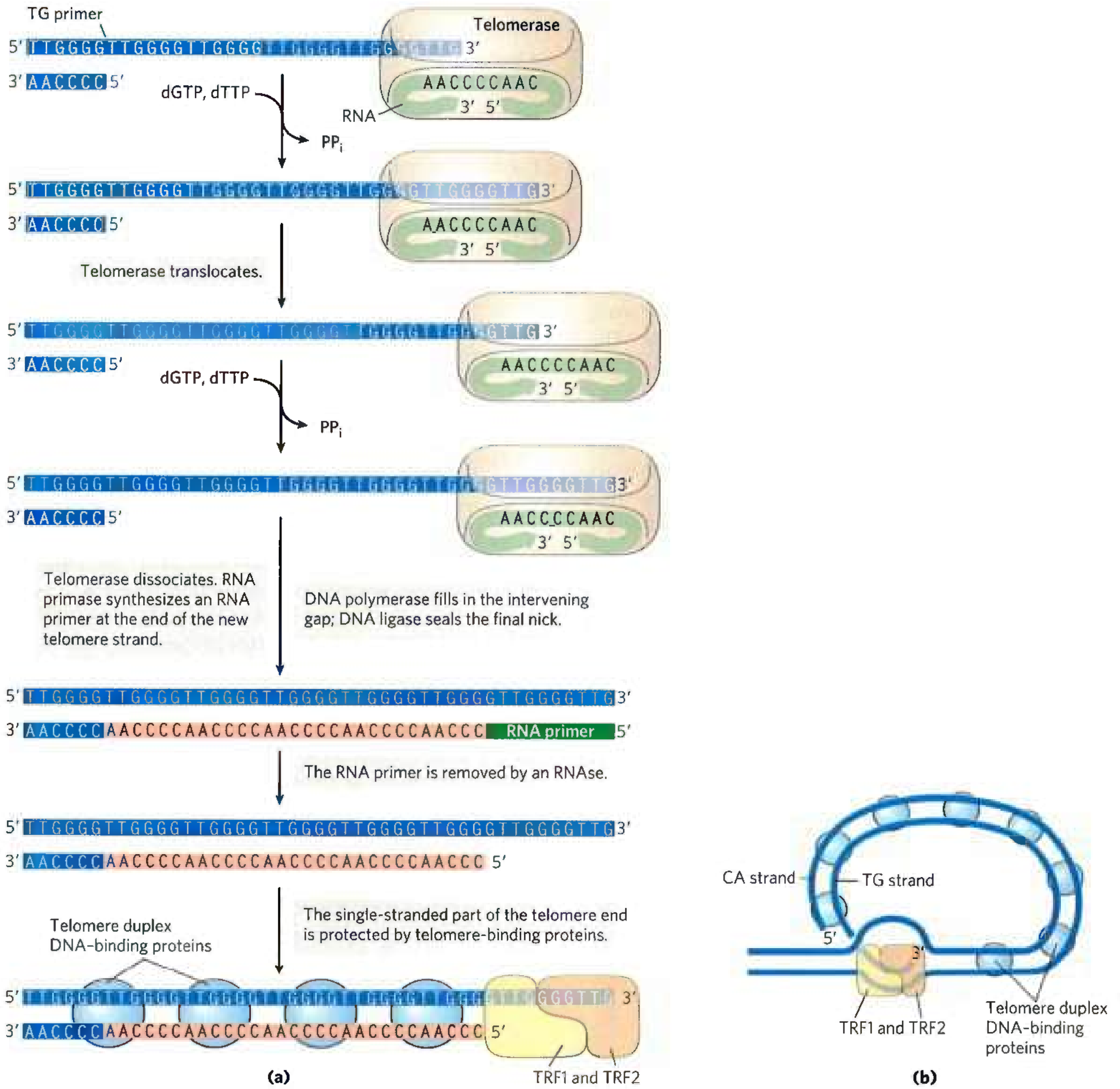
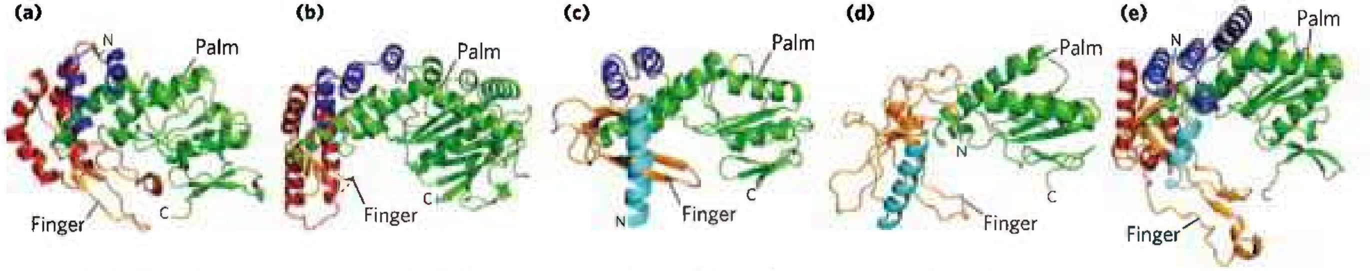

TG primer
Telomerase
5′ TTGGGGTTGGGGTTGGGG 3′
3′ AACCCC 5′
AACCCCAAC
3′ 5′
RNA
dGTP, dTTP
PPᵢ
Telomerase translocates.
Telomerase dissociates. RNA primase synthesizes an RNA primer at the end of the new telomere strand.
DNA polymerase fills in the intervening gap; DNA ligase seals the final nick.
5′ TTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTGGGGTTG 3′
3′ AACCCCAACCCCAACCCCAACCCCAACCCCAACCC RNA primer 5′
The RNA primer is removed by an RNAse.
3′ AACCCCAACCCCAACCCCAACCCCAACCCCAACCC 5′
Telomere duplex DNA-binding proteins
The single-stranded part of the telomere end is protected by telomere-binding proteins.
TRF1 and TRF2
(a)
CA strand
TG strand
5′
3′
TRF1 and TRF2
Telomere duplex DNA-binding proteins
(b)

FIGURE 26-35 **Telomere synthesis and structure.** (a) The internal template RNA of telomerase binds to and base-pairs with the TG primer (T_xG_y) of DNA. Telomerase adds more T and G residues to the TG primer, then repositions the internal template RNA to allow the addition of more T and G residues to generate the TG strand of the telomere. The complementary strand is synthesized by cellular DNA polymerases, after priming by an RNA primase. (b) Proposed structure of T loops in telomeres. The single-stranded tail synthesized by telomerase is folded back and paired with its complement in the duplex portion of the telomere. The telomere is bound by several telomere-binding proteins, including TRF1 and TRF2 (*t*elomere *r*epeat binding *f*actors).

chromosomes of these viruses also function as mRNAs for the synthesis of viral proteins. They are replicated in the host cell by an **RNA-dependent RNA polymerase (RNA replicase)**. All RNA viruses—with the exception of retroviruses—must encode a protein with RNA-dependent RNA polymerase activity, either because the host cells lack such an enzyme or because the RNA genome structure of a virus imposes specialized enzymatic requirements.

The RNA replicase isolated from *E. coli* cells infected with the bacteriophage Qβ catalyzes the formation of an RNA complementary to the viral RNA, in a reaction equivalent to that catalyzed by DNA-dependent RNA polymerases. New RNA strand synthesis proceeds in the 5′→3′ direction by a chemical mechanism identical to that used in all other nucleic acid synthetic reactions that require a template. RNA replicase requires RNA as its template and will not function with DNA. It lacks



Group II intron retrohoming **Nuclear pre-mRNA splicing** **Telomere extension** **Viral reverse transcription** **Viral RNA replication**

FIGURE 26-36 Structural similarities between RNA-dependent polymerases. RNA-dependent polymerases use a common active site architecture in which the duplexed substrate sits in a hand-shaped protein fold with palm and finger domains. This protein fold can be found in protein factors involved in (a) group II intron retrohoming, (b) spliceosome-catalyzed pre-mRNA splicing, (c) telomere synthesis, (d) HIV reverse transcription, and (e) hepatitis C viral genome replication. The retrohoming reverse transcriptase of group II introns is most structurally related to the reverse transcriptase structure present in the spliceosome, supporting their close evolutionary relationship. [Information from C. Zhao and A. M. Pyle, *Nat. Struct. Mol. Biol.* 23:558, 2016, Fig. 2. Data from (a) PDB ID 5HHJ, C. Zhao and A. M. Pyle, *Nat. Struct. Mol. Biol.* 23:558, 2016; (b) PDB ID 4I43, W. P. Galej et al., *Nature* 493:638, 2013; (c) PDB ID 3DU6, A. J. Gillis et al., *Nature* 455:633, 2008; (d) PDB ID 2HMI, J. Ding et al., *J. Mol. Biol.* 284:1095, 1998; (e) PDB ID 1C2P, C. A. Lesburg et al., *Nat. Struct. Biol.* 6:937, 1999.]

a separate proofreading endonuclease activity and has an error rate similar to that of RNA polymerase. Unlike the DNA and RNA polymerases, RNA replicases are specific for the RNA of their own virus; the RNAs of the host cell are generally not replicated. This explains how RNA viruses are preferentially replicated in the host cell, which contains many other types of RNA.

RNA-dependent RNA polymerases are not limited to viruses. Enzymes of this type are found in plants, protists, fungi, and some simpler animals, but not in insects or mammals. Those found in the genomes of eukaryotes generally play a role in the metabolism of another class of small RNAs, called small interfering RNAs (siRNAs), which participate in gene regulation (Chapter 28).

RNA-Dependent RNA Polymerases Share a Common Structural Fold

Even though viral RNA replication and reverse transcription, retrohoming, and telomere synthesis represent a diverse array of biological processes, the polymerases involved in each pathway bear remarkable similarities in structure (**Fig. 26-36**). In all cases, palm and finger domains are used to grip the duplexed template and primer nucleic acids within the active site. Amazingly, the group II intron retrohoming reverse transcriptase is structurally most closely related to a protein component of the spliceosome that helps scaffold its RNA active site. In addition to identical splicing chemistry and active site features (see Fig. 26-17), this provides further evidence that the spliceosome evolved from a group II intron–like ancestor.

SUMMARY 26.3 *RNA-Dependent Synthesis of RNA and DNA*

- RNA-dependent DNA polymerases, also called reverse transcriptases, were first discovered in retroviruses, which must convert their RNA genomes into double-stranded DNA as part of their life cycle. These enzymes transcribe the viral RNA into DNA, a process that can be used experimentally to form cDNA.
- Retroviruses can cause human diseases, including AIDS and cancers. Some of these viruses contain oncogenes, which cause infected cells to grow abnormally.
- Many eukaryotic transposons are related to retroviruses, and their mechanism of transposition includes an RNA intermediate. An RNA intermediate is also present in group II intron retrohoming, in which the RNA intron is inserted into a DNA gene followed by production of a DNA copy by a reverse transcriptase.
- Telomerase, the enzyme that synthesizes the telomere ends of linear chromosomes, is a specialized reverse transcriptase that contains an internal RNA template.
- RNA-dependent RNA polymerases, such as the replicases of RNA bacteriophages, are template-specific for the viral RNA. These enzymes share structural homology with reverse transcriptases involved in retroviral replication, telomere production, and retrohoming.

26.4 Catalytic RNAs and the RNA World Hypothesis

P4 The study of posttranscriptional processing of RNA molecules led to one of the most exciting discoveries in biochemistry—the existence of RNA enzymes or ribozymes. The best-characterized ribozymes are the self-splicing group I introns, RNase P, and the hammerhead ribozyme (discussed below). Most of the activities of these ribozymes are based on two fundamental reactions: transesterification (Fig. 26-14) and phosphodiester bond hydrolysis (cleavage). The substrate for a ribozyme is often an RNA molecule, and it may even be part of the ribozyme itself. When its substrate is RNA, the RNA catalyst can make use of base-pairing interactions to align the substrate for the reaction.

Ribozymes Share Features with Protein Enzymes

Like protein enzymes, ribozymes vary greatly in size. A self-splicing group I intron may have more than 400 nucleotides. In comparison, the hammerhead ribozyme consists of two RNA strands with a total of just 41 nucleotides. **P4** Also as with protein enzymes, the three-dimensional structure of ribozymes is important for function. Ribozymes, like protein enzymes, are inactivated by heating above their melting temperature or by the addition of denaturing agents or complementary oligonucleotides, which disrupt normal base-pairing patterns. Ribozymes can also be inactivated if essential nucleotides are changed. The secondary structure of a self-splicing group I intron from the 26S rRNA precursor of *Tetrahymena* is shown in detail in **Figure 26-37**. This secondary structure highlights the large number of base-pairing and other noncovalent interactions that must occur for the ribozyme to adopt a catalytic structure. Just as amino acid mutations can change activities of protein enzymes, nucleotide mutations can alter the noncovalent interactions required for ribozyme folding and catalysis.

P4 Ribozymes share several properties with enzymes besides accelerating the reaction rate, including kinetic behavior and specificity. Binding of the guanosine cofactor to the *Tetrahymena* group I rRNA intron is saturable ($K_m < 30$ μM) and can be competitively inhibited by 3′-deoxyguanosine. The intron is very precise in its excision reaction, largely due to a segment called the **internal guide sequence** that can base-pair with exon sequences near the 5′ splice site (Fig. 26-37). This pairing promotes the alignment of specific bonds to be cleaved and rejoined.

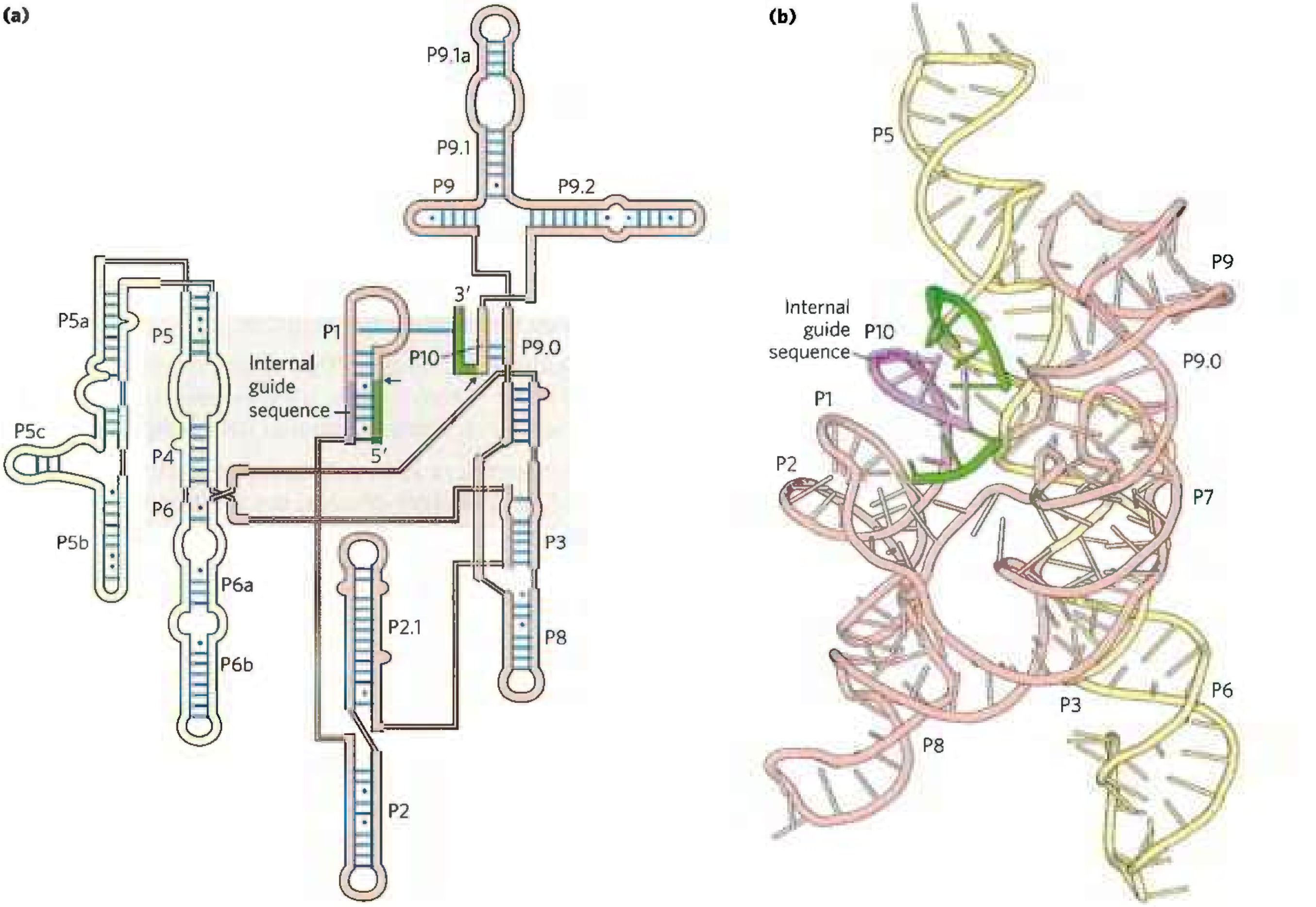


FIGURE 26-37 Secondary structure of the self-splicing rRNA intron of *Tetrahymena*. (a) A two-dimensional representation of secondary structure immediately prior to the initiation of the reaction. Intron sequences are shaded yellow and light red; flanking exon sequences are green; the internal guide sequences that help to align reacting segments at the active site are purple. Each thin, light-red line represents a bond between adjacent nucleotides in a continuous sequence (a device necessitated by showing this complex molecule in two dimensions). Short blue lines represent normal base pairing; blue dots indicate G–U base pairs. All nucleotides are shown. The catalytic core of the self-splicing activity is shaded in gray. Some base-paired regions are labeled (P1, P3, P2.1, P5a, and so forth) according to an established convention for this RNA molecule. The P1 region, which contains the internal guide sequence (purple), is the location of the 5′ splice site (black arrow). Part of the internal guide sequence pairs with the end of the 3′ exon, bringing the 5′ and 3′ splice sites (black arrows) into close proximity. (b) Three-dimensional structure of a reaction intermediate of the same intron, after guanosine-mediated cleavage (Fig. 26-14) and prior to exon ligation. Segments are colored as in (a). [Data from (a) PDB ID 1GID, J. H. Cate et al., *Science* 273:1678, 1996. (b) PDB ID 1U6B, P. L. Adams et al., *Nature* 430:45, 2004.]

Because the intron itself is chemically altered during the splicing reaction—its ends are cleaved—it may seem to lack one key enzymatic property: the ability to catalyze multiple reactions. Closer inspection has shown that after excision, the 414 nucleotide intron from *Tetrahymena* rRNA can, in vitro, act as a true enzyme (but in vivo it is quickly degraded). A series of intramolecular cyclization and cleavage reactions in the excised intron leads to the loss of 19 nucleotides from its 5′ end. The remaining 395 nucleotide, linear RNA—referred to as L-19 IVS (*int*ervening *s*equence)—promotes nucleotidyl transfer reactions in which some oligonucleotides are lengthened at the expense of others (**Fig. 26-38**). The best substrates are oligonucleotides, such as a synthetic $(C)_5$ oligomer, that can base-pair with the same guanylate-rich internal guide sequence that held the 5′ exon in place for self-splicing.

The enzymatic activity of the L-19 IVS ribozyme results from a cycle of transesterification reactions mechanistically similar to self-splicing. Each ribozyme molecule can process about 100 substrate molecules per hour and is not altered in the reaction—that is, the intron acts as a catalyst. It follows Michaelis-Menten kinetics, is specific for RNA oligonucleotide substrates, and can be competitively inhibited. The k_{cat}/K_m (specificity constant) is 10^3 $M^{-1}s^{-1}$, lower than that of many protein enzymes, but the ribozyme accelerates hydrolysis by a factor of 10^{10} relative to the uncatalyzed reaction. **P4** It makes use of substrate orientation, covalent catalysis, and metal-ion catalysis—all strategies shared with protein enzymes.

Ribozymes Participate in a Variety of Biological Processes

E. coli RNase P has both an RNA component (the M1 RNA, with 377 nucleotides) and a protein component (M_r 17,500). In 1983, Sidney Altman and Norman Pace and their coworkers discovered that under some conditions, the M1 RNA alone is capable of catalysis, cleaving tRNA precursors at the correct position. The protein component apparently serves to stabilize the RNA or facilitate its function in vivo. The RNase P ribozyme recognizes the three-dimensional shape of its pre-tRNA substrate, along with the CCA sequence, and thus can cleave the 5′ leaders from diverse tRNAs (Fig. 26-25).

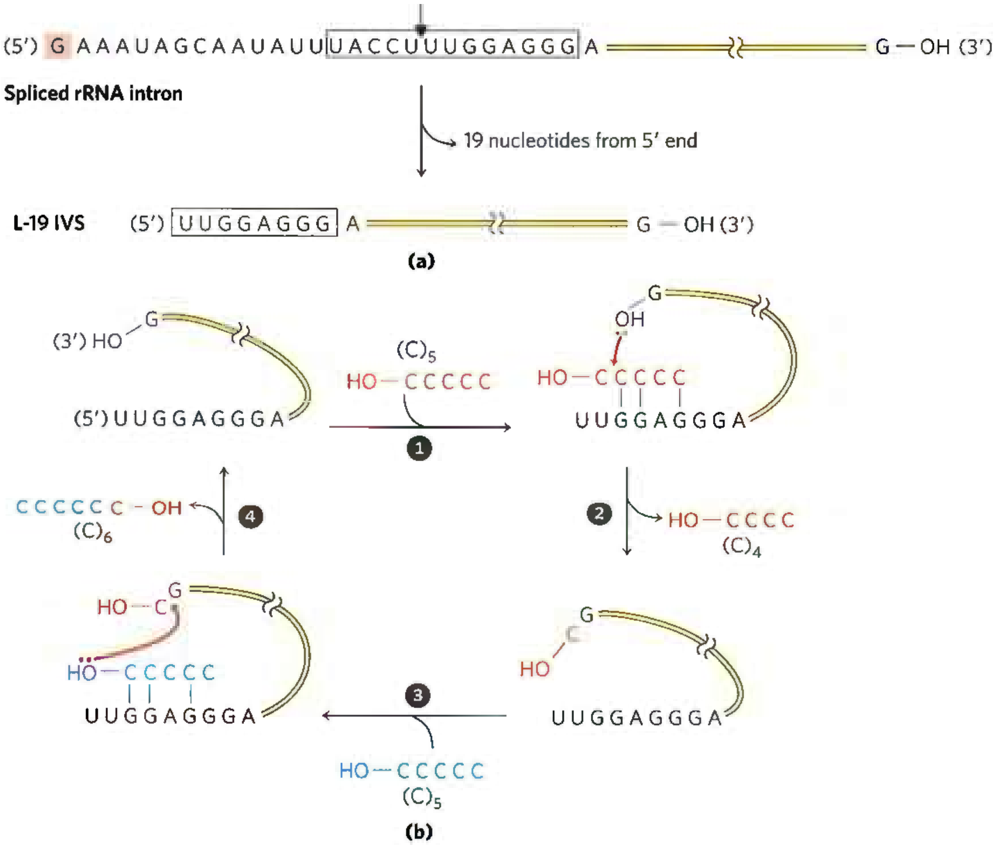


FIGURE 26-38 In vitro catalytic activity of L-19 IVS. (a) L-19 IVS (intervening sequence, the term once used for intron) is generated by the autocatalytic removal of 19 nucleotides from the 5′ end of the spliced *Tetrahymena* intron. The cleavage site is indicated by the arrow in the internal guide sequence (boxed). The G residue (shaded) added in the first step of the splicing reaction is part of the removed sequence. A portion of the internal guide sequence remains at the 5′ end of L-19 IVS. (b) L-19 IVS lengthens some RNA oligonucleotides at the expense of others in a cycle of transesterification reactions (steps ❶ through ❹). The 3′ OH of the G residue at the 3′ end of L-19 IVS plays a key role in this cycle (note that this is *not* the G residue added in the splicing reaction). $(C)_5$ is one of the ribozyme's better substrates because it can base-pair with the guide sequence remaining in the intron.

The known catalytic repertoire of ribozymes continues to expand. Some virusoids, small RNAs associated with plant RNA viruses, include a structure that promotes a self-cleavage reaction; the small hammerhead ribozyme illustrated in **Figure 26-39** is in this class, catalyzing the hydrolysis of an internal phosphodiester bond. There are at least nine structural classes of ribozymes that engage in self-cleavage; all use general acid and base catalysis (Fig. 6-8) to promote the attack of a 2′-hydroxyl group on an adjacent phosphodiester bond. Despite being surrounded by proteins, the splicing reaction that occurs in a spliceosome relies on a catalytic center formed by the U2, U5, and U6 snRNAs and intron (see Figs 26-16 and 26-17). And, as we shall see in Chapter 27, an RNA component of ribosomes catalyzes the synthesis of proteins. Exploring catalytic RNAs has provided new insights into catalytic function in general and has important implications for our understanding of the origin and evolution of life on this planet.

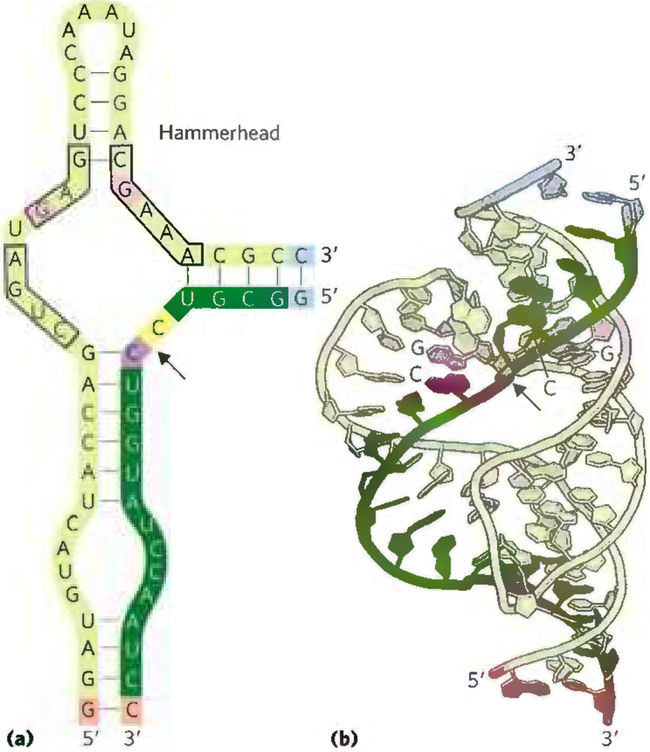
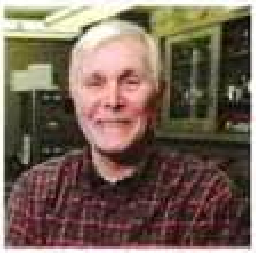


FIGURE 26-39 Hammerhead ribozyme. Some virusoid RNAs include small segments that promote site-specific RNA cleavage reactions associated with replication. These segments are called hammerhead ribozymes, because their secondary structures are shaped like the head of a hammer. (a) The minimal sequences required for catalysis by the ribozyme. The boxed nucleotides are highly conserved and are required for catalytic function. Guanine nucleotides shaded pink form part of the active site. The arrow indicates the site of self-cleavage. (b) Three-dimensional structure of a hammerhead ribozyme (see Fig. 8-25b for a view of another hammerhead ribozyme). The strands are colored as in (a). The hammerhead ribozyme is a metalloenzyme; Mg^{2+} ions are required for activity in vivo. The phosphodiester bond at the site of self-cleavage is indicated by an arrow. [Data from PDB ID 3ZD5, M. Martick and W. G. Scott, *Cell* 126:309, 2006.]

Ribozymes Provide Clues to the Origin of Life in an RNA World

The extraordinary complexity and order that distinguish living from inanimate systems are key manifestations of fundamental life processes. Maintaining the living state requires that *selected* chemical transformations occur very rapidly — especially those that use environmental energy sources and synthesize elaborate or specialized cellular macromolecules. Life depends on powerful and selective catalysts — enzymes — and on informational systems capable of both securely storing the blueprint for these enzymes and accurately reproducing the blueprint for generation after generation. Chromosomes encode the blueprint not for the cell but for the enzymes that construct and maintain the cell. The parallel demands for information and catalysis present a classic conundrum: what came first, the information needed to specify structure or the enzymes needed to maintain and transmit the information?

How might a self-replicating polymer come to be? How might it maintain itself in an environment where the precursors for polymer synthesis are scarce? How could evolution progress from such a polymer to the modern DNA-protein world? These difficult questions can be addressed by careful experimentation, providing clues about how life on Earth began and evolved.



Carl Woese, 1928–2012 [Steve Kagan.]

P4 The unveiling of the structural and functional complexity of RNA led Carl Woese, Francis Crick, and Leslie Orgel to propose in the 1960s that this macromolecule might serve as both information carrier and catalyst. Since that time, at least six lines of evidence have given increasing substance to their **RNA world hypothesis**.

1. Prebiotic Chemistry Experiments The probable origin of purine and pyrimidine bases is suggested by experiments designed to test hypotheses about prebiotic chemistry (pp. 31–32). Beginning with simple molecules thought to be present in the early atmosphere (CH_4, NH_3, H_2O, H_2), electrical discharges mimicking lightning generate, first, more reactive molecules such as HCN and aldehydes, then an array of amino acids and organic acids (see Fig. 1-33). When molecules such as HCN become abundant, purine and pyrimidine bases are synthesized in detectable amounts. Remarkably, a concentrated solution of ammonium cyanide, refluxed for a few days, generates adenine in yields of up to 0.5% (**Fig. 26-40**). Adenine may well have been the first and most abundant nucleotide constituent to appear on Earth. Intriguingly, most enzyme cofactors contain adenosine as part of their structure, although it plays no direct role in the

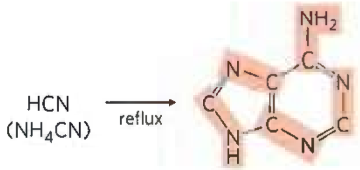


FIGURE 26-40 Experiments supporting prebiotic synthesis of adenine from ammonium cyanide. Adenine is derived from five molecules of cyanide, denoted by shading.

cofactor function (see Fig. 8-41). This may suggest an evolutionary relationship. Based on the simple synthesis of adenine from cyanide, adenine may simply have been abundant and available.

2. The Existence of Catalytic RNAs P4 In an "RNA world," RNAs, not proteins, act as catalysts. Perhaps more than anything else, the discovery of ribozymes gave life to the RNA world hypothesis and led to widespread speculation that an RNA world might have been important in the transition from prebiotic chemistry to life (see Fig. 1-35). The parent of all life on this planet, in the sense that it could reproduce itself across the generations from the origin of life to the present, might have been a self-replicating RNA, or a polymer with equivalent chemical characteristics.

3. The Expanding Catalytic Repertoire of Ribozymes A self-replicating polymer would quickly use up available supplies of precursors provided by the relatively slow processes of prebiotic chemistry. Thus, from an early stage in evolution, metabolic pathways would be required to generate precursors efficiently, with the synthesis of precursors presumably catalyzed by ribozymes. The extant ribozymes found in nature have a limited repertoire of catalytic functions, and of the ribozymes that may once have existed, no trace is left. To explore the RNA world hypothesis more deeply, we need to know whether RNA has the potential to catalyze the many different reactions needed in a primitive system of metabolic pathways.

The search for RNAs with new catalytic functions has been aided by the development of a method that rapidly searches pools of random polymers of RNA and extracts those with particular activities; known as **SELEX**, this is nothing less than accelerated evolution in a test tube (**Box 26-4**). It has been used to generate RNA molecules that bind to amino acids, organic dyes, nucleotides, cyanocobalamin, and other molecules. Researchers have isolated ribozymes that catalyze ester and amide bond formation, S_N2 reactions, metallation of (addition of metal ions to) porphyrins, and carbon–carbon bond formation. The evolution of enzymatic cofactors with nucleotide "handles" that facilitate their binding to ribozymes might have further expanded the repertoire of chemical processes available to primitive metabolic systems.

4. The Structure of the Ribosome As we shall see in Chapter 27, some natural RNA molecules, components of ribosomes, catalyze the formation of peptide bonds, offering a glimpse of how the RNA world might have been transformed by the greater catalytic potential of proteins. The evolution of a capacity to synthesize proteins would have been a major event in the RNA world, allowing the generation of polymers that could greatly stabilize complex RNA structures. However, the onset of peptide synthesis would also have hastened the demise of the RNA world. Proteins simply have more catalytic potential. The information-carrying role of RNA may have passed to DNA because DNA is chemically more stable. RNA replicase and reverse transcriptase may be modern versions of enzymes that once played important roles in making the transition to the modern DNA-based system.

5. Extant Vestiges of an RNA World The known functions of RNA continue to multiply with each decade. Retroviruses, other RNA viruses, and retrotransposons inhabit a semi-independent universe, maintaining a parasitic existence within the biosphere. For evolutionary biologists, these almost-living entities provide a window on key steps in the evolution of life. Transposons may have been an early innovation in an RNA world. With the appearance of the first, inefficient self-replicators, transposition could have been an important alternative to replication as a strategy for successful reproduction and survival. Early parasitic RNAs would simply hop into a self-replicating molecule via catalyzed transesterification, then passively undergo replication. Natural selection would have driven transposition to become site-specific, targeting sequences that did not interfere with the catalytic activities of the host RNA. Replicators and RNA transposons could have existed in a primitive symbiotic relationship, each contributing to the evolution of the other. Modern introns, retroviruses, and transposons may all be vestiges of a "piggyback" strategy pursued by early parasitic RNAs. These elements continue to make major contributions to the evolution of their hosts.

6. Progress in the Search for an RNA Replicator The RNA world hypothesis requires a nucleotide polymer to reproduce itself. Can a ribozyme bring about its own synthesis in a template-directed manner? Researchers are getting closer to finding such a ribozyme or ribozyme system. For example, Gerald Joyce and colleagues, in 2009, reported on the first set of two ribozymes that could cross-catalyze each other's formation (**Fig. 26-41**). One ribozyme, E, catalyzes the joining of two oligonucleotides (A′ and B′) to create a second, complementary

BOX 26-4 METHODS

The SELEX Method for Generating RNA Polymers with New Functions

SELEX (*s*ystematic *e*volution of *l*igands by *ex*ponential enrichment) is used to generate **aptamers**, oligonucleotides selected to tightly bind a specific molecular target. The process is generally automated to allow rapid identification of one or more aptamers with the desired binding specificity.

Figure 1 illustrates how SELEX is used to select an RNA species that binds tightly to ATP. In step ①, a random mixture of RNA polymers is subjected to "unnatural selection" by passing it through a resin to which ATP is attached. The practical limit for the complexity of an RNA mixture is about 10^{15} different sequences, which allows complete randomization of 25 nucleotides ($4^{25} = 10^{15}$). For longer RNAs, the RNA pool used to initiate the search does not include all possible sequences. RNA polymers that pass through the column are discarded (step ②); those that bind to ATP are washed from the column with salt solution and collected (step ③). In step ④, the collected RNA polymers are amplified by reverse transcriptase to make many DNA complements to the selected RNAs, then an RNA polymerase makes many RNA complements of the resulting DNA molecules. Finally, in step ⑤, this new pool of RNA is subjected to the same selection procedure, and the cycle is repeated a dozen or more times. At the end, only a few aptamers—in this case, RNA sequences with considerable affinity for ATP—remain.

Critical sequence features of an RNA aptamer that binds ATP are shown in Figure 2; molecules with this general structure bind ATP (and other adenosine nucleotides) with $K_d < 50\ \mu\text{M}$. Figure 3 presents the three-dimensional structure of a 36 nucleotide RNA aptamer (shown as a complex with AMP) generated by SELEX. This RNA has the backbone structure shown in Figure 2.

In addition to its use in exploring the potential functionality of RNA, SELEX has an important practical side in identifying short RNAs with pharmaceutical uses. Finding an aptamer that binds specifically to every potential therapeutic target may be impossible, but the capacity of SELEX to rapidly select and amplify a specific oligonucleotide sequence from a highly complex pool of sequences makes this a promising approach for generating new therapies. For example, one could select an RNA that binds tightly to a receptor protein prominent in the plasma membrane of cells in a particular cancerous tumor. Blocking the activity of the receptor, or targeting a toxin to the tumor cells by attaching it to the aptamer, would kill the cells. SELEX also has been used to select DNA aptamers that detect anthrax spores. Many other promising applications are under development. ■

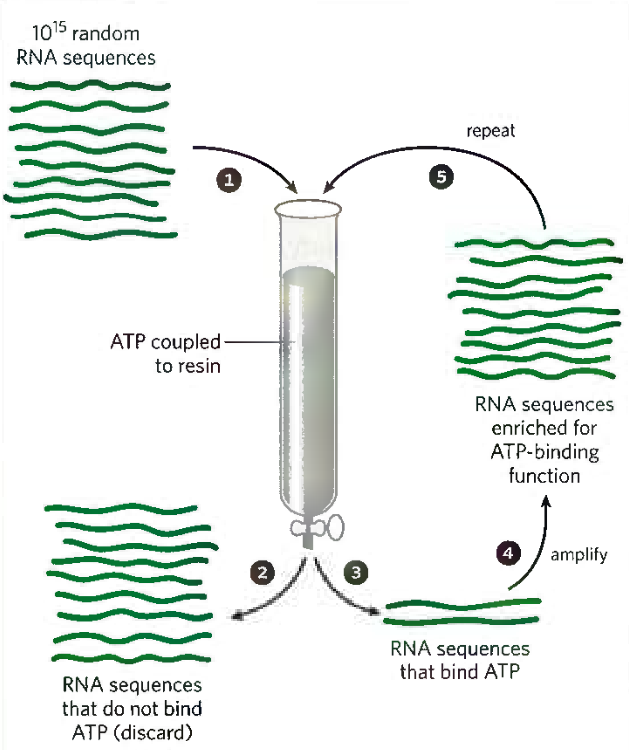

10^{15} random RNA sequences
repeat
①
⑤
ATP coupled to resin
RNA sequences enriched for ATP-binding function
④
amplify
②
③
RNA sequences that do not bind ATP (discard)
RNA sequences that bind ATP

FIGURE 1 The SELEX procedure.

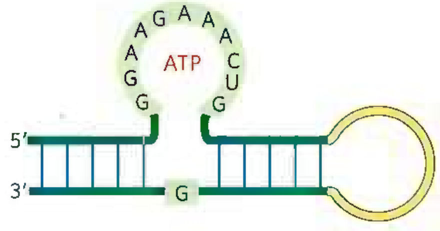


FIGURE 2 RNA aptamer that binds ATP. The shaded nucleotides are those required for the binding activity.

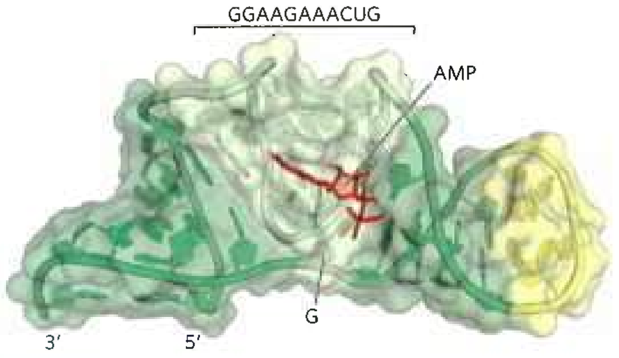


FIGURE 3 RNA aptamer bound to AMP. The bases of the conserved nucleotides (forming the binding pocket) are pale green; the bound AMP is red. [Data from PDB ID 1RAW, T. Dieckmann et al., *RNA* 2:628, 1996.]

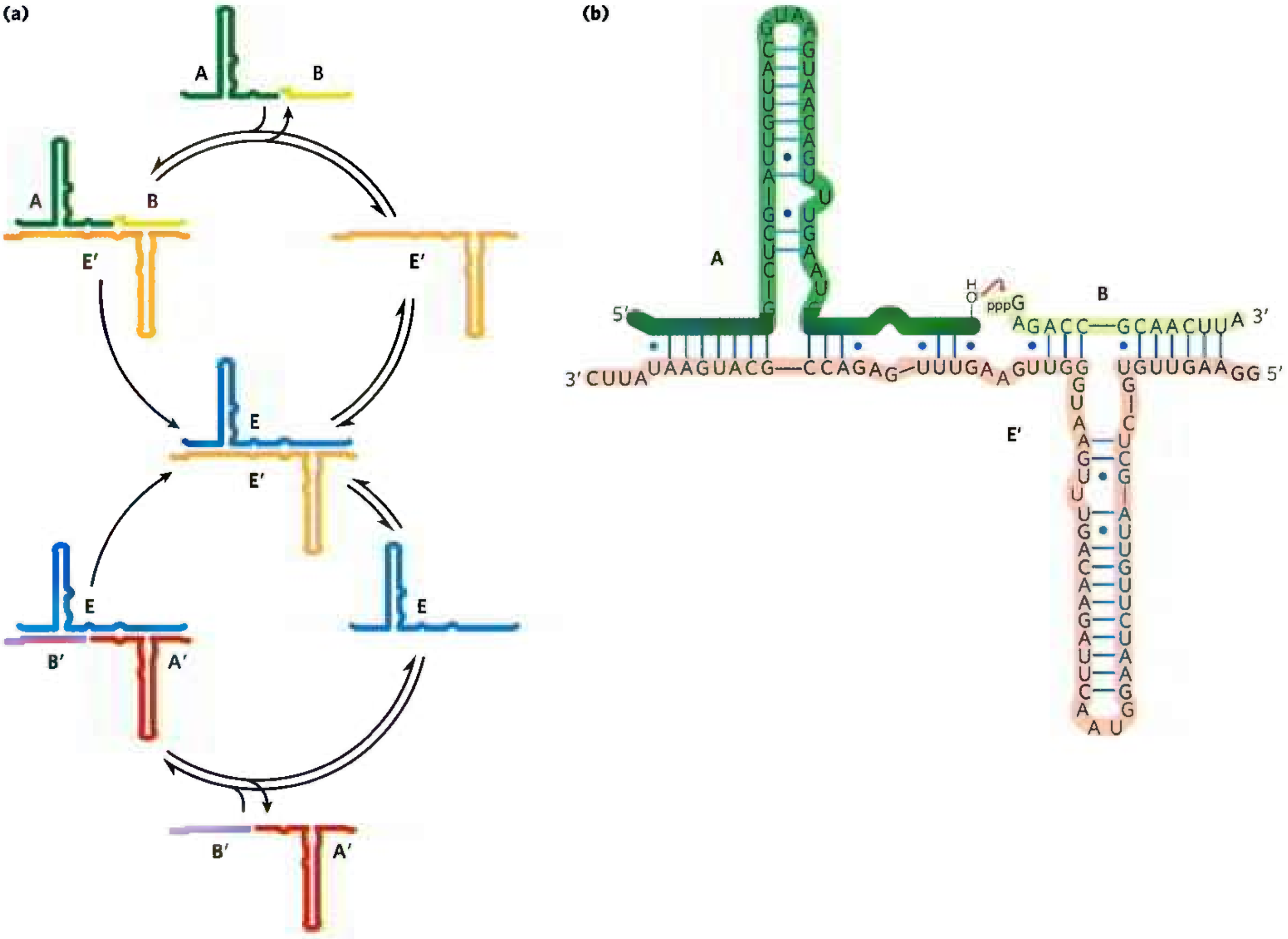


FIGURE 26-41 Self-sustained replication of an RNA enzyme. This system has many of the properties of a living system. The RNA molecules incorporate information and catalytic function, and the reactions produce an exponential increase in the product RNAs. When variants of the RNA substrates are introduced, the system undergoes natural selection such that the best replicators eventually dominate the population. (a) A possible reaction scheme. Oligoribonucleotides A and B anneal to ribozyme E′ and are ligated catalytically to form ribozyme E. The joining of oligoribonucleotides A′ and B′ is similarly catalyzed by ribozyme E. The levels of E and E′ grow exponentially, with a doubling time of about one hour at 42 °C, as long as there is a supply of the precursors A, B, A′, and B′. (b) The ligation reaction involves attack of the 3′ OH of one oligoribonucleotide on the α phosphate of the 5′-triphosphate of the other oligoribonucleotide. Pyrophosphate is released. Base pairing of the substrates with the ribozyme plays a key role in aligning the substrates for the reaction. [Information from T. A. Lincoln and G. F. Joyce, *Science* 323:1229, 2009.]

ribozyme called E′. E′ could then catalyze the joining of two other oligonucleotides (A and B) to form another molecule of E. In this system, the formation of E and E′ was templated, and the amounts grew exponentially as long as substrates were available and proteins were absent. The system evolved so that more-efficient enzymes appeared in the population. A more general RNA-polymerase-like ribozyme was described in 2011 by Philipp Holliger and colleagues.

Although the RNA world remains a hypothesis, with many gaps yet to be explained, experimental evidence supports a growing list of its key elements. Further experimentation should increase our understanding. Important clues to the puzzle will be found in the workings of fundamental chemistry, in living cells, and perhaps on other planets.

SUMMARY 26.4 *Catalytic RNA and the RNA World Hypothesis*

- Ribozymes and protein-based enzymes share common features, including folded three-dimensional structures, inactivation by denaturation, acceleration of reaction rates, saturable kinetics, and reaction specificity.
- Ribozymes and RNA-based catalysts are present in organisms today and are involved in a wide range of activities, including tRNA processing, nuclear pre-mRNA splicing, and translation.
- The evolution of life on Earth may have included an RNA world in which RNA was the central information carrier and catalyst before proteins and DNA emerged as key players. The existence of ribozymes provides a powerful piece of evidence in support of this hypothesis.

KEY TERMS

Terms in bold are defined in the glossary.

ribonucleoprotein (RNP)	960
transcription	960
messenger RNA (mRNA)	960
transfer RNA (tRNA)	960
ribosomal RNA (rRNA)	960
noncoding RNA (ncRNA)	960
transcriptome	960
DNA-dependent RNA polymerase	961
template strand	962
nontemplate strand	962
coding strand	962
promoter	963
consensus sequence	963
footprinting	965
cAMP receptor protein (CRP)	966
repressor	966
carboxyl-terminal domain (CTD)	968
transcription factors	968
general transcription factors	968
preinitiation complex (PIC)	969
ribozymes	972
primary transcript	972
precursor transcript	972
intron	973
exon	973
RNA splicing	973
messenger ribonucleoprotein (mRNP) complex	973
5′ cap	974
spliceosome	977
small nuclear RNA (snRNA)	977
poly(A) tail	980
alternative splicing	981
poly(A) site choice	982
small nucleolar RNA (snoRNA)	984
snoRNA-protein complex (snoRNP)	984
microRNA (miRNA)	985
polynucleotide phosphorylase	987
exosome	987
reverse transcriptase	988
retrovirus	988
complementary DNA (cDNA)	990
homing	992
telomerase	993
T loop	993
RNA-dependent RNA polymerase (RNA replicase)	994
internal guide sequence	996
RNA world hypothesis	998
SELEX	1000
aptamer	1000

PROBLEMS

1. RNA Polymerase (a) How long would it take for the *E. coli* RNA polymerase to synthesize the primary transcript for the *E. coli* genes encoding the enzymes for lactose metabolism, the 5,300 bp *lac* operon? **(b)** How far along the DNA would the transcription "bubble" formed by RNA polymerase move in 10 seconds? **(c)** Assuming that human Pol II transcribes at a similar rate, how long does it take to transcribe the 2,000,000 bp dystrophin gene?

2. Error Correction by RNA Polymerases DNA polymerases are capable of editing and error correction, whereas the capacity for error correction in RNA polymerases seems to be limited. Given that a single base error in either replication or transcription can lead to an error in protein synthesis, suggest a possible biological explanation for this difference.

3. RNA Posttranscriptional Processing Predict the likely effects of a mutation in the sequence (5′)AAUAAA in a eukaryotic mRNA transcript.

4. Coding versus Template Strands The RNA genome of phage Qβ is the nontemplate strand, or coding strand, and when introduced into the cell, it functions as an mRNA. Suppose the RNA replicase of phage Qβ synthesized primarily template-strand RNA and uniquely incorporated this, rather than nontemplate strands, into the viral particles. What would be the fate of the template strands when they entered a new cell? What enzyme would have to be included in the viral particles for successful invasion of a host cell?

5. Transcription The gene encoding the *E. coli* enzyme enolase begins with the sequence ATGTCCAAAATCGTA. What is the sequence of the RNA transcript specified by this part of the gene?

6. The Chemistry of Nucleic Acid Biosynthesis Describe three properties common to the reactions catalyzed by DNA polymerase, RNA polymerase, reverse transcriptase, and RNA replicase. How is the enzyme polynucleotide phosphorylase similar to and different from these four enzymes?

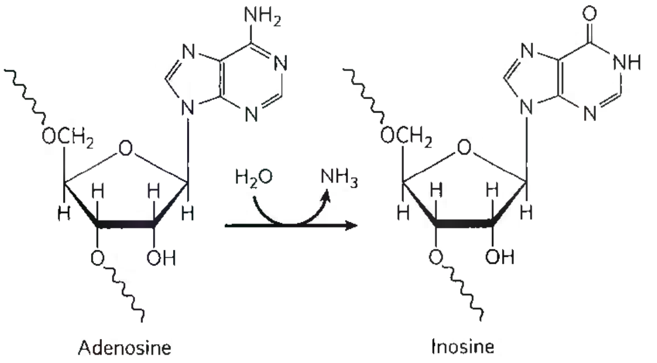
7. RNA Processing I While studying human transcription in the 1960s, James Darnell carried out an experiment that has become a classic in biochemistry, but at the time, it was incredibly perplexing. Darnell and coworkers used radioactive isotopes, such as $[^{32}\text{P}]$-labeled phosphate, to isolate and quantify RNAs from a cultured line of human cancer cells (HeLa). With this approach, they were able to identify those RNAs present in the nucleus and those present in the cytoplasm. The results were puzzling, because it was obvious that a large amount of transcription was occurring in the nucleus, but comparatively little radioactive mRNA was isolated from the cytoplasm. Moreover, the nuclear-isolated RNAs were much longer than those isolated from the cytoplasm. What can account for these observations?

8. The Transcriptome If a cell's genome is completely known, is it possible to determine the cell's transcriptome—the sequence of all the RNAs being produced by the cell—without additional information? Explain.

9. RNA Splicing What is the minimum number of transesterification reactions needed to splice an intron from a pre-mRNA transcript? Explain.

10. RNA Processing II Blocking the splicing of a particular pre-mRNA in a vertebrate cell also blocks a nucleotide modification reaction occurring in the rRNA. Suggest a reason for this.

11. RNA Modification I Researchers Brenda Bass and Harold Weintraub discovered double-stranded RNA-specific adenosine deaminases (ADARs) in 1987. These enzymes recognize double-stranded regions of mRNA and convert adenosine bases to inosine within these regions.



(a) When ADAR encounters a double-stranded RNA substrate in water that also contains $H_2[^{18}O]$, radioactive oxygen incorporates into the RNA. Draw a reaction mechanism for ADAR that accounts for this observation.
(b) After ADAR reacts with a double-stranded RNA, the RNA duplex sometimes spontaneously unwinds to form single strands. Why might this occur?
(c) What is a possible consequence of adenosine to inosine conversion in the coding sequence of an mRNA?

12. Organization of RNA Processing In eukaryotes, pre-mRNA splicing by the spliceosome occurs only in the nucleus and translation of mRNAs occurs only in the cytosol. Why might the separation of these two activities into different cellular compartments be important?

13. RNA Modification II In addition to rRNAs and tRNAs, many human mRNAs also contain modified nucleotides, in particular N^6-methyladenosine.
(a) How might incorporation of N^6-methyladenosine impact RNA processing?
(b) Incorporation of N^6-methyladenosine into the 3′ untranslated region (UTR; see Fig. 9-24) of the MAT2A transcript, which encodes a methionine adenosyltransferase, regulates the metabolism of the cofactor *S*-adenosylmethionine. Why would metabolism of *S*-adenosylmethionine be linked to N^6-methyladenosine formation?

14. RNA Genomes RNA viruses have relatively small genomes. For example, the single-stranded RNAs of retroviruses have about 10,000 nucleotides, and the Qβ RNA is only 4,220 nucleotides long. How might the properties of reverse transcriptase and RNA replicase have contributed to the small size of these viral genomes?

15. Screening of RNAs by SELEX The practical limit for the number of different RNA sequences that can be screened in a SELEX experiment is 10^{15}. **(a)** Suppose you are working with oligonucleotides that are 36 nucleotides long. How many sequences exist in a randomized pool containing every sequence possible? **(b)** What percentage of these can a SELEX experiment screen? **(c)** Suppose you wish to select an RNA molecule that catalyzes the hydrolysis of a particular ester. From what you know about catalysis, propose a SELEX strategy that might allow you to select the appropriate catalyst.

16. Slow Death The death cap mushroom, *Amanita phalloides*, contains several dangerous substances, including the lethal α-amanitin. This toxin blocks RNA elongation in consumers of the mushroom by binding to eukaryotic RNA polymerase II with very high affinity; it is deadly in concentrations as low as 10^{-8} M. The initial reaction to ingestion of the mushroom is gastrointestinal distress (caused by some of the other toxins). These symptoms disappear, but about 48 hours later, the mushroom-eater dies, usually from liver dysfunction. Speculate on why it takes this long for α-amanitin to kill.

17. Detection of Rifampin-Resistant Strains of Tuberculosis Rifampin is an important antibiotic used to treat tuberculosis and other mycobacterial diseases. Some strains of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, are resistant to rifampin. These strains become resistant through mutations that alter the *rpoB* gene, which encodes the β subunit of the RNA polymerase (see Fig. 26-10). Rifampin cannot bind to the mutant RNA polymerase and so is unable to block the initiation of transcription. DNA sequences from a large number of rifampin-resistant *M. tuberculosis* strains have been found to have mutations in a specific 69 bp region of *rpoB*. One well-characterized rifampin-resistant strain has a single base pair alteration in *rpoB* that results in a His residue being replaced by an Asp residue in the β subunit.
(a) Based on your knowledge of protein chemistry, what technique could you use to detect whether a particular strain produces the rpoB His → Asp mutant protein?
(b) Based on your knowledge of nucleic acid chemistry, what technique could you also use to identify the mutant forms of *rpoB*?

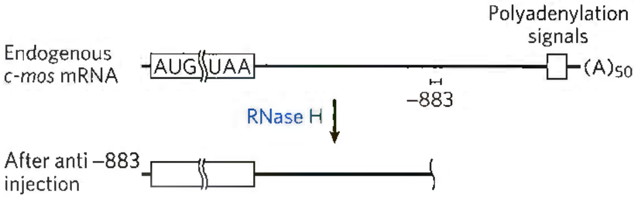
BIOCHEMISTRY ONLINE

18. The Ribonuclease Gene Human pancreatic ribonuclease has 128 amino acid residues.
(a) What is the minimum number of nucleotide pairs required to code for this protein?
(b) The mRNA expressed in human pancreatic cells was copied with reverse transcriptase to create a "library" of human DNA. The sequence of the mRNA coding for human pancreatic ribonuclease was determined by sequencing the complementary DNA (cDNA) from this library that included an open reading frame for the protein. Use the nucleotide database at NCBI (www.ncbi.nlm.nih.gov/nucleotide) to find the published sequence of this mRNA. (Search for accession number D26129.) What is the length of this mRNA?
(c) How can you account for the discrepancy between the size you calculated in (a) and the actual length of the mRNA?

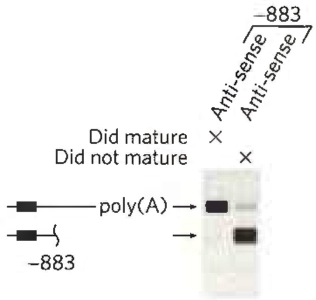
DATA ANALYSIS PROBLEM

19. Amputated RNAs with Prosthetic Tails The Wickens lab studies the dramatic effects of RNA processing on how a cell utilizes mRNAs, such as the c-*mos* (cellular *mo*use *s*arcoma) protooncogene, which controls meiosis and embryonic cell cycles in vertebrates. In frogs (*Xenopus*), expression of the c-*mos* protein is essential for maturation of oocytes after exposure to progesterone and embryo formation.

Specific RNAs can be cleaved (amputated) in *Xenopus* oocytes by injection of antisense DNA oligonucleotides that will hybridize to the mRNA. The formation of an RNA/DNA duplex triggers cleavage of the RNA strand by cellular RNase H. The figure below depicts the results of amputation of the c-*mos* mRNA after oligo injection. In this case, the oligonucleotide targeted the −883 region of the c-*mos* mRNA. This region is downstream of the c-*mos* open reading frame (depicted by the AUG start and UAA stop codons) within the 3′ untranslated region (3′UTR).



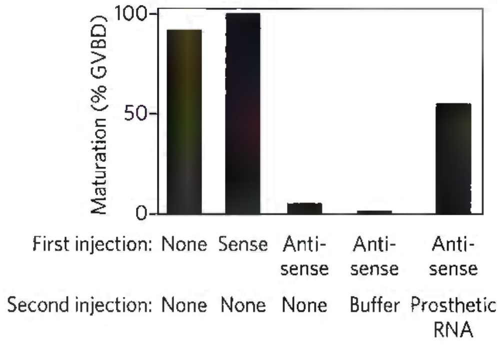
After injection of a sense oligonucleotide, the oocyte maturation percentage was nearly unchanged. However, when an antisense oligonucleotide was injected, the maturation percentage was reduced by ~60%. The c-*mos* mRNA was isolated from oocytes that either did or did not mature after injection of the antisense oligonucleotide. The mRNAs were analyzed by gel electrophoresis followed by northern blotting, as shown below. In a northern blot, RNAs are separated by gel electrophoresis according to their size, transferred to a membrane, and then detected by hybridization to radioactive or fluorescent complementary DNA probes.



(a) What can you conclude about the importance of the c-*mos* mRNA poly(A) tail on oocyte maturation?

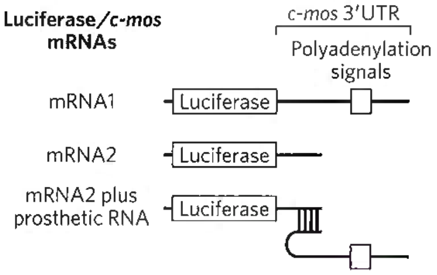
(b) Why was a sense oligonucleotide used in some experiments?

Members of the Wickens lab then decided to inject a prosthetic RNA after amputation of the c-*mos* mRNA. The prosthetic RNA contained the c-*mos* mRNA 3′UTR as well as a region complementary to the amputated c-*mos* mRNA. Their observations are shown below. In these experiments, oocyte maturation was measured by the percentage of cells in which germinal vesicle breakdown (% GVBD) was observed. GVBD occurs when the oocyte nucleus (called the germinal vesicle) dissolves and the cell resumes meiosis.



(c) How do you explain these results?

Wickens lab scientists then tried attaching the c-*mos* mRNA 3′UTR to an unrelated reporter enzyme. In this case, they used luciferase, whose enzymatic activity can easily be measured by luminescence (see Box 13-2). The total luciferase activity is also proportional to the amount of luciferase protein being produced by the cell. They tried attaching the entire c-*mos* mRNA 3′UTR (mRNA1), the amputated 3′UTR (mRNA2), or a combination of the amputated 3′UTR with the prosthetic poly(A) tail to luciferase. High luciferase activity was observed only when either the entire c-*mos* mRNA 3′UTR was used or when the prosthetic poly(A) tail was included along with the amputated 3′UTR.

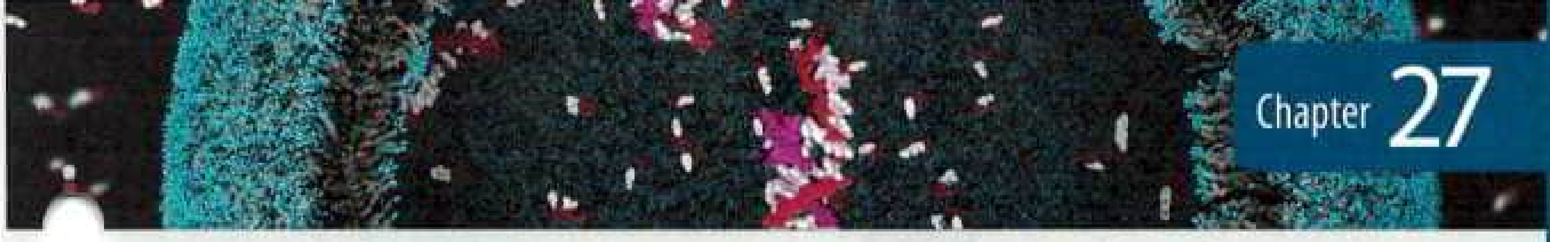


(d) What does this experiment tell you about the likely function of the 3′UTR and poly(A) tail for the c-*mos* mRNA and in oocyte maturation?

(e) How might these results be useful for controlling cellular gene expression?

Reference

Sheets, M.D., M. Wu, and M. Wickens. 1995. Polyadenylation of c-*mos* mRNA as a control point in *Xenopus* meiotic maturation. *Nature* 374:511–516.



PROTEIN METABOLISM

27.1 The Genetic Code *1006*
27.2 Protein Synthesis *1015*
27.3 Protein Targeting and Degradation *1041*

Almost every biological process requires one or more proteins. A typical cell requires thousands of different proteins at any given moment. These proteins must be synthesized in response to the cell's current needs, modified to alter their activity or fate, transported (targeted) to their appropriate cellular locations, and degraded when no longer needed. Dozens of separate processes contribute to cellular **proteostasis**, the steady-state complement of proteins that enable the life of a cell at any given moment.

Many of the fundamental components and mechanisms used by the protein biosynthetic machinery are remarkably well conserved in all life-forms, from bacteria to higher eukaryotes, indicating that they were present in the last universal common ancestor (LUCA) of all extant organisms. Whereas the chapter focuses on protein biosynthesis, all aspects of proteostasis are considered. The principles that guide our approach are interrelated, reflecting all of these realities and more.

P1 **Information is expensive.** This principle, noted in earlier chapters, is particularly well illustrated by protein synthesis. Protein synthesis consumes more cellular resources than any other process in most cells. The 15,000 ribosomes, 100,000 molecules of protein synthesis–related protein factors and enzymes, and 200,000 tRNA molecules in a typical bacterial cell can account for more than 35% of the cell's dry weight. Overall, almost 300 *different* macromolecules cooperate to synthesize polypeptides. Protein synthesis can account for up to 90% of the chemical energy used by a cell for all biosynthetic reactions. Why? The enzymatic synthesis of an amide bond should require little energetic input. However, the sequence of amino acids in a protein is a form of biological information. Synthesis of each amide (peptide) bond between two *particular* amino acids is ensured by the investment of more than four nucleoside triphosphates (NTPs).

P2 **The genetic code is nearly universal and arose early in evolution.** This is one of the many characteristics of living systems that ties all of them to a common ancestor. Even the rare exceptions to code universality reinforce this rule.

P3 **The genetic code functions via linker molecules.** The tRNAs are the crucial adaptor, matching amino acids with DNA codons.

P4 **Proteins are synthesized by RNAs.** The study of protein synthesis offers another important reward: a look at a world of RNA catalysts that may have existed in an RNA world before the dawn of life "as we know it." Proteins are synthesized by a gigantic RNA enzyme.

P5 **Protein metabolism is regulated at many levels.** The resource investment in protein synthesis ensures that many layers of regulation work together to determine which proteins are synthesized at any given moment. However, proteins often function only in particular cellular locations and at particular times. Mechanisms for spatial and temporal regulation—protein targeting, activation, and eventual degradation—exhibit complexities that can approach those of the biosynthetic processes.

Several major advances set the stage for our present knowledge of protein biosynthesis (**Fig. 27-1**). First, in the early 1950s, Paul Zamecnik and Elizabeth Keller discovered the ribonucleoprotein particles in which protein synthesis occurs. These particles, visible in animal tissues by electron microscopy, were later named ribosomes. Soon after, Francis Crick considered how the genetic information encoded in the 4-letter language of nucleic acids could be translated into the 20-letter language of proteins. In 1955, Crick postulated that a small nucleic acid could serve the role of an adaptor, with one part of the adaptor molecule binding a specific

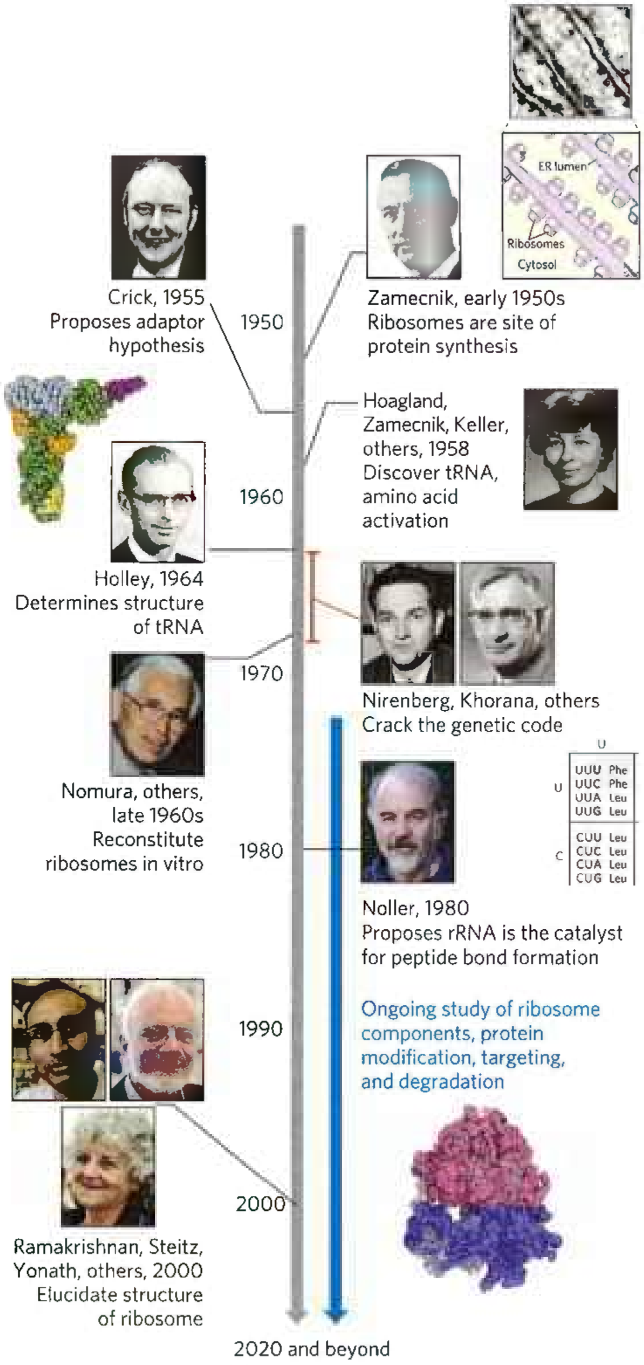

Crick, 1955
Proposes adaptor hypothesis
1950
Zamecnik, early 1950s
Ribosomes are site of protein synthesis
ER lumen
Ribosomes
Cytosol
Hoagland, Zamecnik, Keller, others, 1958
Discover tRNA, amino acid activation
1960
Holley, 1964
Determines structure of tRNA
1970
Nirenberg, Khorana, others
Crack the genetic code
U
U
UUU Phe
UUC Phe
UUA Leu
UUG Leu
C
CUU Leu
CUC Leu
CUA Leu
CUG Leu
Nomura, others, late 1960s
Reconstitute ribosomes in vitro
1980
Noller, 1980
Proposes rRNA is the catalyst for peptide bond formation
1990
Ongoing study of ribosome components, protein modification, targeting, and degradation
2000
Ramakrishnan, Steitz, Yonath, others, 2000
Elucidate structure of ribosome
2020 and beyond

FIGURE 27-1 Timeline for the elucidation of protein biosynthetic pathways. Some key contributions are highlighted. However, our current understanding of the genetic code and protein biosynthetic pathways comes as the result of international endeavors involving hundreds of laboratories. [Top left to bottom left: Bettmann/Getty Images; data from PDB ID 4TRA, E. Westhof et al., *Acta Crystallogr. A* 44:112, 1988; Bettmann/Getty Images; Courtesy Archives, University of Wisconsin, Madison; Alastair Grant/AP Images; Lucas Jackson/Reuters/Newscom; Xinhua/ZUMAPRESS.com. Top right to bottom right: Joseph F. Gennaro Jr./Science Source; Archives and Special Collections, Massachusetts General Hospital; Cornell University Media Services PhotoSection records, #21-37-2227, Division of Rare and Manuscript Collections, Cornell University Library; Bob Schutz/AP Images; Courtesy Archives, University of Wisconsin, Madison; Courtesy Harry Noller; data from PDB ID 4V7R, A. Ben-Shem et al., *Science* 330:1203, 2010.]

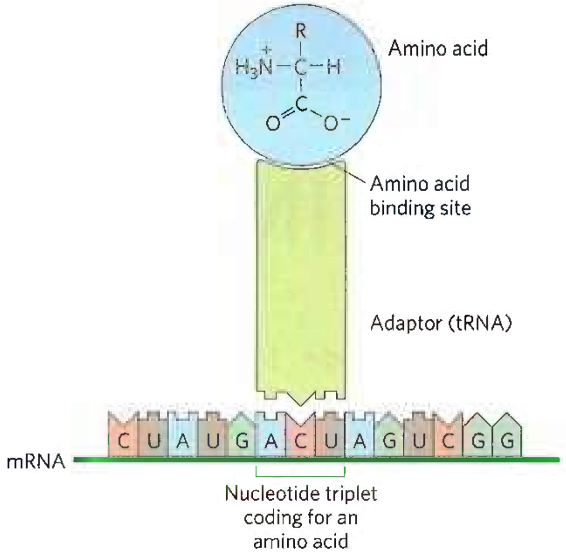

R
$H_3\overset{+}{N}$–C–H
C
O
O⁻
Amino acid
Amino acid binding site
Adaptor (tRNA)
C U A U G A C U A G U C G G
mRNA
Nucleotide triplet coding for an amino acid

FIGURE 27-2 Crick's adaptor hypothesis. Francis Crick proposed that one end of a small nucleotide adaptor could bind a specific amino acid and the other end could recognize a nucleotide sequence in the mRNA. Today we know that the amino acid is covalently bound at the 3′ end of a tRNA molecule and that a specific nucleotide triplet elsewhere in the tRNA interacts with a particular triplet codon in mRNA through hydrogen bonding of complementary bases.

amino acid and another part recognizing the nucleotide sequence encoding that amino acid in an mRNA (**Fig. 27-2**). Crick's adaptor hypothesis was soon verified when Mahlon Hoagland and Zamecnik discovered tRNA. The structure of alanyl-tRNA was reported by Robert Holley in 1964. The tRNA adaptor "translates" the nucleotide sequence of an mRNA into the amino acid sequence of a polypeptide. The overall process of mRNA-guided protein synthesis is often referred to simply as **translation**. Hoagland, Zamecnik, and Elizabeth Keller also discovered that amino acids were "activated" for protein synthesis when incubated with ATP and the cytosolic fraction of liver cells. The amino acids became attached to a heat-stable soluble RNA—the tRNA—to form **aminoacyl-tRNAs**. The enzymes that catalyze this process are the **aminoacyl-tRNA synthetases**.

These developments soon led to recognition of the major stages of protein synthesis and ultimately to elucidation of the genetic code that specifies each amino acid. In subsequent decades, ribosomes were purified and their protein and rRNA components were dissected. Elucidation of the three-dimensional structures of ribosomes was completed by 2000, confirming a hypothesis first put forward by Harry Noller two decades earlier: it is the rRNA, rather than ribosomal proteins, that catalyzes peptide bond formation.

27.1 The Genetic Code

By the 1960s, it was apparent that at least three nucleotide residues of DNA are necessary to encode each amino acid. The four code letters of DNA (A, T, G, and C) in groups of two can yield only $4^2 = 16$ different combinations, insufficient to encode 20 amino acids. Groups of three, however, yield $4^3 = 64$ different combinations. Deciphering the genetic code quickly became a major goal.

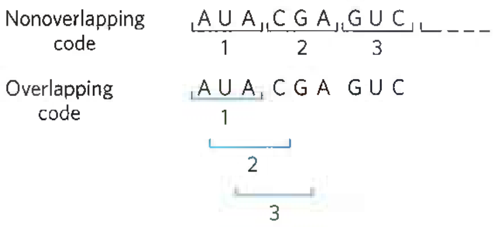


FIGURE 27-3 Overlapping versus nonoverlapping genetic codes. In a nonoverlapping code, codons (numbered consecutively) do not share nucleotides. In an overlapping code, some nucleotides in the mRNA are shared by different codons. In a triplet code with maximum overlap, many nucleotides, such as the third nucleotide from the left (A), are shared by three codons. A nonoverlapping code provides much more flexibility in the triplet sequence of neighboring codons and therefore in the possible amino acid sequences designated by the code.

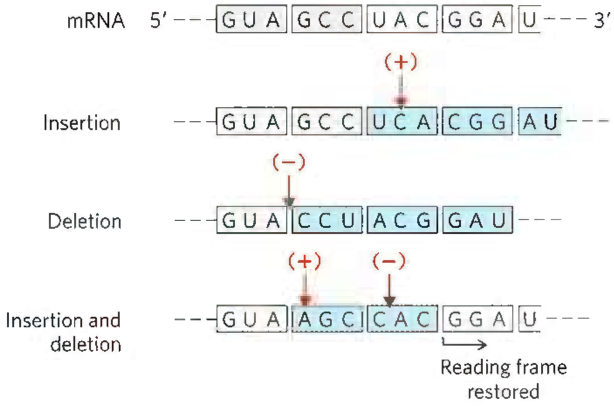

mRNA 5′ - - - G U A G C C U A C G G A U - - - 3′
(+)
Insertion - - - G U A G C C U C A C G G A U - - -
(−)
Deletion - - - G U A C C U A C G G A U - - -
(+) (−)
Insertion and deletion - - - G U A A G C C A C G G A U - - -
Reading frame restored

FIGURE 27-4 The triplet, nonoverlapping code. Evidence for the general nature of the genetic code came from many types of experiments, including genetic experiments on the effects of deletion and insertion mutations. Inserting or deleting one base pair (shown here in the mRNA transcript) alters the sequence of all amino acids coded by the mRNA following the change. Combining insertion and deletion mutations affects some amino acids but can eventually restore the correct amino acid sequence. Adding or subtracting three nucleotides (not shown) leaves the remaining triplets intact, providing evidence that a codon has three, rather than four or five, nucleotides. The triplet codons shaded in gray are those transcribed from the original gene; codons shaded in blue are new codons resulting from the insertion or deletion mutations.

The Genetic Code Was Cracked Using Artificial mRNA Templates

Several key properties of the genetic code were established in early genetic studies. A **codon** is a triplet of nucleotides that codes for a specific amino acid. In all living systems, translation occurs in such a way that these nucleotide triplets are read in a successive, nonoverlapping fashion (Figs. 27-3, 27-4). A specific first codon in the sequence establishes the **reading frame**, in which a new codon begins every three nucleotide residues. There is no punctuation between codons for successive amino acid residues. The amino acid sequence of a protein is defined by a linear sequence of contiguous triplets. In principle, any given single-stranded DNA or mRNA sequence has three possible reading frames. Each reading frame gives a different sequence of codons (Fig. 27-5), but only one is likely to encode a given protein. A key question remained: what were the three-letter code words for each amino acid?

In 1961, Marshall Nirenberg and J. Heinrich Matthaei reported the first breakthrough. They incubated synthetic polyuridylate, poly(U), with an *Escherichia coli* extract, GTP, ATP, and a mixture of the 20 amino acids in 20 different tubes, each tube containing a different radioactively labeled amino acid. Because poly(U) mRNA is made up of many successive UUU triplets, it should promote the synthesis of a polypeptide containing only the amino acid encoded by UUU. A radioactive polypeptide was formed only in the tube containing radioactive phenylalanine. Nirenberg and Matthaei therefore concluded that the triplet codon UUU encodes phenylalanine. The same approach soon revealed that polycytidylate, poly(C), encodes a polypeptide containing only proline (polyproline), and that polyadenylate, poly(A), encodes polylysine. Polyguanylate did not generate any polypeptide in this experiment because it spontaneously forms tetraplexes (see Fig. 8-20d) that cannot be bound by ribosomes.

The synthetic polynucleotides used in such experiments were prepared by using polynucleotide phosphorylase (p. 987), which catalyzes the formation of RNA polymers starting from ADP, UDP, CDP, and GDP. This enzyme, discovered by physician/biochemist Severo Ochoa, requires no template and makes polymers with a base composition that directly reflects the relative concentrations of the nucleoside 5′-diphosphate precursors in the medium. If polynucleotide phosphorylase is presented with only UDP, it makes only poly(U). If it is presented with a mixture of five parts ADP and one part CDP, it makes a polymer in which about five-sixths of the residues are adenylate and one-sixth is cytidylate. This random polymer is likely to have many triplets of the sequence AAA; smaller numbers of AAC, ACA, and CAA triplets; relatively few ACC, CCA, and CAC triplets; and very few CCC triplets (Table 27-1). Using a variety of artificial mRNAs made by polynucleotide phosphorylase

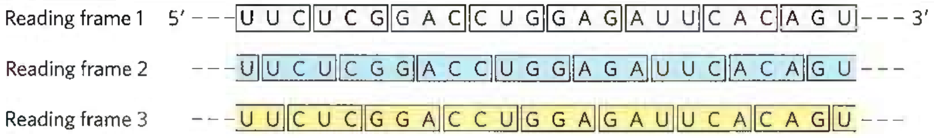

Reading frame 1 5′ - - - U U C U C G G A C C U G G A G A U U C A C A G U - - - 3′
Reading frame 2 - - - U U C U C G G A C C U G G A G A U U C A C A G U - - -
Reading frame 3 - - - U U C U C G G A C C U G G A G A U U C A C A G U - - -

FIGURE 27-5 Reading frames in the genetic code. In a triplet, nonoverlapping code, all mRNAs have three potential reading frames, shaded here in different colors. The triplets, and hence the amino acids specified, are different in each reading frame.

TABLE 27-1 Incorporation of Amino Acids into Polypeptides in Response to Random Polymers of RNA

Amino acid	Observed frequency of incorporation (Lys = 100)	Tentative assignment for nucleotide composition of corresponding codon[a]	Expected frequency of incorporation based on assignment (Lys = 100)
Asparagine	24	A_2C	20
Glutamine	24	A_2C	20
Histidine	6	AC_2	4
Lysine	100	AAA	100
Proline	7	AC_2, CCC	4.8
Threonine	26	A_2C, AC_2	24

Note: Presented here is a summary of data from one of the early experiments designed to elucidate the genetic code. A synthetic RNA containing only A and C residues in 5:1 ratio directed polypeptide synthesis, and both the identity and the quantity of incorporated amino acids were determined. Based on the relative abundance of A and C residues in the synthetic RNA, and assigning the codon AAA (the most likely codon) a frequency of 100, there should be three different codons of composition A_2C, each at a relative frequency of 20; three of composition AC_2, each at a relative frequency of 4.0; and CCC at a relative frequency of 0.8. The CCC assignment was based on information derived from prior studies with poly(C). Where two tentative codon assignments are made, both are proposed to code for the same amino acid.

[a] These designations of nucleotide composition contain no information on nucleotide sequence (except, of course, AAA and CCC).

TABLE 27-2 Trinucleotides That Induce Specific Binding of Aminoacyl-tRNAs to Ribosomes

Trinucleotide	Relative increase in ^{14}C-labeled aminoacyl-tRNA bound to ribosome[a] Phe-tRNA^{Phe}	 Lys-tRNA^{Lys}	 Pro-tRNA^{Pro}
UUU	4.6	0	0
AAA	0	7.7	0
CCC	0	0	3.1

Information from M. Nirenberg and P. Leder, *Science* 145:1399, 1964.

[a] Each number represents the factor by which the amount of bound ^{14}C increased when the indicated trinucleotide was present, relative to a control with no trinucleotide.

from different starting mixtures of ADP, GDP, UDP, and CDP, the Nirenberg and Ochoa groups soon identified the base compositions of the triplets coding for almost all the amino acids. Although these experiments revealed the base composition of the coding triplets, they usually could not reveal the sequence of the bases.

KEY CONVENTION Much of the following discussion deals with tRNAs. The amino acid specified by a tRNA is indicated by a superscript, such as tRNA^{Ala}, and the aminoacylated tRNA is designated by a hyphenated name, such as alanyl-tRNA^{Ala} or Ala-tRNA^{Ala}. ■

In 1964, Nirenberg and Philip Leder achieved another experimental breakthrough. Isolated *E. coli* ribosomes would bind a specific aminoacyl-tRNA in the presence of the corresponding synthetic polynucleotide messenger. For example, ribosomes incubated with poly(U) and phenylalanyl-tRNA^{Phe} (Phe-tRNA^{Phe}) bind both RNAs, but if the ribosomes are incubated with poly(U) and some other aminoacyl-tRNA, the aminoacyl-tRNA is not bound, because it does not recognize the UUU triplets in poly(U) (**Table 27-2**). Even trinucleotides could promote specific binding of appropriate tRNAs, so these experiments could be carried out with chemically synthesized small oligonucleotides. With this technique, researchers determined which aminoacyl-tRNA bound to 54 of the 64 possible triplet codons. For some codons, either no aminoacyl-tRNA or more than one would bind. Another method was needed to complete and confirm the entire genetic code.

At about this time, a complementary approach was provided by H. Gobind Khorana, who developed chemical methods to synthesize polyribonucleotides with defined, repeating sequences of two to four bases. The polypeptides produced by these mRNAs had one or a few amino acids in repeating patterns. These patterns, when combined with information from the random polymers used by Nirenberg and colleagues, permitted unambiguous codon assignments. The copolymer $(AC)_n$, for example, has alternating ACA and CAC codons: ACACACACACACACA. The polypeptide synthesized on this messenger contained equal amounts of threonine and histidine. Given that a histidine codon has one A and two Cs (Table 27-1), CAC must code for histidine and ACA for threonine.

Consolidation of the results from many experiments permitted assignment of 61 of the 64 possible codons. The other three were identified as termination codons, in part because they disrupted amino acid coding patterns when they occurred in a synthetic RNA polymer (**Fig. 27-6**). Meanings for all the triplet codons (tabulated in **Fig. 27-7**) were established by 1966 and have been verified in many different ways. The cracking of the genetic code is regarded as one of the most important scientific discoveries of the twentieth century.

P2 Codons are the key to the translation of genetic information, directing the synthesis of specific proteins. The reading frame is set when translation of an mRNA molecule begins, and it is maintained as the synthetic machinery reads sequentially from one triplet to the next. If the initial

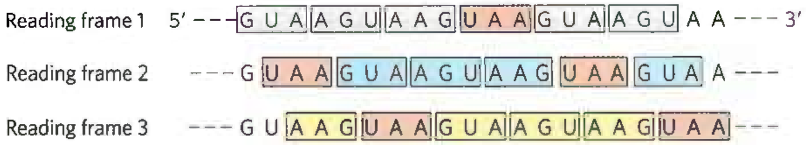
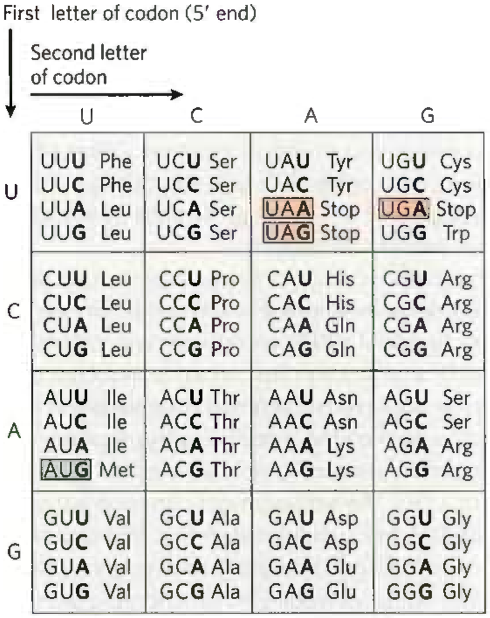

Reading frame 1 5′ - - - G U A A G U A A G U A A G U A A G U A A - - - 3′
Reading frame 2 - - - G U A A G U A A G U A A G U A A G U A A - - -
Reading frame 3 - - - G U A A G U A A G U A A G U A A G U A A - - -

FIGURE 27-6 Effect of a termination codon in a repeating tetranucleotide. Termination codons (light red) are encountered every fourth codon in three different reading frames (shown in different colors). Dipeptides or tripeptides are synthesized, depending on where the ribosome initially binds.


First letter of codon (5′ end)
Second letter of codon

	U	C	A	G
U	UU**U** Phe UU**C** Phe UU**A** Leu UU**G** Leu	UC**U** Ser UC**C** Ser UC**A** Ser UC**G** Ser	UA**U** Tyr UA**C** Tyr UA**A** Stop UA**G** Stop	UG**U** Cys UG**C** Cys UG**A** Stop UG**G** Trp
C	CU**U** Leu CU**C** Leu CU**A** Leu CU**G** Leu	CC**U** Pro CC**C** Pro CC**A** Pro CC**G** Pro	CA**U** His CA**C** His CA**A** Gln CA**G** Gln	CG**U** Arg CG**C** Arg CG**A** Arg CG**G** Arg
A	AU**U** Ile AU**C** Ile AU**A** Ile AU**G** Met	AC**U** Thr AC**C** Thr AC**A** Thr AC**G** Thr	AA**U** Asn AA**C** Asn AA**A** Lys AA**G** Lys	AG**U** Ser AG**C** Ser AG**A** Arg AG**G** Arg
G	GU**U** Val GU**C** Val GU**A** Val GU**G** Val	GC**U** Ala GC**C** Ala GC**A** Ala GC**G** Ala	GA**U** Asp GA**C** Asp GA**A** Glu GA**G** Glu	GG**U** Gly GG**C** Gly GG**A** Gly GG**G** Gly

FIGURE 27-7 "Dictionary" of amino acid code words in mRNAs. The codons are written in the 5′→3′ direction. The third base of each codon (in bold type) plays a lesser role in specifying an amino acid than the first two. The three termination codons are shaded in light red, the initiation codon AUG in green. All the amino acids except methionine and tryptophan have more than one codon. In most cases, codons that specify the same amino acid differ only at the third base.

reading frame is off by one or two bases, or if translation somehow skips a nucleotide in the mRNA, all the subsequent codons will be out of register; the result is usually a "missense" protein with a garbled amino acid sequence.

Several codons serve special functions (Fig. 27-7). The **initiation codon** AUG is the most common signal for the beginning of a polypeptide in all cells, in addition to coding for Met residues in internal positions of polypeptides. The **termination codons** (UAA, UAG, and UGA), also called stop codons or nonsense codons, normally signal the end of polypeptide synthesis and do not code for any known amino acids.

As described in Section 27.2, initiation of protein synthesis in the cell is an elaborate process that relies on initiation codons and other signals in the mRNA. **P1** In retrospect, the experiments of Nirenberg, Khorana, and others to identify codon function should not have worked in the absence of initiation codons. Serendipitously, experimental conditions caused the normally complex initiation requirements for protein synthesis (unknown at the time) to be relaxed. Diligence combined with chance to produce a breakthrough—a common occurrence in the history of biochemistry.

In a random sequence of nucleotides, 1 in every 20 codons in each reading frame is, on average, a termination codon. In general, a reading frame without a termination codon among 50 or more consecutive codons is referred to as an **open reading frame (ORF)**. Long ORFs usually correspond to genes that encode proteins. In the analysis of sequence databases, sophisticated programs are used to search for ORFs in order to find genes among the often-huge background of nongenic DNA. An uninterrupted gene coding for a typical protein with a molecular weight of 60,000 would require an ORF with 500 or more codons.

A striking feature of the genetic code is that an amino acid may be specified by more than one codon, so the code is described as **degenerate**. This does *not* suggest that the code is flawed: although an amino acid may have two or more codons, each codon specifies only one amino acid. The degeneracy of the code is not uniform. Whereas methionine and tryptophan have single codons, for example, three amino acids (Arg, Leu, Ser) have six codons, five amino acids have four, isoleucine has three, and nine amino acids have two (**Table 27-3**).

The genetic code is nearly universal. With the intriguing exception of a few minor variations in mitochondria, some bacteria, and some single-celled eukaryotes, amino acid codons are identical in all species examined so far. Human beings, *E. coli*, tobacco plants, amphibians, and viruses share the same genetic code. This suggests that all life-forms have a common evolutionary ancestor, whose genetic code has been preserved throughout biological evolution. Even the variations (**Box 27-1**) reinforce this theme.

TABLE 27-3 Degeneracy of the Genetic Code

Amino acid	Number of codons	Amino acid	Number of codons
Met	1	Tyr	2
Trp	1	Ile	3
Asn	2	Ala	4
Asp	2	Gly	4
Cys	2	Pro	4
Gln	2	Thr	4
Glu	2	Val	4
His	2	Arg	6
Lys	2	Leu	6
Phe	2	Ser	6

BOX 27-1

Exceptions That Prove the Rule: Natural Variations in the Genetic Code

In biochemistry, as in other disciplines, exceptions to general rules can be problematic for instructors and frustrating for students. At the same time, though, they teach us that life is complex and they inspire us to search for more surprises. Understanding the exceptions can even reinforce the original rule in unpredictable ways.

One would expect little room for variation in the genetic code. Even a single amino acid substitution can have profoundly deleterious effects on the structure of a protein. Nevertheless, variations in the code do occur in some organisms, and they are both interesting and instructive. The types of variation and their rarity provide powerful evidence for a common evolutionary origin of all living things.

To alter the code, changes must occur in the gene(s) encoding one or more tRNAs, with the obvious target for alteration being the anticodon. Such a change would lead to the systematic insertion of an amino acid at a codon that, according to the standard code (see Fig. 27-7), does not specify that amino acid. The genetic code, in effect, is defined by two elements: (1) the anticodons on tRNAs, which determine where an amino acid is placed in a growing polypeptide, and (2) the specificity of the enzymes — the aminoacyl-tRNA synthetases — that charge the tRNAs, which determines the identity of the amino acid attached to a given tRNA.

Most sudden changes in the code would have catastrophic effects on cellular proteins, so code alterations are more likely to persist where relatively few proteins would be affected — such as in small genomes encoding only a few proteins. The biological consequences of a code change could also be limited by restricting changes to the three termination codons, which do not generally occur *within* genes. This pattern is, in fact, observed.

Of the very few variations in the genetic code that we know of, most occur in mitochondrial DNA (mtDNA), which encodes only 10 to 20 proteins. Mitochondria have their own tRNAs, so their code variations do not affect the much larger cellular genome. The most common changes in mitochondria involve termination codons. These changes affect termination in the products of only a subset of genes, and sometimes the effects are minor, because the genes have multiple (redundant) termination codons.

Vertebrate mtDNAs have genes that encode 13 proteins, 2 rRNAs, and 22 tRNAs (see Fig. 19-40). Given the small number of codon reassignments, along with an unusual set of wobble rules (p. 1012), the 22 tRNAs are sufficient to decode the protein-coding genes, as opposed to the 32 tRNAs required for the standard code. In mitochondria, these changes can be viewed as a kind of genomic streamlining, as a smaller genome confers a replication advantage on the organelle. Four codon families (in which the amino acid is determined entirely by the first two nucleotides) are decoded by a single tRNA with a U residue in the first (or wobble) position in the anticodon. Either the U pairs somehow with any of the four possible bases in the third position of the codon or a "two out of three" mechanism is used — that is, no base pairing is needed at the third position. Other tRNAs recognize codons with either A or G in the third position, and yet others recognize U or C, so that virtually all the tRNAs recognize either two or four codons.

In the standard code, only two amino acids are specified by single codons: methionine and tryptophan (see Table 27-3). If all mitochondrial tRNAs recognize two codons, we would expect additional Met and Trp codons in mitochondria. And we find that the single most common code variation is UGA, usually a termination codon, specifying tryptophan. The tRNA^{Trp} recognizes and inserts a Trp residue at either UGA or the usual Trp codon, UGG. The second most common variation is conversion of AUA from an Ile codon to a Met codon; the usual Met codon is AUG, and a single tRNA recognizes both codons. The known coding variations in mitochondria are summarized in Table 1.

Turning to the much rarer changes in the codes for cellular (as distinct from mitochondrial) genomes, we find that the only known variation in a bacterium is again the use of UGA to encode Trp residues, occurring in the simplest free-living cell, *Mycoplasma capricolum*. Among eukaryotes, rare extramitochondrial coding changes occur in a few species of ciliated protists, in which both termination codons UAA and UAG can specify glutamine. There are also rare but interesting cases in which stop codons have been adapted to encode amino acids that are not among the standard 20, as detailed in Box 27-2.

Changes in the code need not be absolute; a codon might not always encode the same amino acid. For example, in many bacteria — including *E. coli* — GUG (Val) is sometimes used as an initiation codon that specifies Met. This occurs only for those genes in which the GUG is properly located relative to particular mRNA sequences that affect the initiation of translation (as discussed in Section 27.2).

Wobble Allows Some tRNAs to Recognize More than One Codon

When several different codons specify one amino acid, the difference between them usually lies at the third base position (at the 3′ end). For example, alanine is encoded by the triplets GCU, GCC, GCA, and GCG. The codons for most amino acids can be symbolized by XY$^{A}_{G}$ or XY$^{U}_{C}$. The first two letters of each codon are the primary determinants of specificity, a feature that has some interesting consequences.

P3 Transfer RNAs base-pair with mRNA codons at a three-base sequence on the tRNA called the **anticodon**.

TABLE 1 **Known Variant Codon Assignments in Mitochondria**

	Codons[a]				
	UGA	AUA	AGA AGG	CUN	CGG
Normal (cellular) code assignment	Stop	Ile	Arg	Leu	Arg
Animals					
Vertebrates	Trp	Met	Stop	+	+
Drosophila	Trp	Met	Ser	+	+
Yeasts					
Saccharomyces cerevisiae	Trp	Met	+	Thr	+
Torulopsis glabrata	Trp	Met	+	Thr	?
Schizosaccharomyces pombe	Trp	+	+	+	+
Filamentous fungi	Trp	+	+	+	+
Trypanosomes	Trp	+	+	+	+
Higher plants	+	+	+	+	Trp
Chlamydomonas reinhardtii	?	+	+	+	?

[a] N indicates any nucleotide; + indicates that a codon has the same meaning as in the cellular code; ? indicates that a codon was not observed in this mitochondrial genome.

The most surprising alteration in the genetic code occurs in some fungal species of the genus *Candida*, as originally discovered for *Candida albicans*. *C. albicans* is an organism of high genomic complexity, yet its genetic code has undergone a dramatic change: the CUG codon, which usually encodes Leu residues, encodes Ser instead. The natural selection pressure for this change is completely unknown. Furthermore, Ser and Leu are quite different in chemical structure. However, even this change can be understood based on the properties of a universal code. When several codons encode the same amino acid and use multiple tRNAs, not all of the codons are used with equal frequency. In a phenomenon called **codon bias**, some codons for a particular amino acid are used more frequently (sometimes much more frequently) than others. The tRNAs for the frequently used codons are often present at much higher concentrations than the tRNAs required for the rarely used codons. Code degeneracy leads to the presence of six codons for Leu. In bacteria, CUG often encodes Leu. However, in fungi of genera that are very closely related to *Candida* but do not have the coding change, CUG only rarely encodes Leu and is often entirely absent in highly expressed proteins. A change in the coding sense of CUG would thus have a much smaller effect on fungal cell metabolism than might be expected if all codons were used equally. The coding change may have occurred by a gradual loss of CUG codons in genes and of the tRNA that recognizes CUG as a Leu codon, followed by a capture event — a mutation in the anticodon of a tRNA^{Ser} that allowed it to recognize CUG. Alternatively, there may have been an intermediate stage in which CUG was recognized as encoding both Leu and Ser, perhaps with contextual signals in the mRNAs that helped one tRNA or another recognize specific CUG codons (see Box 27-2). Phylogenetic analysis indicates that the reassignment of CUG as a Ser codon occurred in *Candida* ancestors about 150 million to 170 million years ago.

These variations tell us that the code is not quite as universal as once believed, but that its flexibility is severely constrained. The variations are obviously derivatives of the cellular code, and no example of a completely different code has been found. The limited scope of code variants strengthens the principle that all life on this planet evolved on the basis of a single (slightly flexible) genetic code.

The first base of the codon in mRNA (read in the 5′→3′ direction) pairs with the third base of the anticodon (**Fig. 27-8a**). If the anticodon triplet of a tRNA recognized only one codon triplet through Watson-Crick base pairing at all three positions, cells would have a different tRNA for each amino acid codon. This is not the case, however, because the anticodons in some tRNAs include the nucleotide inosinate (designated I), which contains the uncommon base hypoxanthine (see Fig. 8-5b). Inosinate can form hydrogen bonds with three different nucleotides — A, U, and C (Fig. 27-8b) — although these pairings are much weaker than the hydrogen bonds of Watson-Crick base pairs G≡C and A=U. In yeast, one tRNA^{Arg} has the anticodon (5′)ICG, which recognizes three arginine codons: (5′)CGA, (5′)CGU, and (5′)CGC. The first two bases are identical (CG) and form strong Watson-Crick base pairs with the corresponding bases of the anticodon, but the third base (A, U, or C) forms rather weak hydrogen bonds with the I residue at the first position of the anticodon.

Examination of these and other codon-anticodon pairings led Crick to conclude that the third base of most codons pairs rather loosely with the corresponding base of its anticodon; to use his picturesque word, the third base of such codons (and the first base of their corresponding anticodons) "wobbles." Crick proposed a set of four relationships called the **wobble hypothesis**:

1. The first two bases of an mRNA codon always form strong Watson-Crick base pairs with the corresponding bases of the tRNA anticodon and confer most of the coding specificity.
2. The first base of the anticodon (reading in the 5′→3′ direction; this pairs with the third base of the codon) determines the number of codons recognized by the tRNA. When the first base of the anticodon is C or A, base pairing is specific and only one codon is recognized by that tRNA. When the first base is U or G, binding is less specific and two different codons may be read. When inosine (I) is the first (wobble) nucleotide of an anticodon, three different codons can be recognized—the maximum number for any tRNA. These relationships are summarized in **Table 27-4**.
3. When an amino acid is specified by several different codons, the codons that differ in either of the first two bases require different tRNAs.
4. A minimum of 32 tRNAs are required to translate all 61 codons (31 to encode the amino acids, 1 for initiation).

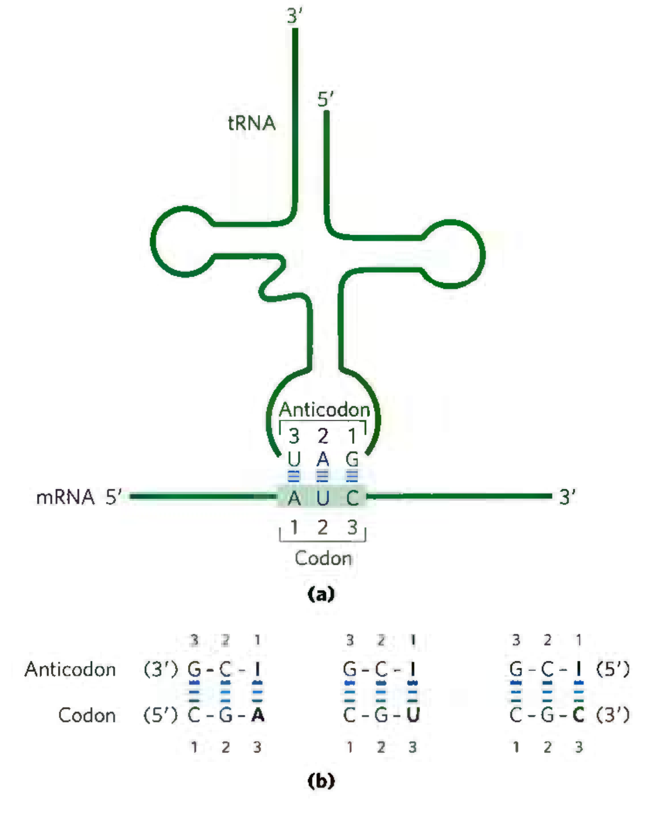


FIGURE 27-8 Pairing relationship of codon and anticodon. (a) Alignment of the two RNAs is antiparallel. The tRNA is shown in the traditional cloverleaf configuration. (b) Three different codon pairing relationships are possible when the tRNA anticodon contains inosinate.

The wobble (or third) base of the codon contributes to specificity, but because it pairs only loosely with its corresponding base in the anticodon, it permits rapid dissociation of the tRNA from its codon during protein synthesis. If all three bases of a codon engaged in strong Watson-Crick pairing with the three bases of the anticodon, tRNAs would dissociate too slowly and this would limit the rate of protein synthesis. Codon-anticodon interactions balance the requirements for accuracy and speed.

P3 Although only 32 tRNAs are required to translate all codons, most cells have more than that. The bacterium *E. coli* has 47 different tRNA genes. Many of these are present in multiple copies, such that there are 86 total tRNA genes in the *E. coli* genome.

The Genetic Code Is Mutation-Resistant

The genetic code plays an interesting role in safeguarding the genomic integrity of every living organism. Evolution did not produce a code in which codon assignments appeared at random. Instead, the code is strikingly resistant to the deleterious effects of the most common kinds

TABLE 27-4 How the Wobble Base of the Anticodon Determines the Number of Codons a tRNA Can Recognize

1. One codon recognized:			
	Anticodon	(3′) X—Y—**C** (5′)	(3′) X—Y—**A** (5′)
	Codon	(5′) X′—Y′—**G** (3′)	(5′) X′—Y′—**U** (3′)
2. Two codons recognized:			
	Anticodon	(3′) X—Y—**U** (5′)	(3′) X—Y—**G** (5′)
	Codon	(5′) X′—Y′—$\substack{A\\G}$ (3′)	(5′) X′—Y′—$\substack{C\\U}$ (3′)
3. Three codons recognized:			
	Anticodon	(3′) X—Y—**I** (5′)	
	Codon	(5′) X′—Y′—$\substack{A\\U\\C}$ (3′)	

Note: X and Y denote bases complementary to and capable of strong Watson-Crick base pairing with X′ and Y′, respectively. Wobble bases—in the 3′ position of codons and 5′ position of anticodons—are shaded.

of mutations—**missense mutations**, in which a single new base pair replaces another. In the third, or wobble, position of the codon, single base substitutions produce a change in the encoded amino acid only about 25% of the time. Most such changes are thus **silent mutations**, in which the nucleotide is different but the encoded amino acid remains the same.

Due to the types of spontaneous DNA damage that affect genomes (see Chapter 8), the most frequent missense mutation is a **transition mutation**, in which a purine is replaced by a purine, or a pyrimidine by a pyrimidine (for example, G≡C changed to A=T). All three codon positions have evolved so that there is some resistance to transition mutations. A mutation in the first position of the codon will usually produce an amino acid coding change, but the change often results in an amino acid with similar chemical properties. This is especially true for the hydrophobic amino acids that dominate the first column of the code shown in Figure 27-7. Consider the Val codon GUU. A change to AUU would substitute Ile for Val. A change to CUU would replace Val with Leu. The resulting changes in the structure and/or function of the protein encoded by that gene would often (but not always) be small.

P2 Computational studies have shown that alternative genetic codes, delineated at random, are almost always less resistant to mutation than the existing code. The results indicate that the code underwent considerable streamlining before the appearance of LUCA, the ancestral cell.

The genetic code tells us how protein sequence information is stored in nucleic acids and provides some clues about how that information is translated into protein.

Translational Frameshifting Affects How the Code Is Read

Once the reading frame has been set during protein synthesis, codons are translated without overlap or punctuation until the ribosomal complex encounters a termination codon. The other two possible reading frames usually contain no useful genetic information. Overlap between two genes would necessarily constrain the possible amino acid sequences encoded by one or both genes in the overlap region. However, to make maximal use of limited (and expensive) genetic information, a few genes are structured so that ribosomes "hiccup" at a certain point in the translation of their mRNAs, changing the reading frame from that point on. This allows two or more related but distinct proteins to be produced from a single transcript.

One of the best-documented examples of **translational frameshifting** occurs during translation of the mRNA for the overlapping *gag* and *pol* genes of the Rous sarcoma virus, a retrovirus (see Fig. 26-31). The reading frame for *pol* is offset to the left by one base pair (−1 reading frame) relative to the reading frame for *gag* (**Fig. 27-9**).

The product of the retroviral *pol* gene (reverse transcriptase) is translated as a larger polyprotein, on the same mRNA that is used for the Gag protein alone (see Fig. 26-30). The polyprotein, or Gag-Pol protein, is then trimmed to the mature reverse transcriptase by proteolytic digestion. Production of the polyprotein requires a translational frameshift in the overlap region to allow the ribosome to bypass the UAG termination codon at the end of the *gag* gene (shaded light red in Fig. 27-9).

Frameshifts occur during about 5% of translations of this mRNA, and the Gag-Pol polyprotein (and ultimately reverse transcriptase) is synthesized at about one-twentieth the frequency of the Gag protein, a level that suffices for efficient reproduction of the virus. A similar mechanism produces both the τ and γ subunits of *E. coli* DNA polymerase III from a single *dnaX* gene transcript (see footnote to Table 25-2).

Some mRNAs Are Edited before Translation

RNA editing can involve the addition, deletion, or alteration of nucleotides in the RNA in a manner that affects the meaning of the transcript when it is translated. Addition or deletion of nucleotides has been most commonly observed in RNAs originating from the mitochondrial and chloroplast genomes. The initial transcripts of the genes that encode cytochrome oxidase subunit II in some protist mitochondria provide an example of editing by insertion. These transcripts do not correspond precisely to the sequence needed at the carboxyl terminus of the protein product. A posttranscriptional editing process inserts four U residues that shift the translational reading frame of the transcript. The insertions require a special class of **guide RNAs (gRNAs**; **Fig. 27-10**) that act as templates for the editing process. The added U residues are all located in a small part of the transcript. Note that the base pairing between the initial transcript and the guide RNA includes several G=U base pairs (blue dots), which are common in RNA molecules.

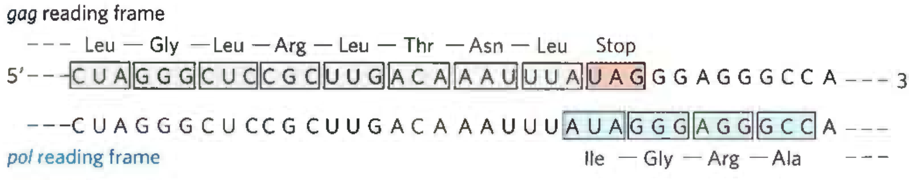

gag reading frame
--- Leu — Gly — Leu — Arg — Leu — Thr — Asn — Leu Stop
5′---C U A G G G C U C C G C U U G A C A A A U U U A U A G G G A G G G C C A --- 3′
---C U A G G G C U C C G C U U G A C A A A U U U A U A G G G A G G G C C A ---
pol reading frame
Ile — Gly — Arg — Ala ---

FIGURE 27-9 Translational frameshifting in a retroviral transcript. The *gag-pol* overlap region in Rous sarcoma virus RNA is shown.

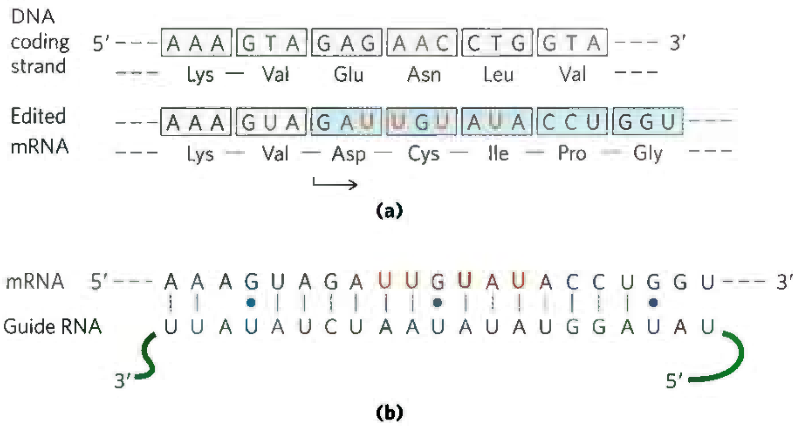

DNA coding strand
5′--- A A A G T A G A G A A C C T G G T A --- 3′
--- Lys — Val Glu Asn Leu Val ---
Edited mRNA
--- A A A G U A G A U U G U A U A C C U G G U ---
--- Lys — Val — Asp — Cys — Ile — Pro — Gly ---
(a)
mRNA 5′--- A A A G U A G A U U G U A U A C C U G G U --- 3′
Guide RNA U U A U A U C U A A U A U A U G G A U A U
3′
5′
(b)

FIGURE 27-10 RNA editing of the transcript of the cytochrome oxidase subunit II gene from *Trypanosoma brucei* mitochondria. (a) Insertion of four U residues (red) produces a revised reading frame. (b) A special class of guide RNAs, complementary to the edited product, acts as templates for the editing process. Note the presence of three G=U base pairs, signified by blue dots to indicate non-Watson-Crick pairing.

RNA editing by alteration of nucleotides most commonly involves the enzymatic deamination of adenosine or cytidine residues, forming inosine or uridine, respectively (**Fig. 27-11**), although other base changes have been described. Inosine is interpreted as a G residue during translation. The adenosine deamination reactions are carried out by *a*denosine *d*eaminases that *a*ct on *R*NA **(ADARs)**. The cytidine deaminations are carried out by the *apoB* mRNA *e*diting *c*atalytic peptide **(APOBEC)** family of enzymes, which includes the *a*ctivation-*i*nduced *d*eaminase **(AID)** enzymes. Both the ADAR and APOBEC groups of deaminase enzymes have a homologous zinc-coordinating catalytic domain.

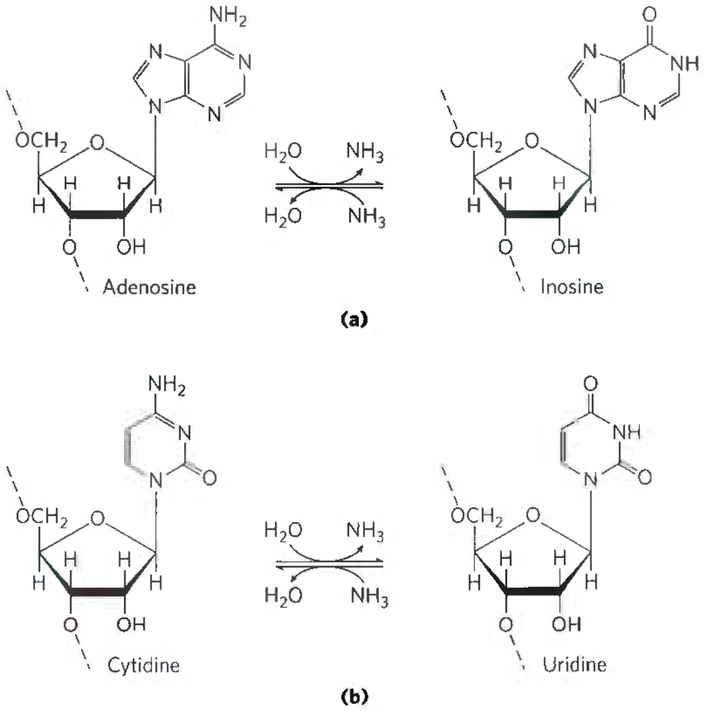


FIGURE 27-11 Deamination reactions that result in RNA editing. (a) Conversion of adenosine nucleotides to inosine nucleotides is catalyzed by ADAR enzymes. (b) Cytidine-to-uridine conversions are catalyzed by the APOBEC family of enzymes.

The ADAR-promoted A-to-I editing of RNA transcripts is particularly common in primates. Most of the editing occurs in Alu elements, a subset of short interspersed elements (SINEs), eukaryotic transposons that are particularly common in primate genomes. Human DNA contains more than a million 300 bp Alu elements, making up about 10% of the genome. These elements are concentrated near protein-coding genes, often in introns and untranslated regions at the 3′ and 5′ ends of transcripts. When it is first synthesized (before processing), the *average* human mRNA includes 10 to 20 Alu elements. Certain microRNAs are also targeted by ADARs. The microRNA (miRNA) alterations generally reduce expression and/or function.

The ADAR enzymes bind to and promote A-to-I editing only in duplex regions of RNA. The abundant Alu elements offer many opportunities for intramolecular base pairing within the transcripts, providing the duplex targets required by ADARs. Some of the editing affects the coding sequences of genes. Defects in ADAR function have been associated with a variety of human neurological conditions, including amyotrophic lateral sclerosis (ALS), epilepsy, and major depression.

There are six general classes of APOBEC cytidine deaminases: APOBEC1–APOBEC5 and AID. The AID proteins function in increasing antibody diversity during immunoglobulin gene maturation (see Figs. 25-42 and 25-43). APOBEC1 and some of the APOBEC3 proteins (seven APOBEC paralogs are encoded by the human genome) edit mRNAs. A well-studied example of RNA editing by APOBEC1-mediated deamination occurs in the gene for the apolipoprotein B component of low-density lipoprotein in vertebrates. One form of apolipoprotein B, apoB-100 (M_r 513,000), is synthesized in the liver; a second form, apoB-48 (M_r 250,000), is synthesized in the intestine. Both are encoded by an mRNA produced from the gene for apoB-100. An APOBEC cytidine deaminase found only in the intestine binds to the mRNA at the codon for amino acid residue 2,153 (CAA=Gln) and converts the C to a U to create the termination codon UAA. The apoB-48 produced in the intestine from this modified

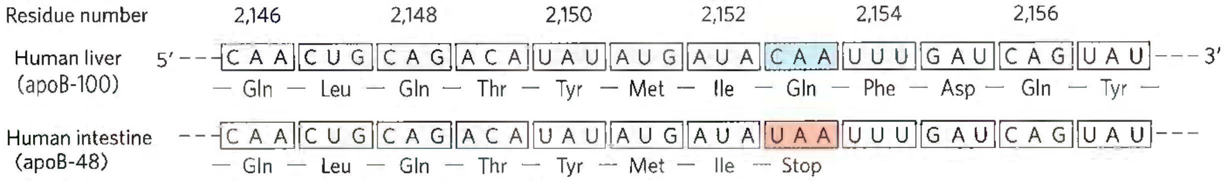

Residue number
2,146
2,148
2,150
2,152
2,154
2,156
Human liver (apoB-100)
5′---CAA CUG CAG ACA UAU AUG AUA CAA UUU GAU CAG UAU---3′
— Gln — Leu — Gln — Thr — Tyr — Met — Ile — Gln — Phe — Asp — Gln — Tyr —
Human intestine (apoB-48)
---CAA CUG CAG ACA UAU AUG AUA UAA UUU GAU CAG UAU---
— Gln — Leu — Gln — Thr — Tyr — Met — Ile — Stop

FIGURE 27-12 RNA editing of the transcript of the gene for the apoB-100 component of LDL. Deamination, which occurs only in the intestine, converts a specific cytidine to uridine, changing a Gln codon to a stop codon and producing a truncated protein.

mRNA is simply an abbreviated form (corresponding to the amino-terminal half) of apoB-100 (**Fig. 27-12**). **P1** This reaction permits tissue-specific synthesis of two different proteins from one gene.

APOBEC2, 4, and 5 act on DNA rather than RNA, and their functions are poorly understood. However, their ability to cause genomic mutations can make them a liability to the cell. One or more APOBEC enzymes are often overexpressed in tumor cells, and their mutagenic ability can contribute to the formation of tumors. They also provide a mechanism for introducing multiple mutations into a targeted segment of a chromosome, leading to selective and more rapid evolution of that DNA region.

SUMMARY 27.1 *The Genetic Code*

- The particular amino acid sequence of a protein is constructed through the translation of information encoded in mRNA. This process is carried out by ribosomes. Amino acids are specified by mRNA codons consisting of nucleotide triplets. Translation requires adaptor molecules, the tRNAs, that recognize codons and insert amino acids into their appropriate sequential positions in the polypeptide.
- The base sequences of the codons were deduced from experiments using synthetic mRNAs of known composition and sequence. The codon AUG signals initiation of translation. The triplets UAA, UAG, and UGA are signals for termination. The genetic code is degenerate: it has multiple codons for almost every amino acid. The standard genetic code is universal in all species, with some minor deviations in mitochondria and a few single-celled organisms. The deviations occur in a pattern that reinforces the concept of a universal code.
- The third position in each codon is much less specific than the first and second positions and is said to wobble. This property allows certain tRNAs to recognize more than one codon.
- The genetic code is resistant to the effects of missense mutations. The code evolved so that many nucleotide changes in a DNA codon do not alter the encoded amino acid, or they result in a very conservative alteration.
- Translational frameshifting and RNA editing affect how the genetic code is read during translation.
- RNA editing by ADARs (adenosine deaminases) and APOBECs (cytidine deaminases) also alters the coding sequence of some mRNAs. Many APOBEC enzymes target DNA, where they function in facilitating antibody diversity and suppression of retroviruses and retrotransposons.

27.2 Protein Synthesis

As we have seen for DNA and RNA (Chapters 25 and 26), the synthesis of polymeric biomolecules can be considered in terms of initiation, elongation, and termination stages. These fundamental processes are typically bracketed by two additional stages: activation of precursors before synthesis and postsynthetic processing of the completed polymer. Protein synthesis follows the same pattern. The activation of amino acids before their incorporation into polypeptides and the posttranslational processing of the completed polypeptide play particularly important roles in ensuring both the fidelity of synthesis and the proper function of the protein product. The process is outlined in **Figure 27-13**. **P1** The cellular components involved in the five stages of protein synthesis in *E. coli* and other bacteria are listed in **Table 27-5**; the requirements in eukaryotic cells are similar, although the components are usually more numerous. Before looking at these five stages in detail, we must examine two key components of protein biosynthesis: the ribosome and tRNAs.

The Ribosome Is a Complex Supramolecular Machine

P1 Each *E. coli* cell contains 15,000 or more ribosomes, which comprise nearly a quarter of the dry weight of the cell. Bacterial ribosomes contain about 65% rRNA and 35% protein; they have a diameter of about 18 nm and are composed of two unequal subunits with sedimentation coefficients of 30S and 50S and a combined sedimentation coefficient of 70S. Both subunits contain dozens of ribosomal proteins (r-proteins) and at least one large rRNA (**Table 27-6**).

As it became clear that ribosomes are the complexes responsible for protein synthesis, and following elucidation of the genetic code, the study of ribosomes accelerated. In the late 1960s Masayasu Nomura and colleagues demonstrated that both ribosomal subunits can be broken down into their RNA and protein components, then reconstituted in vitro. Ribosomal subunits are identified by their S (Svedberg unit) values, sedimentation coefficients that refer to their rate of sedimentation in a centrifuge.

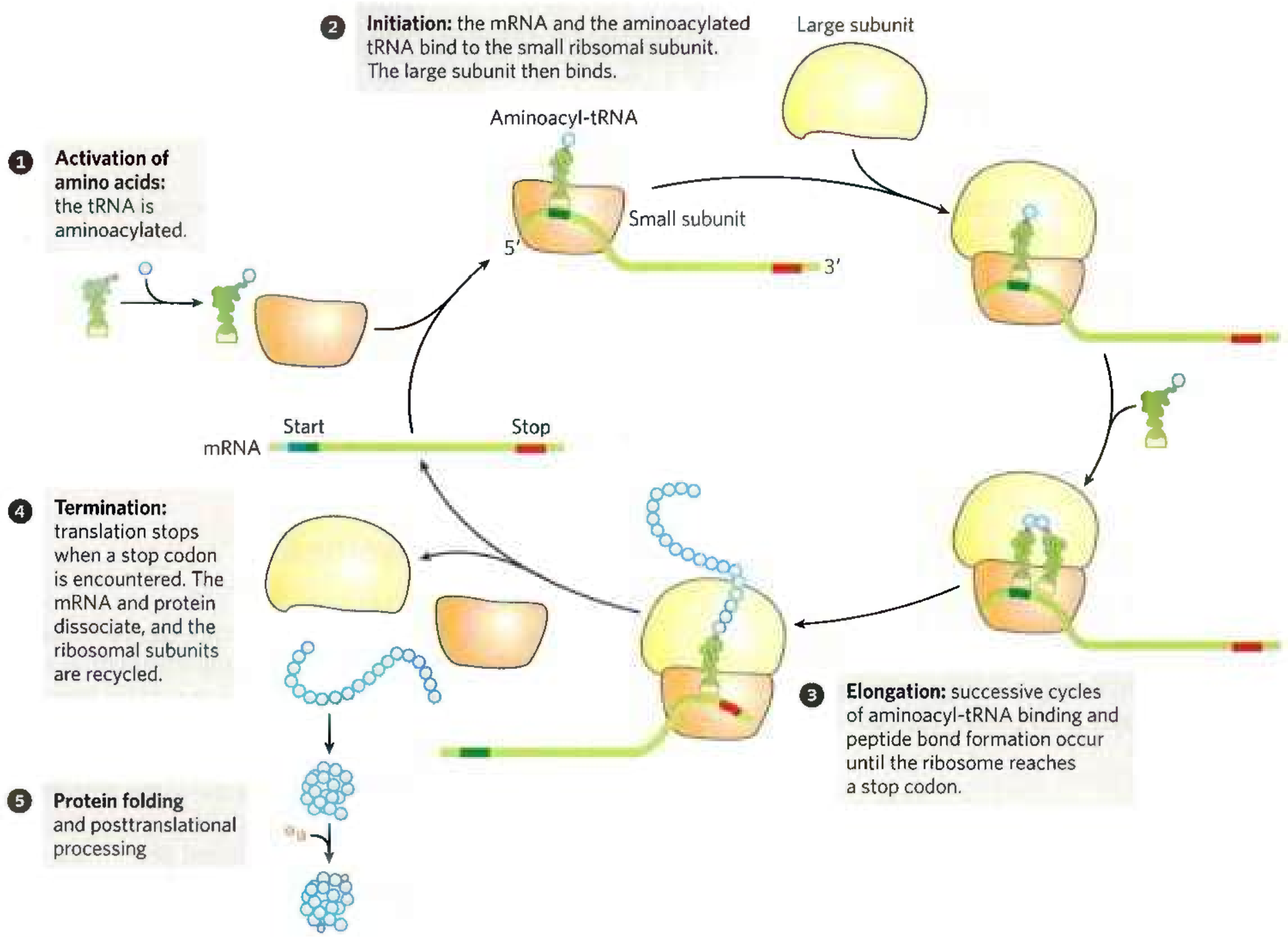

② Initiation: the mRNA and the aminoacylated tRNA bind to the small ribosomal subunit. The large subunit then binds.
Large subunit
Aminoacyl-tRNA
① Activation of amino acids: the tRNA is aminoacylated.
Small subunit
5′
3′
Start
Stop
mRNA
④ Termination: translation stops when a stop codon is encountered. The mRNA and protein dissociate, and the ribosomal subunits are recycled.
③ Elongation: successive cycles of aminoacyl-tRNA binding and peptide bond formation occur until the ribosome reaches a stop codon.
⑤ Protein folding and posttranslational processing

FIGURE 27-13 An overview of the five stages of protein synthesis.

TABLE 27-5 Components Required for the Five Major Stages of Protein Synthesis in *E. coli*

Stage	Essential components	
1. Activation of amino acids	20 amino acids 20 aminoacyl-tRNA synthases 32 or more tRNAs	ATP Mg^{2+}
2. Initiation	mRNA *N*-Formylmethionyl-$tRNA^{fMet}$ Initiation codon in mRNA (AUG) 30S ribosomal subunit	50S ribosomal subunit Initiation factors (IF1, IF2, IF3) GTP Mg^{2+}
3. Elongation	Functional 70S ribosomes (initiation complex) Aminoacyl-tRNAs specified by codons	Elongation factors (EF-Tu, EF-Ts, EF-G) GTP Mg^{2+}
4. Termination and ribosome recycling	Termination codon in mRNA Release factors (RF1, RF2, RF3, RRF)	EF-G IF3
5. Folding and posttranslational processing	Chaperones and folding enzymes (PPI, PDI); specific enzymes, cofactors, and other components for removal of initiating residues and signal sequences, additional proteolytic processing, modification of terminal residues, and attachment of acetyl, phosphoryl, methyl, carboxyl, carbohydrate, or prosthetic groups	

Under appropriate experimental conditions, the RNA and protein spontaneously reassemble to form 30S or 50S subunits nearly identical in structure and activity to native subunits. This breakthrough fueled decades of research into the function and structure of ribosomal RNAs and proteins. At the same time, increasingly sophisticated structural methods revealed more and more details about ribosome structure.

TABLE 27-6 **RNA and Protein Components of the *E. coli* Ribosome**

Subunit	Number of different proteins	Total number of proteins	Protein designations	Number and type of rRNAs
30S	21	21	S1–S21	1 (16S rRNA)
50S	33	36	L1–L36[a]	2 (5S and 23S rRNAs)

[a] The L1 to L36 protein designations do not correspond to 36 different proteins. The protein originally designated L7 is a modified form of L12, and L8 is a complex of three other proteins. Also, L26 proved to be the same protein as S20 (and not part of the 50S subunit). This gives 33 different proteins in the large subunit. There are four copies of the L7/L12 protein, with the three extra copies bringing the total protein count to 36.

The dawn of a new millennium illuminated the first high-resolution structures of bacterial ribosomal subunits by Venkatraman Ramakrishnan, Thomas Steitz, Ada Yonath, Harry Noller, and others. This work yielded a wealth of surprises (**Fig. 27-14a**). First, a traditional focus on the protein components of ribosomes was shifted. The ribosomal subunits are huge RNA molecules. In the 50S subunit, the 5S and 23S rRNAs form the structural core. The proteins are secondary elements in the complex, decorating the surface. Second, and most important, there is no protein within 18 Å of the active site for peptide bond formation. **P4** The high-resolution structure thus confirms what Noller had predicted much earlier: the ribosome is a ribozyme. In addition to the insight that the detailed structures of the ribosome and its subunits provide into the mechanism of protein synthesis (as elaborated below), these findings have stimulated a new look at the evolution of life (Section 26.4). The ribosomes of eukaryotic cells have also yielded to structural analysis (Fig. 27-14b).

The bacterial ribosome is complex, with a combined molecular weight of ~2.7 million. The two irregularly shaped ribosomal subunits fit together to form a cleft through which the mRNA passes as the ribosome moves along it during translation (Fig. 27-14a). The 57 proteins in bacterial ribosomes vary enormously in size and structure. Molecular weights range from about 6,000 to 75,000. Most of the proteins have globular domains arranged on the ribosome surface. Some also have snakelike extensions that protrude into the rRNA core of the ribosome, stabilizing its structure. The functions of some of these proteins have not yet been elucidated in detail, although a structural role seems evident for many of them.

The sequences of the rRNAs of countless thousands of organisms are now known due to genomic sequencing. Each of the three single-stranded rRNAs of *E. coli* has a specific three-dimensional conformation with extensive intrachain base pairing. The folding patterns of the rRNAs are highly conserved in all organisms, particularly the regions implicated in key functions (**Fig. 27-15**). The predicted secondary structure of the rRNAs has largely been confirmed by structural analysis but fails to convey the extensive network of tertiary interactions apparent in the complete structure.

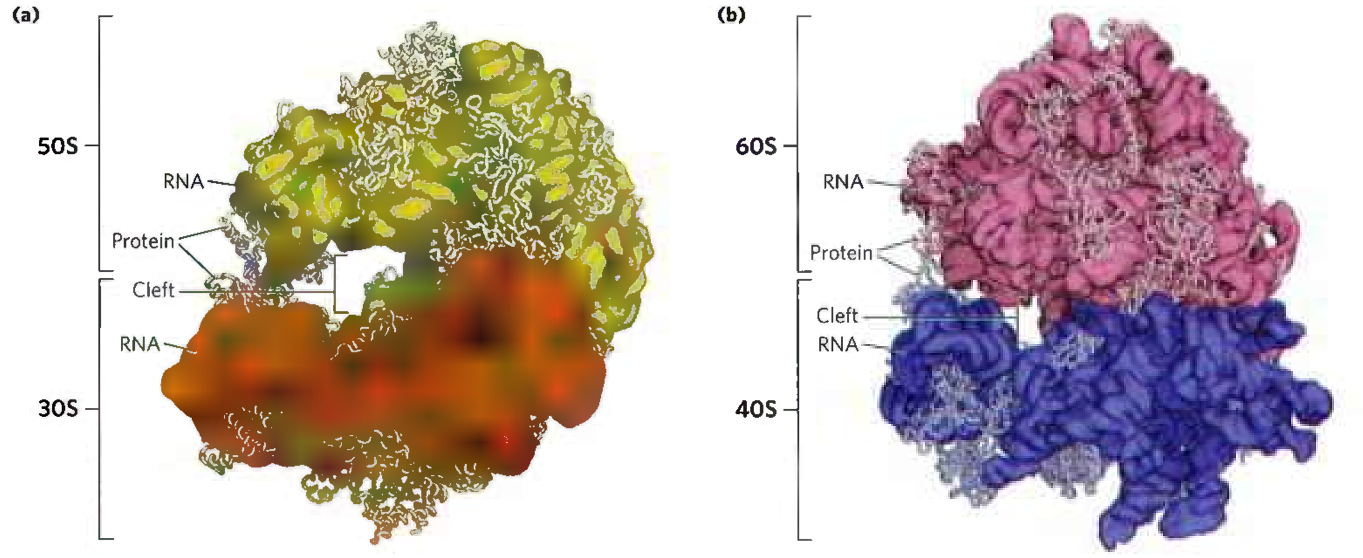


FIGURE 27-14 **The structure of ribosomes.** Our understanding of ribosome structure has been greatly enhanced by multiple high-resolution images of the ribosomes from bacteria and yeast. (a) The bacterial ribosome. The 50S and 30S subunits come together to form the 70S ribosome. The cleft between them is where protein synthesis occurs. (b) The yeast ribosome has a similar structure with somewhat increased complexity. [Data from (a) PDB ID 4V4I, A. Korostelev et al., *Cell* 126:1065, 2006; (b) PDB ID 4V7R, A. Ben-Shem et al., *Science* 330:1203, 2010.]

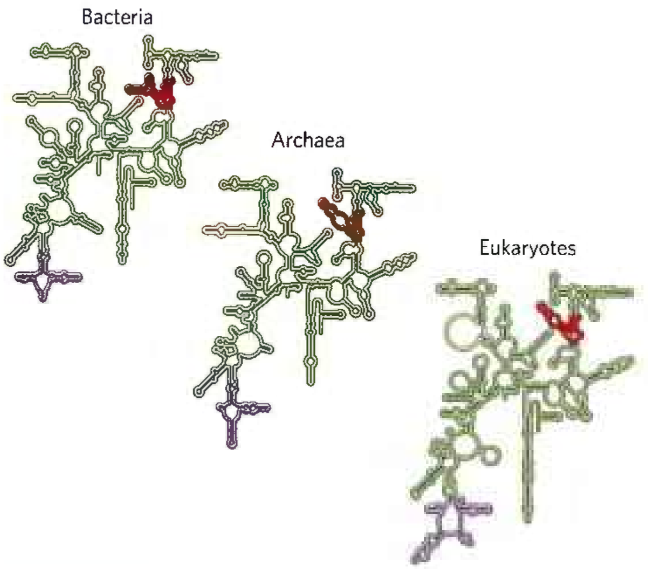

Bacteria
Archaea
Eukaryotes

FIGURE 27-15 Conservation of secondary structure in the small subunit rRNAs from the three domains of life. The red, yellow, and purple indicate areas where the structures of the rRNAs from bacteria, archaea, and eukaryotes have diverged. Conserved regions are shown in green. [Information originally from the Comparative RNA Web, University of Texas.]

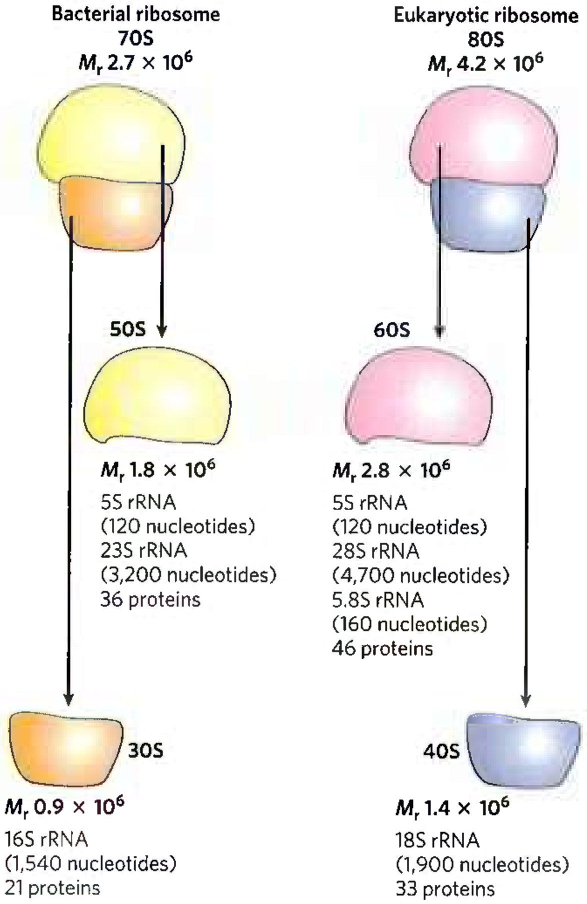

Bacterial ribosome
70S
M_r 2.7 × 10^6
Eukaryotic ribosome
80S
M_r 4.2 × 10^6
50S
60S
M_r 1.8 × 10^6
5S rRNA
(120 nucleotides)
23S rRNA
(3,200 nucleotides)
36 proteins
M_r 2.8 × 10^6
5S rRNA
(120 nucleotides)
28S rRNA
(4,700 nucleotides)
5.8S rRNA
(160 nucleotides)
46 proteins
30S
40S
M_r 0.9 × 10^6
16S rRNA
(1,540 nucleotides)
21 proteins
M_r 1.4 × 10^6
18S rRNA
(1,900 nucleotides)
33 proteins

FIGURE 27-16 Summary of the composition and mass of ribosomes in bacteria and eukaryotes. The S values associated with the subunits are not additive when subunits are combined, because S values are approximately proportional to the two-thirds power of molecular weight and are also slightly affected by shape.

The ribosomes of eukaryotic cells (other than mitochondrial and chloroplast ribosomes) are larger and more complex than bacterial ribosomes (**Fig. 27-16**; compare Fig. 27-14b), with a diameter of about 23 nm and a sedimentation coefficient of about 80S. They also have two subunits, which vary in size among species but on average are 60S and 40S. Altogether, a ribosome of the yeast *Saccharomyces cerevisiae* contains 79 different proteins and 4 ribosomal RNAs. The ribosomes of mitochondria and chloroplasts are somewhat smaller and simpler than bacterial ribosomes. Nevertheless, ribosomal structure and function are strikingly similar in all organisms and organelles.

In both bacteria and eukaryotes, ribosomes are assembled through a hierarchical incorporation of r-proteins as the rRNAs are synthesized. Much of the processing of pre-rRNAs occurs within large ribonucleoprotein complexes. The composition of these complexes changes as new r-proteins are added, the rRNAs acquire their final form, and some proteins required for rRNA processing dissociate. In eukaryotes, the early stages of assembly occur in the nucleolus, with the final maturation of the ribosome completed after export to the cytosol. P1 Dozens of assembly factors, both proteins and some small RNA molecules (snoRNAs; Fig. 26-24), participate in this process (**Fig. 27-17**).

Transfer RNAs Have Characteristic Structural Features

To understand how tRNAs can serve as adaptors in translating the language of nucleic acids into the language of proteins, we must first examine their structure in more detail. Transfer RNAs are relatively small and consist of a single strand of RNA folded into a precise three-dimensional structure (see Fig. 8-25a). The tRNAs of bacteria and in the cytosol of eukaryotes have between 73 and 93 nucleotide residues, corresponding to molecular weights of 24,000 to 31,000. Mitochondria and chloroplasts contain distinctive, somewhat smaller tRNAs. Cells have at least one kind of tRNA for each amino acid; at least 32 tRNAs are required to recognize all the amino acid codons (some recognize more than one codon), but some cells use more than 32.

Yeast alanine tRNA (tRNA^{Ala}) was the first nucleic acid to be completely sequenced, by Robert Holley in 1965. It contains 76 nucleotide residues, 10 of which have modified bases. Comparisons of tRNAs from various species have revealed many common structural features (**Fig. 27-18a**). Eight or more of the nucleotide residues have modified bases and sugars, many of which are methylated derivatives of the principal bases. Most tRNAs have a guanylate (pG) residue at the 5′ end, and all have the trinucleotide sequence CCA(3′) at the

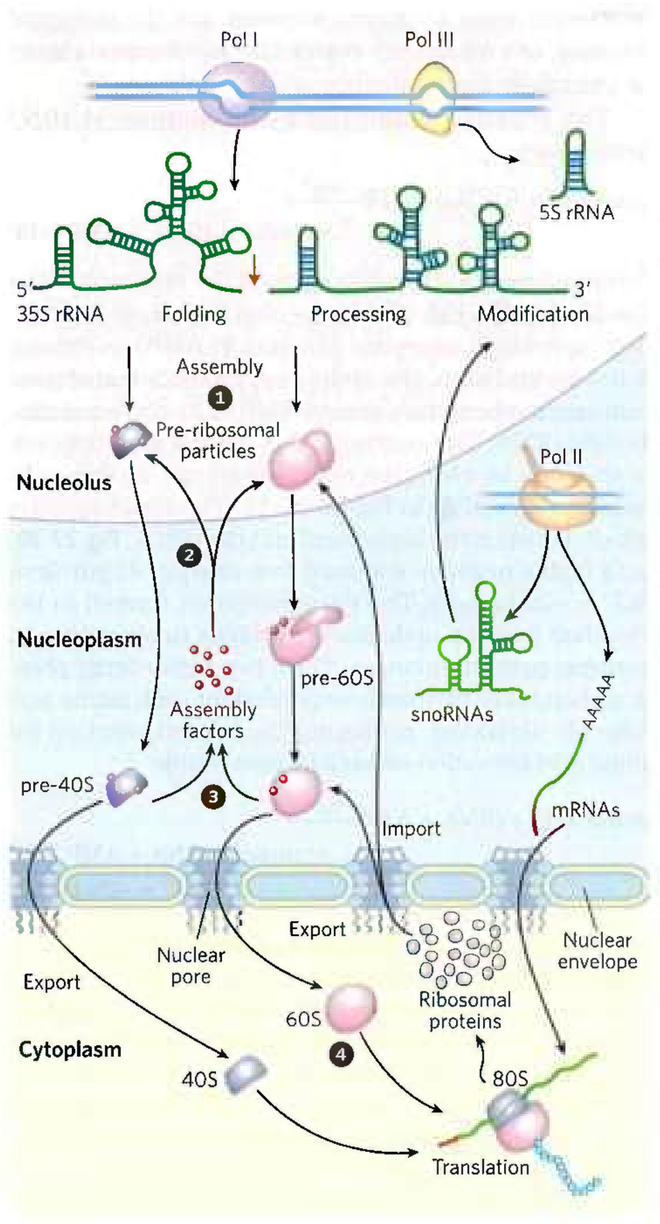
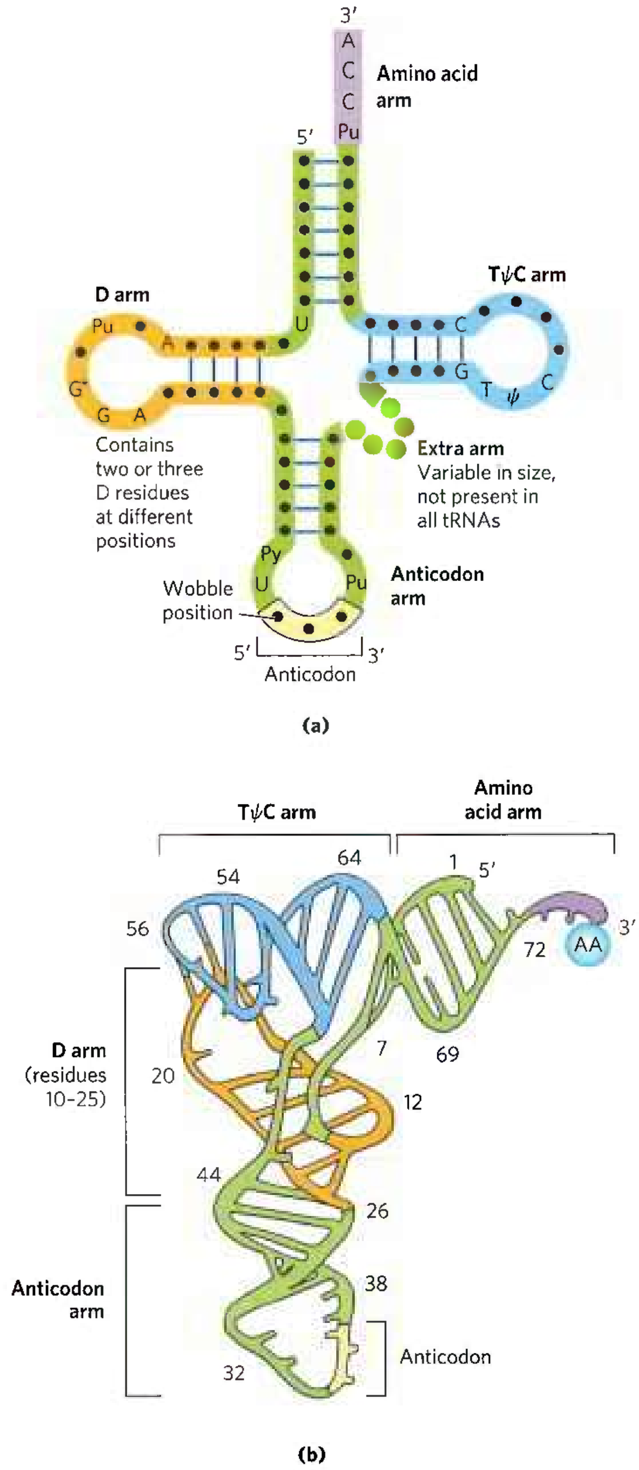

Pol I
Pol III
5S rRNA
5′
3′
35S rRNA
Folding
Processing
Modification
Assembly
❶
Pre-ribosomal particles
Nucleolus
❷
Pol II
Nucleoplasm
pre-60S
snoRNAs
Assembly factors
AAAAA
pre-40S
❸
mRNAs
Import
Export
Nuclear pore
Nuclear envelope
Export
Ribosomal proteins
60S
Cytoplasm
❹
80S
40S
Translation

FIGURE 27-17 Assembly of ribosomes in eukaryotes. ❶ Most of the early steps of ribosome assembly occur in the nucleolus, an organelle inside the nucleus. Ribonucleases and specialized RNAs, including some snoRNAs, process the initial rRNA transcript. ❷ Large pre-ribosomal particles are formed and additional processing of the large complex occurs with the aid of proteins called assembly factors. The pre-40S and pre-60S complexes move into the nucleoplasm. ❸ The 40S and 60S subunits are exported to the cytoplasm, coupled with the ejection of assembly factors. ❹ Final maturation of the ribosome occurs in the cytoplasm.

3′ end. When drawn in two dimensions, all tRNAs have a hydrogen-bonding pattern that forms a cloverleaf structure with four arms, as first proposed by Elizabeth Keller. The longer tRNAs have a short fifth arm, or extra arm. In three dimensions, a tRNA has the form of a twisted L (Fig. 27-18b).

P3 Two of the arms of a tRNA are critical for its adaptor function. The **amino acid arm** can carry a specific


3′
A
C
C
Pu
5′
Amino acid arm
D arm
Pu
A
U
TψC arm
C
G
T ψ
C
G*
G A
Contains two or three D residues at different positions
Extra arm Variable in size, not present in all tRNAs
Py
U
Pu
Wobble position
Anticodon arm
5′
3′
Anticodon
TψC arm
Amino acid arm
64
1
5′
54
56
3′
72
AA
D arm (residues 10-25)
7
69
20
12
44
26
Anticodon arm
38
Anticodon
32

(a)

(b)

FIGURE 27-18 General structure of tRNAs. (a) The cloverleaf structure. The large dots on the backbone represent nucleotide residues; the blue lines represent base pairs. Characteristic and/or invariant residues common to all tRNAs are specified. Transfer RNAs vary in length from 73 to 93 nucleotides. Extra nucleotides occur in the extra arm or in the D arm. At the end of the anticodon arm is the anticodon loop, which always contains seven unpaired nucleotides. The D arm contains two or three D (5,6-dihydrouridine) residues, depending on the tRNA. In some tRNAs, the D arm has only three hydrogen-bonded base pairs. Pu represents purine nucleotide; Py, pyrimidine nucleotide; ψ, pseudouridylate; G*, guanylate or 2′-*O*-methylguanylate. (b) Schematic diagram of the folded tRNA, which resembles a twisted L.

amino acid esterified by its carboxyl group to the 2′- or 3′-hydroxyl group of the A residue at the 3′ end of the tRNA. The **anticodon arm** contains the anticodon. The other major arms are the **D arm**, which contains the unusual nucleotide dihydrouridine (D), and the **TψC arm**, which contains ribothymidine (T), not usually present in RNAs, and pseudouridine (ψ), which has an unusual carbon–carbon bond between the base and ribose (see Fig. 26-22). The D and TψC arms contribute important interactions for the overall folding of tRNA molecules, and the TψC arm interacts with the large-subunit rRNA.

Having looked at the structures of ribosomes and tRNAs, we now consider in detail the five stages of protein synthesis.

Stage 1: Aminoacyl-tRNA Synthetases Attach the Correct Amino Acids to Their tRNAs

P3 For the synthesis of a polypeptide with a defined sequence, two fundamental chemical requirements must be met: (1) the carboxyl group of each amino acid must be activated to facilitate formation of a peptide bond, and (2) a link must be established between each new amino acid and the information in the mRNA that encodes it. Both these requirements are met by attaching the amino acid to a tRNA in the first stage of protein synthesis. When attached to their amino acid (aminoacylated), the tRNAs are said to be "charged."

This first stage of protein synthesis takes place in the cytosol. Aminoacyl-tRNA synthetases esterify the 20 amino acids to their corresponding tRNAs. Each enzyme is specific for one amino acid and one or more corresponding tRNAs. Most organisms have one aminoacyl-tRNA synthetase for each amino acid. For amino acids with two or more corresponding tRNAs, the same enzyme usually aminoacylates all of them.

In all organisms, the aminoacyl-tRNA synthetases fall into two classes (**Table 27-7**), based on substantial differences in primary and tertiary structure and in reaction mechanism (**Fig. 27-19**). There is no evidence that the two classes share a common ancestor, and the biological, chemical, or evolutionary reasons for two enzyme classes for essentially identical processes remain obscure.

TABLE 27-7 **The Two Classes of Aminoacyl-tRNA Synthetases**

Class I		Class II	
Arg	Leu	Ala	Lys
Cys	Met	Asn	Phe
Gln	Trp	Asp	Pro
Glu	Tyr	Gly	Ser
Ile	Val	His	Thr

Note: Here, Arg represents arginyl-tRNA synthetase, and so forth. The classification applies to all organisms for which tRNA synthetases have been analyzed and is based on protein structural distinctions and on the mechanistic distinction outlined in Figure 27-19.

The reaction catalyzed by an aminoacyl-tRNA synthetase is

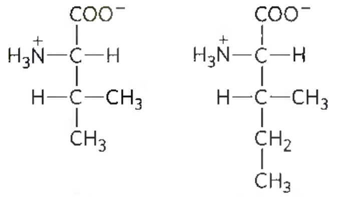
$$\text{Amino acid} + \text{tRNA} + \text{ATP} \xrightarrow{\text{Mg}^{2+}} \text{aminoacyl-tRNA} + \text{AMP} + \text{PP}_i$$

This reaction occurs in two steps in the enzyme's active site. In step ❶ (Fig. 27-19), an enzyme-bound intermediate, aminoacyl adenylate (aminoacyl-AMP), is formed. In the second step, the aminoacyl group is transferred from enzyme-bound aminoacyl-AMP to its corresponding specific tRNA. The course of this second step depends on the class to which the enzyme belongs, as shown by pathways (2a) and (2b) in Figure 27-19. The resulting ester linkage between the amino acid and the tRNA (**Fig. 27-20**) has a highly negative standard free energy of hydrolysis ($\Delta G'^\circ = -29$ kJ/mol). The pyrophosphate formed in the activation reaction undergoes hydrolysis to phosphate by inorganic pyrophosphatase. Thus, *two* high-energy phosphate bonds are ultimately expended for each amino acid molecule activated, rendering the overall reaction for amino acid activation essentially irreversible:

$$\text{Amino acid} + \text{tRNA} + \text{ATP} \xrightarrow{\text{Mg}^{2+}} \text{aminoacyl-tRNA} + \text{AMP} + 2\text{P}_i \qquad \Delta G'^\circ \approx -29 \text{ kJ/mol}$$

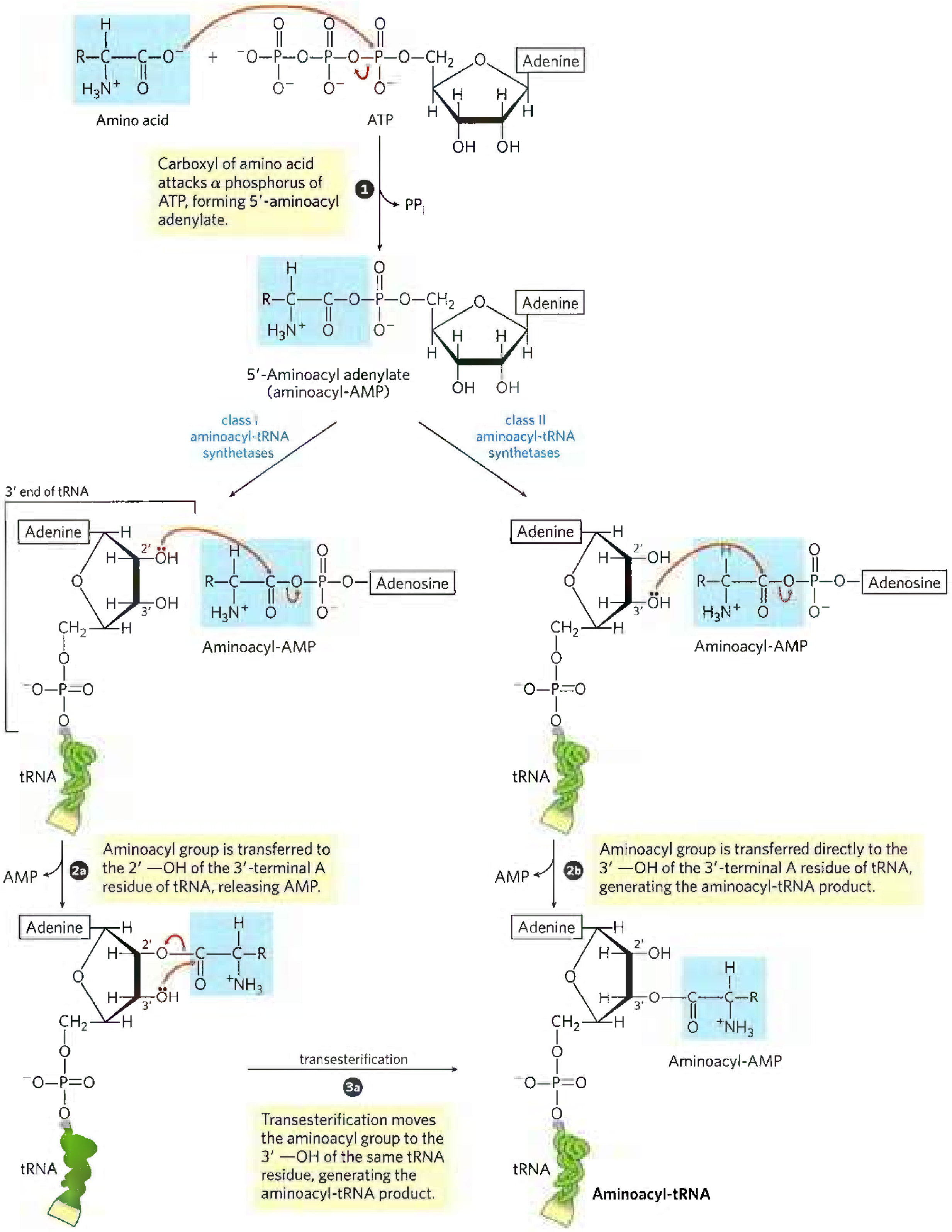
Proofreading by Aminoacyl-tRNA Synthetases The aminoacylation of tRNA accomplishes two ends: (1) it activates an amino acid for peptide bond formation and (2) it ensures appropriate placement of the amino acid in a growing polypeptide. The identity of the amino acid attached to a tRNA is not checked on the ribosome, so attachment of the correct amino acid to the tRNA is essential to the fidelity of protein synthesis.

As you will recall from Chapter 6, enzyme specificity is limited by the binding energy available from enzyme-substrate interactions. Discrimination between two similar amino acid substrates has been studied in detail in the case of Ile-tRNA synthetase, which distinguishes between valine and isoleucine, amino acids that differ by only a single methylene group (—CH_2—):



Valine Isoleucine

Ile-tRNA synthetase favors activation of isoleucine (to form Ile-AMP) over valine by a factor of 200 — as we


Amino acid
ATP
Adenine
PP$_i$
5′-Aminoacyl adenylate (aminoacyl-AMP)
class I aminoacyl-tRNA synthetases
class II aminoacyl-tRNA synthetases
3′ end of tRNA
Adenosine
Aminoacyl-AMP
tRNA
AMP
transesterification
Aminoacyl-AMP
Aminoacyl-tRNA

❶ Carboxyl of amino acid attacks α phosphorus of ATP, forming 5′-aminoacyl adenylate.

②a Aminoacyl group is transferred to the 2′ —OH of the 3′-terminal A residue of tRNA, releasing AMP.

②b Aminoacyl group is transferred directly to the 3′ —OH of the 3′-terminal A residue of tRNA, generating the aminoacyl-tRNA product.

③a Transesterification moves the aminoacyl group to the 3′ —OH of the same tRNA residue, generating the aminoacyl-tRNA product.

MECHANISM FIGURE 27-19 Aminoacylation of tRNA by aminoacyl-tRNA synthetases. Step ❶ is formation of an aminoacyl adenylate, which remains bound to the active site. In the second step, the aminoacyl group is transferred to the tRNA. The mechanism of this step is somewhat different for the two classes of aminoacyl-tRNA synthetases. For class I enzymes, ②a the aminoacyl group is transferred first to the 2′-hydroxyl group of the 3′-terminal A residue, then ③a to the 3′-hydroxyl group by a transesterification reaction. For class II enzymes, ②b the aminoacyl group is transferred directly to the 3′-hydroxyl group of the terminal adenylate.

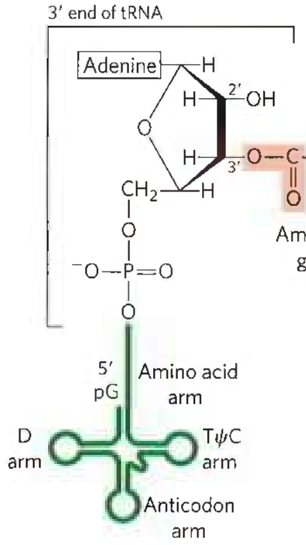


FIGURE 27-20 General structure of aminoacyl-tRNAs. The aminoacyl group is esterified to the 3′ position of the terminal A residue. The ester linkage that both activates the amino acid and joins it to the tRNA is shaded light red.

would expect, given the amount by which a methylene group (in Ile) could enhance substrate binding. Yet valine is erroneously incorporated into proteins in positions normally occupied by an Ile residue at a frequency of only about 1 in 3,000. How is this greater-than-10-fold increase in accuracy brought about? Ile-tRNA synthetase, like some other aminoacyl-tRNA synthetases, has a proofreading function.

Recall a general principle from the discussion of proofreading by DNA polymerases (see Fig. 25-6): if available binding interactions do not provide sufficient discrimination between two substrates, the necessary specificity can be achieved by substrate-specific binding in *two successive* steps. The effect of forcing the system through two successive filters is multiplicative. In the case of Ile-tRNA synthetase, the first filter is the initial binding of the amino acid to the enzyme and its activation to aminoacyl-AMP. The second is the binding of any *incorrect* aminoacyl-AMP products to a separate active site on the enzyme; a substrate that binds in this second active site is hydrolyzed. The R group of valine is slightly smaller than that of isoleucine, so Val-AMP fits the hydrolytic (proofreading) site of the Ile-tRNA synthetase, but Ile-AMP does not. P1 Thus Val-AMP is hydrolyzed to valine and AMP in the proofreading active site, and tRNA bound to the synthetase does not become aminoacylated to the wrong amino acid.

In addition to proofreading after formation of the aminoacyl-AMP intermediate, most aminoacyl-tRNA synthetases can hydrolyze the ester linkage between amino acids and tRNAs in the aminoacyl-tRNAs. This hydrolysis is greatly accelerated for incorrectly charged tRNAs, providing yet a third filter to enhance the fidelity of the overall process. The few aminoacyl-tRNA synthetases that activate amino acids with no close structural relatives (Cys-tRNA synthetase, for example) demonstrate little or no proofreading activity; in these cases, the active site for aminoacylation can sufficiently discriminate between the proper substrate and any incorrect amino acid.

The overall error rate of protein synthesis (~1 mistake per 10^4 amino acids incorporated) is not nearly as low as that of DNA replication. Because flaws in a protein are eliminated when the protein is degraded and are not passed on to future generations, they have less biological significance. The degree of fidelity in protein synthesis is sufficient to ensure that most proteins contain no mistakes and that the large amount of energy required to synthesize a protein is rarely wasted. One defective protein molecule is usually unimportant when many correct copies of the same protein are present.

A “Second Genetic Code” P3 An individual aminoacyl-tRNA synthetase must be specific not only for a single amino acid but for certain tRNAs as well. Discriminating among dozens of tRNAs is just as important for the overall fidelity of protein biosynthesis as is distinguishing among amino acids. The interaction between aminoacyl-tRNA synthetases and tRNAs has been referred to as the “second genetic code,” reflecting its critical role in maintaining the accuracy of protein synthesis. The “coding” rules appear to be more complex than those in the “first” code.

Figure 27-21 summarizes what we know about the nucleotides involved in recognition by some aminoacyl-tRNA synthetases. Some nucleotides are conserved in all tRNAs and therefore cannot be used for discrimination. Nucleotide positions necessary for discrimination by the aminoacyl-tRNA synthetases seem to be concentrated in the amino acid arm and the anticodon arm, including the nucleotides of the anticodon itself. A few are located in other parts of the tRNA molecule. Determination of the crystal structures of aminoacyl-tRNA synthetases complexed with their cognate tRNAs and ATP has added a great deal to our understanding of these interactions (**Fig. 27-22**).

Ten or more specific nucleotides may be involved in recognition of a tRNA by its specific aminoacyl-tRNA synthetase. But in a few cases the recognition mechanism is quite simple. Across a range of organisms from bacteria to humans, the primary determinant of tRNA recognition by the Ala-tRNA synthetases is a single G═U base pair in the amino acid arm of tRNA^{Ala} (**Fig. 27-23a**). A short synthetic RNA with as few as 7 bp arranged in a simple hairpin minihelix is efficiently aminoacylated by the Ala-tRNA synthetase, as long as the RNA contains the critical G═U (Fig. 27-23b). This relatively simple alanine system may be an evolutionary relic of a period when RNA oligonucleotides, ancestors to tRNA, were aminoacylated in a primitive system for protein synthesis.

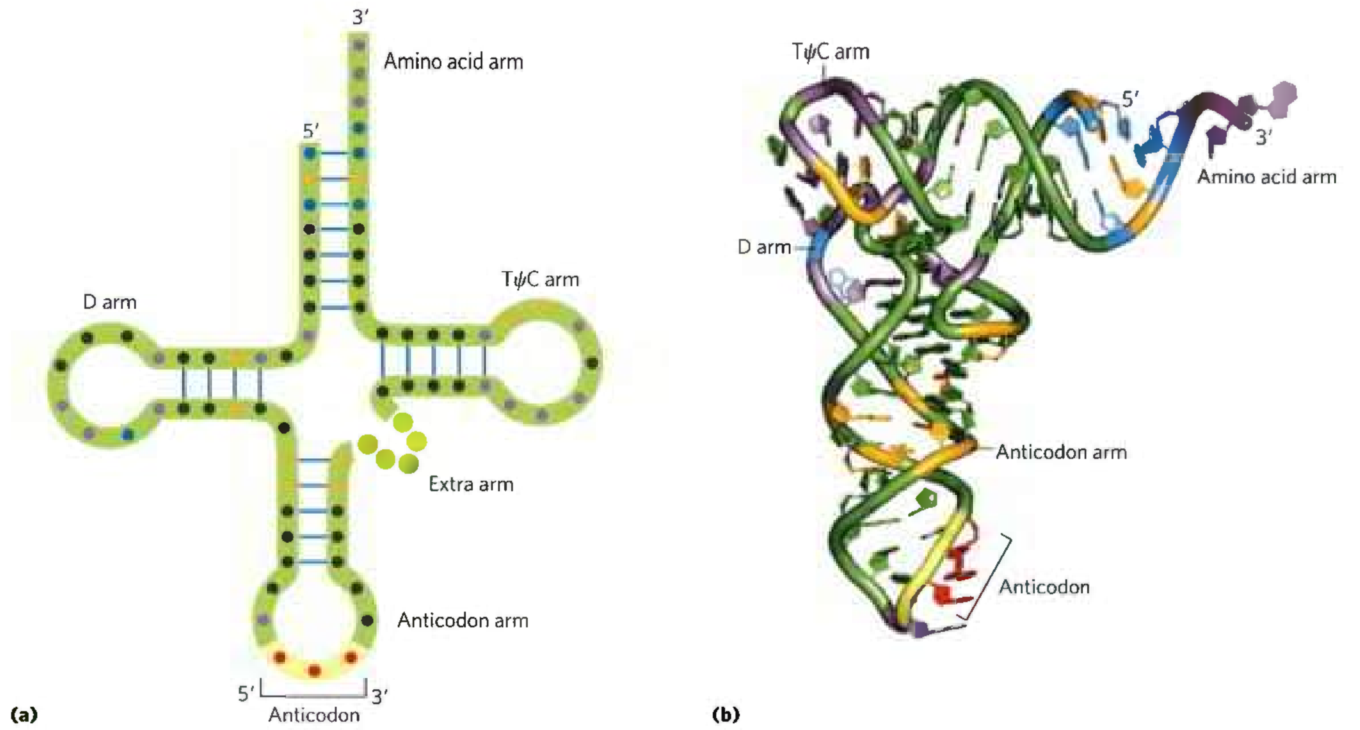

3′
Amino acid arm
5′
TψC arm
D arm
Extra arm
Anticodon arm
5′
3′
Anticodon
(a)
TψC arm
5′
3′
Amino acid arm
D arm
Anticodon arm
Anticodon
(b)

FIGURE 27-21 Nucleotide positions in a tRNA that are recognized by aminoacyl-tRNA synthetases. (a) Some positions (purple dots) are the same in all tRNAs and therefore cannot be used to discriminate one from another. Other positions are known recognition points for one (orange) or more (blue) aminoacyl-tRNA synthetases. Structural features other than sequence are important for recognition by some of the synthetases. (b) The same structural features are shown in three dimensions, with the orange and blue residues again representing positions recognized by one or more aminoacyl-tRNA synthetases, respectively. [(b) Data from PDB ID 1EHZ, H. Shi and P. B. Moore, *RNA* 6:1091, 2000.]

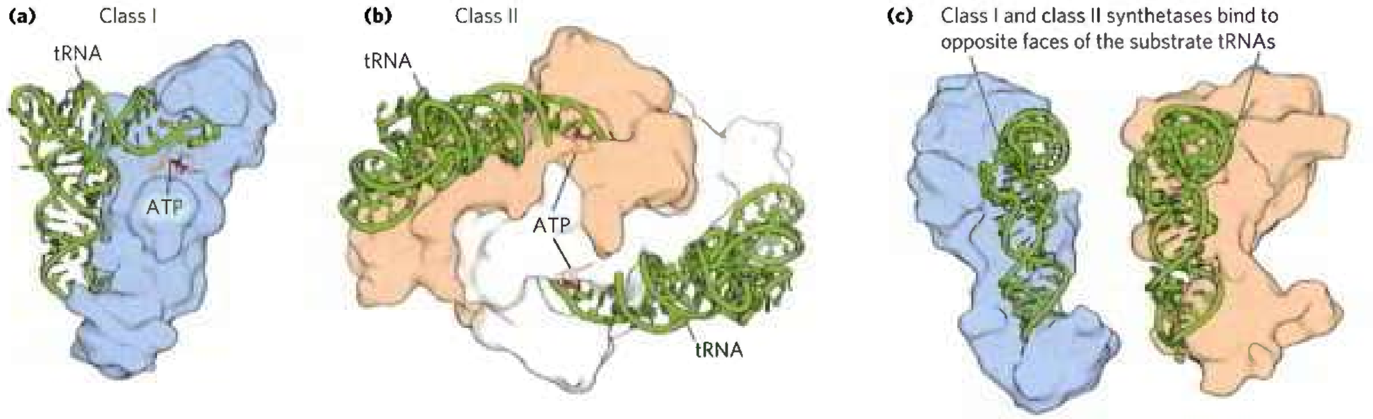

(a) Class I
tRNA
ATP
(b) Class II
tRNA
ATP
tRNA
(c) Class I and class II synthetases bind to opposite faces of the substrate tRNAs

FIGURE 27-22 Aminoacyl-tRNA synthetases. The synthetases are complexed with their cognate tRNAs (green). Bound ATP (red) pinpoints the active site near the end of the aminoacyl arm. (a) Gln-tRNA synthetase of *E. coli*, a typical monomeric class I synthetase. (b) Asp-tRNA synthetase of yeast, a typical dimeric class II synthetase. (c) The two classes of aminoacyl-tRNA synthetases recognize different faces of their tRNA substrates. [Data from (a, c (left)) PDB ID 1QRT, J. G. Arnez and T. A. Steitz, *Biochemistry* 35:14,725, 1996; (b, c (right)) PDB ID 1ASZ, J. Cavarelli et al., *EMBO J.* 13:327, 1994.]

The interaction of aminoacyl-tRNA synthetases and their cognate tRNAs is critical to accurate reading of the genetic code. Any expansion of the code to include new amino acids would necessarily require a new aminoacyl-tRNA synthetase–tRNA pair. A limited expansion of the genetic code has been observed in nature; a more extensive expansion has been accomplished in the laboratory (**Box 27-2**).

Stage 2: A Specific Amino Acid Initiates Protein Synthesis

Protein synthesis begins at the amino-terminal end and proceeds by the stepwise addition of amino acids to the carboxyl-terminal end of the growing polypeptide. The AUG initiation codon thus specifies an *amino-terminal* methionine residue. Although methionine has only one

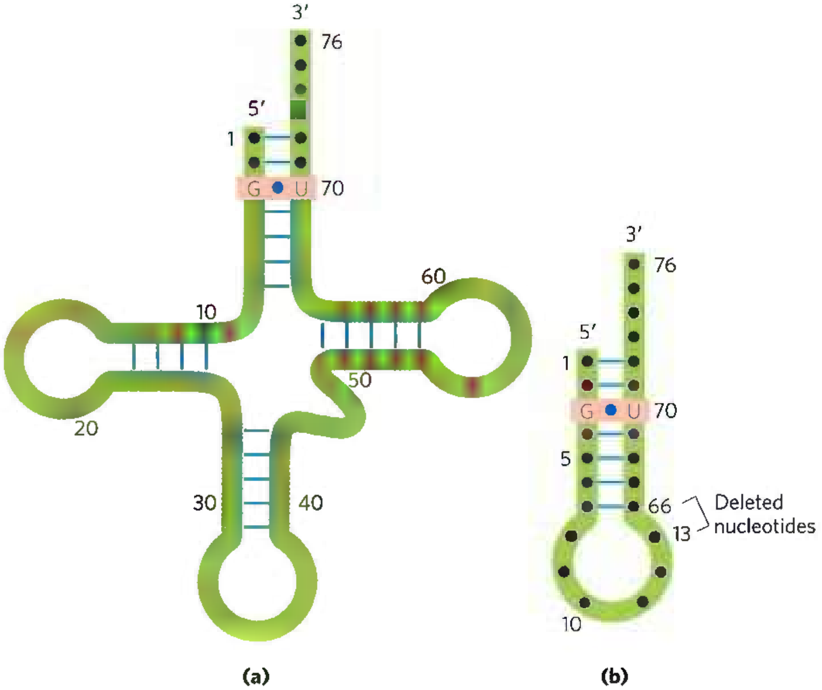


FIGURE 27-23 Structural elements of tRNA^{Ala} that are required for recognition by Ala-tRNA synthetase. (a) The tRNA^{Ala} structural elements recognized by the Ala-tRNA synthetase are unusually simple. A single G=U base pair (light red) is the only element needed for specific binding and aminoacylation. (b) A short synthetic RNA minihelix, with the critical G=U base pair but lacking most of the remaining tRNA structure. This is aminoacylated specifically with alanine almost as efficiently as the complete tRNA^{Ala}.

codon, (5′)AUG, all organisms have two tRNAs for methionine. One is used exclusively when (5′)AUG is the initiation codon for protein synthesis. The other is used to code for a Met residue in an internal position in a polypeptide.

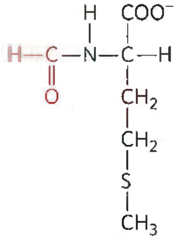
The distinction between an initiating (5′)AUG and an internal one is straightforward. In bacteria, the two types of tRNA specific for methionine are designated tRNA^{Met} and $\text{tRNA}^{\text{fMet}}$. The amino acid incorporated in response to the (5′)AUG initiation codon is *N*-formylmethionine (fMet). It arrives at the ribosome as *N*-formylmethionyl-$\text{tRNA}^{\text{fMet}}$ (fMet-$\text{tRNA}^{\text{fMet}}$), which is formed in two successive reactions. First, methionine is attached to $\text{tRNA}^{\text{fMet}}$ by the Met-tRNA synthetase (which in *E. coli* aminoacylates both $\text{tRNA}^{\text{fMet}}$ and tRNA^{Met}):

$$\text{Methionine} + \text{tRNA}^{\text{fMet}} + \text{ATP} \longrightarrow \text{Met-tRNA}^{\text{fMet}} + \text{AMP} + \text{PP}_i$$

Next, a transformylase transfers a formyl group from N^{10}-formyltetrahydrofolate to the amino group of the Met residue:

$$N^{10}\text{-Formyltetrahydrofolate} + \text{Met-tRNA}^{\text{fMet}} \longrightarrow \text{tetrahydrofolate} + \text{fMet-tRNA}^{\text{fMet}}$$

The transformylase is more selective than the Met-tRNA synthetase; it is specific for Met residues attached to $\text{tRNA}^{\text{fMet}}$, presumably recognizing some unique structural feature of that tRNA. By contrast, Met-$\text{tRNA}^{\text{fMet}}$ inserts methionine in interior positions in polypeptides.



N-Formylmethionine

Addition of the *N*-formyl group to the amino group of methionine by the transformylase prevents fMet from entering interior positions in a polypeptide while also allowing fMet-$\text{tRNA}^{\text{fMet}}$ to be bound at a specific ribosomal initiation site that accepts neither Met-tRNA^{Met} nor any other aminoacyl-tRNA.

In eukaryotic cells, all polypeptides synthesized by cytosolic ribosomes begin with a Met residue (rather than fMet), but, again, the cell uses a specialized initiating tRNA that is distinct from the tRNA^{Met} used at (5′)AUG codons at interior positions in the mRNA. Polypeptides synthesized by mitochondrial and chloroplast ribosomes, however, begin with *N*-formylmethionine. This strongly supports the view that mitochondria and chloroplasts originated from bacterial ancestors that were symbiotically incorporated into precursor eukaryotic cells at an early stage of evolution (see Fig. 1-37).

How can the single (5′)AUG codon determine whether a starting *N*-formylmethionine (or methionine, in eukaryotes) or an interior Met residue is ultimately inserted? The details of the initiation process provide the answer.

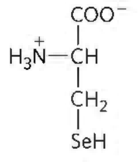
BOX 27-2

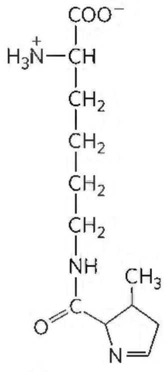
Natural and Unnatural Expansion of the Genetic Code

As we have seen, the 20 standard amino acids found in proteins offer only limited chemical functionality. Living systems generally overcome these limitations by using enzymatic cofactors or by modifying particular amino acids after they have been incorporated into proteins. In principle, expansion of the genetic code to introduce new amino acids into proteins offers another route to new functionality, but it is a very difficult route to exploit. Such a change might just as easily result in inactivation of thousands of cellular proteins.

Expanding the genetic code to include a new amino acid requires several cellular changes. A new aminoacyl-tRNA synthetase must generally be present, along with a cognate tRNA. Both of these components must be highly specific, interacting only with each other and the new amino acid. Significant concentrations of the new amino acid must be present in the cell, which may entail the evolution of new metabolic pathways. As outlined in Box 27-1, the anticodon on the tRNA would most likely pair with a codon that usually specifies termination. Making all of this work in a living cell seems unlikely, but it has happened both in nature and in the laboratory.

There are actually 22 rather than 20 amino acids specified by the known genetic code. The two extra amino acids are selenocysteine and pyrrolysine, each found in only very few proteins but both offering a glimpse into the intricacies of code evolution.


Selenocysteine


Pyrrolysine

A few proteins in all cells (such as formate dehydrogenase in bacteria and glutathione peroxidase in mammals) require selenocysteine for their activity. In *E. coli*, selenocysteine is introduced into the enzyme formate dehydrogenase during translation, in response to an in-frame UGA codon. A special type of Ser-tRNA, present at lower levels than other Ser-tRNAs, recognizes UGA and no other codons. This tRNA is charged with serine by the normal serine aminoacyl-tRNA synthetase, and the serine is enzymatically converted to selenocysteine by a separate enzyme before its use at the ribosome. The charged tRNA does not recognize just any UGA codon; some contextual signal in the mRNA, still to be identified, ensures that this tRNA recognizes only the few UGA codons, within certain genes, that specify selenocysteine. In effect, UGA doubles as a codon for both termination and (very occasionally) selenocysteine. This particular code expansion has a dedicated tRNA, as described above, but it lacks a dedicated cognate aminoacyl-tRNA synthetase. The process works for selenocysteine, but one might consider it an intermediate step in the evolution of a complete new codon definition.

Pyrrolysine is found in a group of anaerobic archaea called methanogens (see Box 22-1). These organisms produce methane as a required part of their metabolism, and the Methanosarcinaceae family can use methylamines as substrates for methanogenesis. Producing methane from monomethylamine requires the enzyme monomethylamine methyltransferase. The gene encoding this enzyme has an in-frame UAG termination codon. The structure of the methyltransferase was elucidated in 2002, revealing the presence of the novel amino acid pyrrolysine at the position specified by the UAG codon. Subsequent experiments demonstrated that — unlike selenocysteine — pyrrolysine is attached directly to a dedicated tRNA by a cognate pyrrolysyl-tRNA synthetase. These methanogens produce pyrrolysine via a metabolic pathway that remains to be elucidated. The overall system has all the hallmarks of an established codon assignment, although it works only for UAG codons in this particular gene. As in the case of selenocysteine, there are probably contextual signals that direct this tRNA to the correct UAG codon.

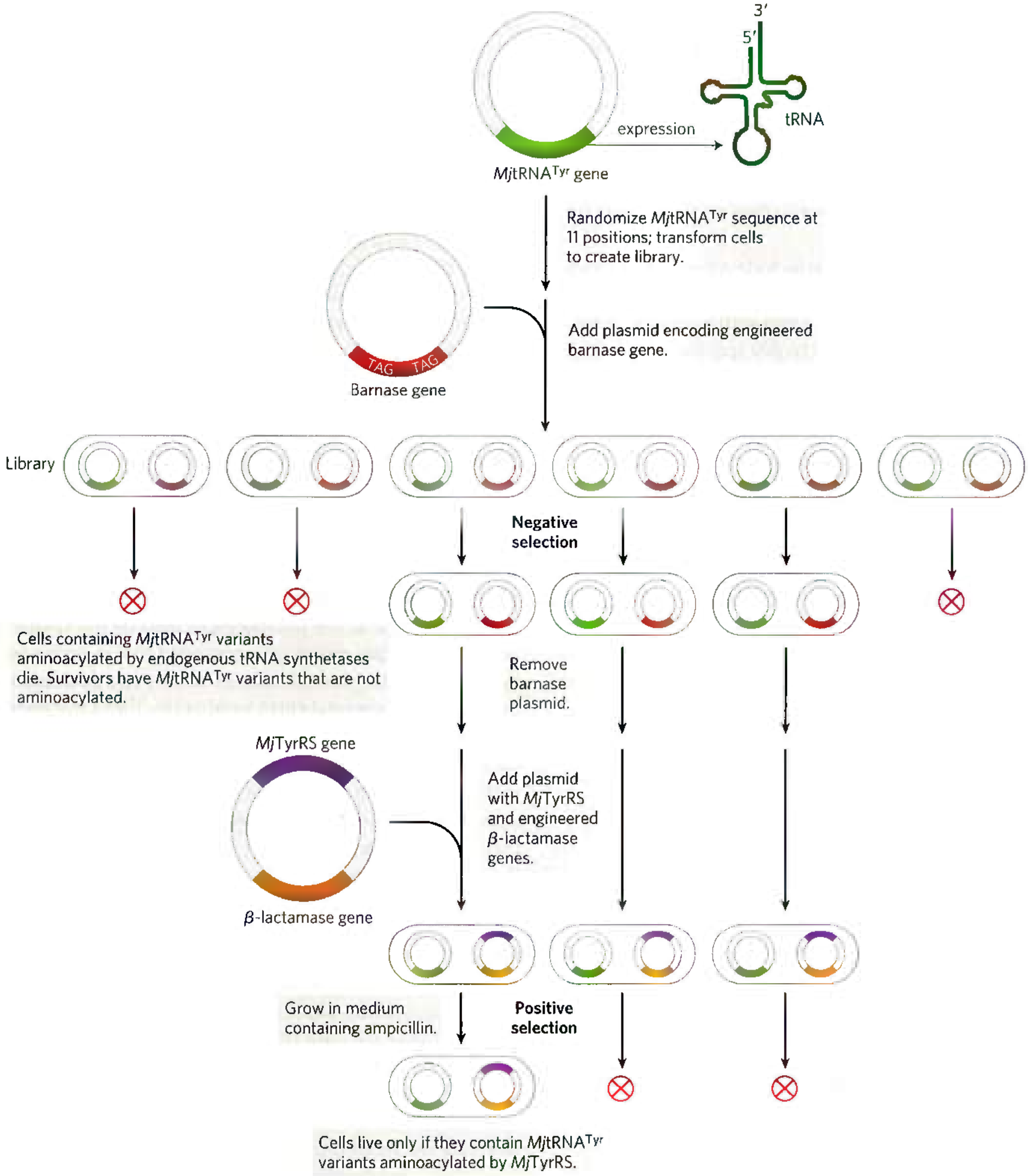
Can scientists match this evolutionary feat? Modification of proteins with various functional groups can provide important insights into the activity and/or structure of the proteins. However, protein modification is often laborious. For example, an investigator who wishes to attach a new group to a particular Cys residue will have to somehow block other Cys residues that may be present on the same protein. If one could instead adapt the genetic code to enable a cell to insert a modified amino acid at a particular location in a protein, the process could be rendered much more convenient. Peter Schultz and coworkers have done just that.

To develop a new codon assignment, one again needs a new aminoacyl-tRNA synthetase and a novel cognate tRNA, both adapted to work only with a particular new amino acid. Efforts to create such an "unnatural" code expansion initially focused on *E. coli*. The codon UAG was chosen as the best target for encoding a new amino acid. UAG is the least used of the three termination codons, and strains with tRNAs selected

(*Continued on next page*)

BOX 27-2

Natural and Unnatural Expansion of the Genetic Code (*Continued*)



Randomize *Mj*tRNA$^{\text{Tyr}}$ sequence at 11 positions; transform cells to create library.

Add plasmid encoding engineered barnase gene.

Cells containing *Mj*tRNA$^{\text{Tyr}}$ variants aminoacylated by endogenous tRNA synthetases die. Survivors have *Mj*tRNA$^{\text{Tyr}}$ variants that are not aminoacylated.

Remove barnase plasmid.

Add plasmid with *Mj*TyrRS and engineered β-lactamase genes.

Grow in medium containing ampicillin.

Cells live only if they contain *Mj*tRNA$^{\text{Tyr}}$ variants aminoacylated by *Mj*TyrRS.

FIGURE 1 Selecting *Mj*tRNA$^{\text{Tyr}}$ variants that function only with the tyrosyl-tRNA synthetase *Mj*TyrRS. The sequence of the gene encoding *Mj*tRNA$^{\text{Tyr}}$, on a plasmid, is randomized at 11 positions that do not interact with *Mj*TyrRS (red dots). The mutagenized plasmids are introduced into *E. coli* cells to create a library of millions of *Mj*tRNA$^{\text{Tyr}}$ variants, represented by the six cells shown here. The toxic barnase gene, engineered to include the sequence TAG so that its transcript includes UAG codons, is present on a separate plasmid, providing a negative selection. If this gene is expressed, the cells die. It can be successfully expressed only if the *Mj*tRNA$^{\text{Tyr}}$ variant expressed by that particular cell is aminoacylated by endogenous (*E. coli*) aminoacyl-tRNA synthetases, inserting an amino acid instead of stopping translation. Another gene, encoding β-lactamase, and also engineered with TAG sequences to produce UAG stop codons, is provided on yet another plasmid that also expresses the gene encoding *Mj*TyrRS. This serves as a means of positive selection for the remaining *Mj*tRNA$^{\text{Tyr}}$ variants. Those variants that are aminoacylated by *Mj*TyrRS allow expression of the β-lactamase gene, so these cells can grow on ampicillin. Multiple rounds of negative and positive selection yield the best *Mj*tRNA$^{\text{Tyr}}$ variants that are aminoacylated uniquely by *Mj*TyrRS and used efficiently in translation.

to recognize UAG do not exhibit growth defects. To create the new tRNA and tRNA synthetase, the genes for a $tRNA^{Tyr}$ and its cognate tyrosyl-tRNA synthetase were taken from the archaeon *Methanococcus jannaschii* (*Mj*$tRNA^{Tyr}$ and *Mj*TyrRS, respectively). *Mj*TyrRS does not bind to the anticodon loop of *Mj*$tRNA^{Tyr}$, so the anticodon loop can be modified to CUA (complementary to UAG) without affecting the interaction. Because the archaeal and bacterial systems are orthologous, the modified archaeal components could be transferred to *E. coli* without disrupting the intrinsic translation system of the bacterial cells.

First, the gene encoding *Mj*$tRNA^{Tyr}$ had to be modified to generate an ideal product tRNA—one that was not recognized by any aminoacyl-tRNA synthetases endogenous to *E. coli* but was aminoacylated by *Mj*TyrRS. Finding such a variant could be accomplished through a series of negative and positive selection cycles designed to efficiently sift through variants of the tRNA gene (Fig. 1). Parts of the *Mj*$tRNA^{Tyr}$ sequence were randomized, allowing creation of a library of cells that each expressed a different version of the tRNA. A gene encoding barnase (a ribonuclease toxic to *E. coli*) was engineered so that its mRNA transcript contained several UAG codons, and this gene was also introduced into the cells on a plasmid. If the *Mj*$tRNA^{Tyr}$ variant expressed in a particular cell in the library were aminoacylated by an endogenous tRNA synthetase, it would express the barnase gene and that cell would die (negative selection). Surviving cells would contain tRNA variants that were not aminoacylated by endogenous tRNA synthetases, but could potentially be aminoacylated by *Mj*TyrRS. A positive selection (Fig. 1) was then set up by engineering the β-lactamase gene (which confers resistance to the antibiotic ampicillin) so that its transcript contained several UAG codons, and introducing this gene into the cells along with the gene encoding *Mj*TyrRS. Those *Mj*$tRNA^{Tyr}$ variants that could be aminoacylated by *Mj*TyrRS allowed growth on ampicillin only when *Mj*TyrRS was also expressed in the cell. Several rounds of this negative and positive selection scheme identified a new *Mj*$tRNA^{Tyr}$ variant that was not affected by endogenous enzymes, was aminoacylated by *Mj*TyrRS, and functioned well in translation.

Next, the *Mj*TyrRS had to be modified to recognize the new amino acid. The gene encoding *Mj*TyrRS was mutagenized to create a large library of variants. Variants that would aminoacylate the new *Mj*$tRNA^{Tyr}$ variant with endogenous amino acids were eliminated using the barnase gene selection. A second positive selection (similar to the ampicillin selection described above) was carried out so that cells would survive only if the *Mj*$tRNA^{Tyr}$ variant were aminoacylated only in the presence of the unnatural amino acid. Several rounds of negative and positive selection generated a cognate tRNA synthetase–tRNA pair that recognized only the unnatural amino acid.

Using this approach, researchers have constructed many *E. coli* strains, each capable of incorporating one particular unnatural amino acid into a protein in response to a UAG codon. The same approach has been used to artificially expand the genetic code of yeast and even mammalian cells. More than 30 different amino acids (Fig. 2) can be introduced site-specifically and efficiently into cloned proteins in this way. The result is an increasingly useful and flexible tool kit with which to advance the study of protein structure and function.

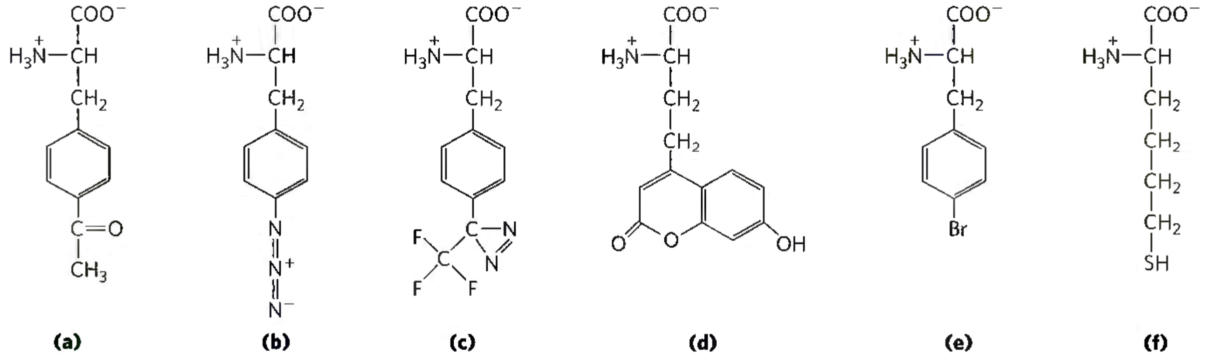


FIGURE 2 A sampling of unnatural amino acids that have been added to the genetic code. These unnatural amino acids add uniquely reactive chemical groups such as (a) a ketone; (b) an azide; (c) a photocrosslinker, a functional group designed to form a covalent bond with a nearby group when activated by light; (d) a highly fluorescent amino acid; (e) an amino acid with a heavy atom (Br) for use in crystallography; and (f) a long-chain cysteine analog that can form extended disulfide bonds. [Information from J. Xie and P. G. Schultz, *Nat. Rev. Mol. Cell Biol.* 7:775, 2006.]

The Three Steps of Initiation The **initiation** of polypeptide synthesis in bacteria requires (1) the 30S ribosomal subunit, (2) the mRNA coding for the polypeptide to be made, (3) the initiating fMet-tRNA$^{\text{fMet}}$, (4) a set of three proteins called initiation factors (IF1, IF2, and IF3), (5) GTP, (6) the 50S ribosomal subunit, and (7) Mg^{2+}. Formation of the initiation complex takes place in three steps (**Fig. 27-24**).

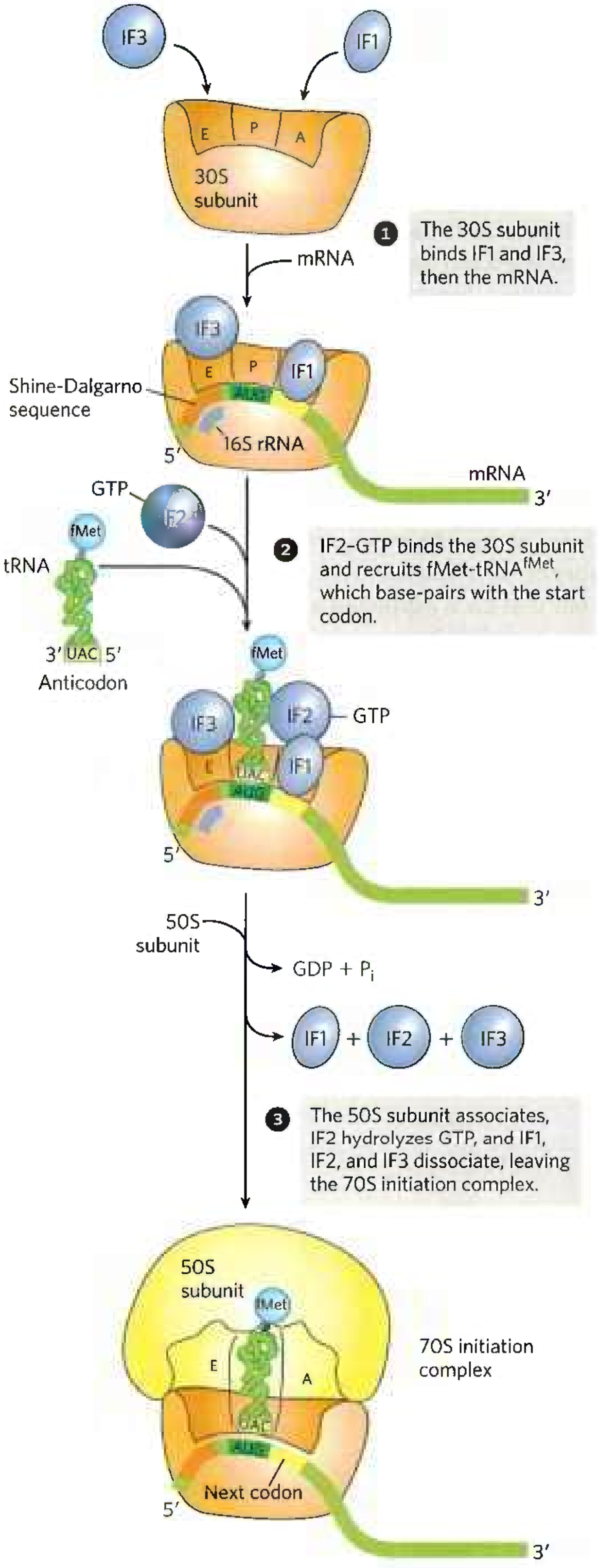
In step ①, the 30S ribosomal subunit binds two initiation factors, IF1 and IF3. Factor IF3 prevents the 30S and 50S subunits from combining prematurely. The mRNA then binds to the 30S subunit. The initiating (5′)AUG is guided to its correct position by the **Shine-Dalgarno sequence** (named for Australian researchers John Shine and Lynn Dalgarno, who identified it) in the mRNA. This consensus sequence is an initiation signal of four to nine purine residues, 8 to 13 bp to the 5′ side of the initiation codon (**Fig. 27-25a**). The sequence base-pairs with a complementary pyrimidine-rich sequence near the 3′ end of the 16S rRNA of the 30S ribosomal subunit (Fig. 27-25b). This mRNA-rRNA interaction positions the initiating (5′)AUG sequence of the mRNA in the precise position on the 30S subunit where it is required for initiation of translation. The particular (5′)AUG where fMet-tRNA$^{\text{fMet}}$ is to be bound is distinguished from other methionine codons by its proximity to the Shine-Dalgarno sequence in the mRNA.

Bacterial ribosomes have three sites that bind tRNAs, the **aminoacyl (A) site**, the **peptidyl (P) site**, and the **exit (E) site**. The A and P sites bind aminoacyl-tRNAs, whereas the E site binds only uncharged tRNAs that have completed their task on the ribosome. Factor IF1 binds at the A site and prevents tRNA binding at this site during initiation. The initiating (5′)AUG is positioned at the P site, the only site to which fMet-tRNA$^{\text{fMet}}$ can bind (Fig. 27-24). The fMet-tRNA$^{\text{fMet}}$ is the only aminoacyl-tRNA that binds first to the P site; during the subsequent elongation stage, all other incoming aminoacyl-tRNAs (including the Met-tRNA$^{\text{fMet}}$ that binds to interior AUG codons) bind first to the A site and only subsequently to the P and E sites. The E site is the site from which the "uncharged" tRNAs leave during elongation. Both the 30S and the 50S subunits contribute to the characteristics of the A and P sites, whereas the E site is largely confined to the 50S subunit.

In step ② of the initiation process (Fig. 27-24), the complex consisting of the 30S ribosomal subunit, IF3, and mRNA is joined by both GTP-bound IF2 and the initiating fMet-tRNA$^{\text{fMet}}$. The anticodon of this tRNA now pairs correctly with the mRNA's initiation codon.

In step ③, this large complex combines with the 50S ribosomal subunit; simultaneously, the GTP bound to IF2 is hydrolyzed to GDP and P_i, which are released from the complex. All three initiation factors leave the ribosome at this point.

P1 Completion of the steps in Figure 27-24 produces a functional 70S ribosome called the **initiation complex**,



① The 30S subunit binds IF1 and IF3, then the mRNA.

② IF2-GTP binds the 30S subunit and recruits fMet-tRNA$^{\text{fMet}}$, which base-pairs with the start codon.

③ The 50S subunit associates, IF2 hydrolyzes GTP, and IF1, IF2, and IF3 dissociate, leaving the 70S initiation complex.

FIGURE 27-24 Formation of the initiation complex in bacteria. The complex forms in three steps (described in the text) at the expense of the hydrolysis of GTP to GDP and P_i. IF1, IF2, and IF3 are initiation factors. E designates the exit site; P, the peptidyl site; and A, the aminoacyl site. Here the anticodon of the tRNA is oriented 3′ to 5′, left to right, as in Figure 27-8, but opposite to the orientation in Figures 27-21 and 27-23.

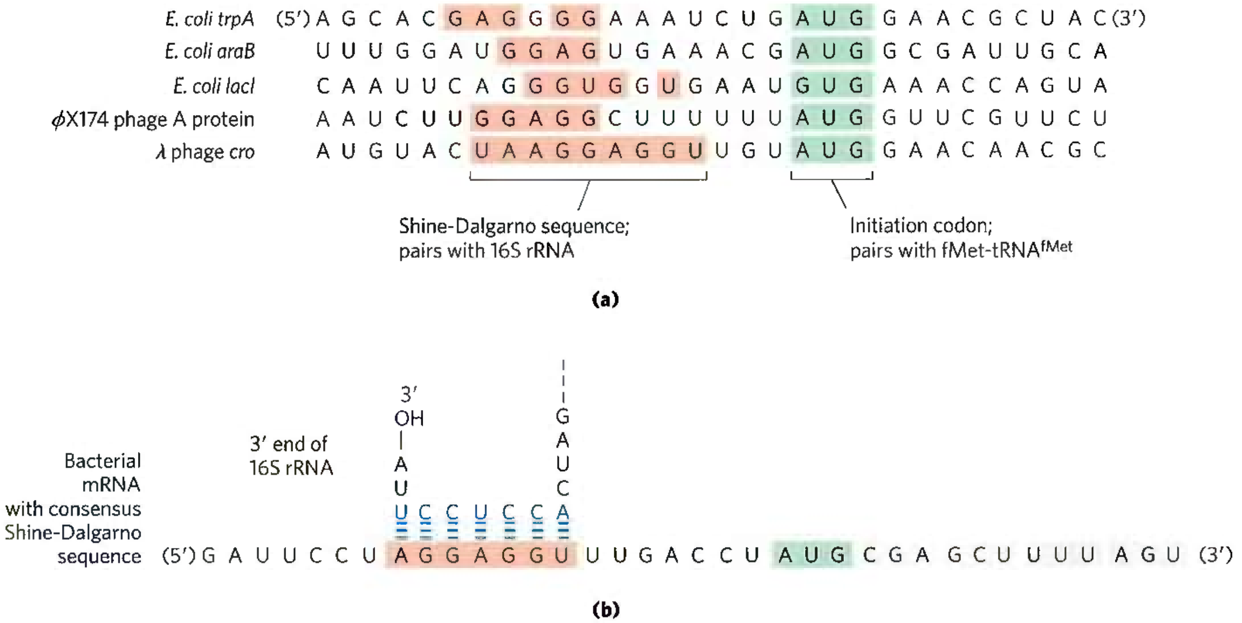

E. coli trpA (5′) A G C A C G A G G G G A A A U C U G A U G G A A C G C U A C (3′)
E. coli araB U U U G G A U G G A G U G A A A C G A U G G C G A U U G C A
E. coli lacI C A A U U C A G G G U G G U G A A U G U G A A A C C A G U A
ϕX174 phage A protein A A U C U U G G A G G C U U U U U U A U G G U U C G U U C U
λ phage cro A U G U A C U A A G G A G G U U G U A U G G A A C A A C G C
Shine-Dalgarno sequence; pairs with 16S rRNA
Initiation codon; pairs with fMet-tRNA^fMet
(a)
3′ end of 16S rRNA
3′ OH A U U C C U C C A C U A G
Bacterial mRNA with consensus Shine-Dalgarno sequence (5′) G A U U C C U A G G A G G U U U G A C C U A U G C G A G C U U U U A G U (3′)
(b)

FIGURE 27-25 Messenger RNA sequences that serve as signals for initiation of protein synthesis in bacteria. (a) Alignment of the initiating AUG (shaded in green) at its correct location on the 30S ribosomal subunit depends in part on upstream Shine-Dalgarno sequences (light red). Portions of the mRNA transcripts of five bacterial genes are shown. Note the unusual example of the *E. coli* LacI protein, which initiates with a GUG (Val) codon. In *E. coli*, AUG is the start codon in approximately 91% of genes, with GUG (7%) and UUG (2%) assuming this role more rarely. (b) The Shine-Dalgarno sequence of the mRNA pairs with a sequence near the 3′ end of the 16S rRNA.

containing the mRNA and the initiating fMet-tRNA$^{\text{fMet}}$. The correct binding of the fMet-tRNA$^{\text{fMet}}$ to the P site in the complete 70S initiation complex is ensured by at least three points of recognition and attachment: the codon-anticodon interaction involving the initiation AUG fixed in the P site, the interaction between the Shine-Dalgarno sequence in the mRNA and the 16S rRNA, and the binding interactions between the ribosomal P site and the fMet-tRNA$^{\text{fMet}}$. The initiation complex is now ready for elongation.

Initiation in Eukaryotic Cells Translation is generally similar in eukaryotic and bacterial cells; most of the significant differences are in the number of components and the mechanistic details. The initiation process in eukaryotes is outlined in **Figure 27-26**. Eukaryotic mRNAs are bound to the ribosome as a complex with a number of specific binding proteins. Eukaryotic cells have at least 12 initiation factors. Initiation factors eIF1A and eIF3 are the functional homologs of the bacterial IF1 and IF3, binding to the 40S subunit in step ①, blocking tRNA binding to the A site and premature joining of the large and small ribosomal subunits, respectively. The factor eIF1 binds to the E site. The charged initiator tRNA is bound by the initiation factor eIF2, which also has bound GTP. In step ②, this ternary complex binds to the 40S ribosomal subunit, along with two other proteins involved in later steps, eIF5 (not shown in Fig. 27-26) and eIF5B. This creates a 43S preinitiation complex. The mRNA binds to the eIF4F complex, which, in step ③, mediates its association with the 43S preinitiation complex. The eIF4F complex is made up of eIF4E (binding to the 5′ cap), eIF4A (an ATPase and RNA helicase), and eIF4G (a linker protein). The eIF4G protein binds to eIF3 and eIF4E to provide the first link between the 43S preinitiation complex and the mRNA. The eIF4G also binds to the poly(A) binding protein (PABP) at the 3′ end of the mRNA, circularizing the mRNA (**Fig. 27-27**) and facilitating the translational regulation of gene expression, as described in Chapter 28.

Addition of the mRNA and its associated factors creates a 48S complex. This complex scans the bound mRNA, starting at the 5′ cap, until an AUG codon is encountered. The scanning process (step ④ in Fig. 27-26) may be facilitated by the RNA helicase of eIF4A, which unwinds RNA secondary structures while in a transient complex with another factor, eIF4B (not shown in Fig. 27-26).

Once the initiating AUG site is encountered, the 60S ribosomal subunit associates with the complex in step ⑤, which is accompanied by release of many of the initiation factors. This requires the activity of eIF5 and eIF5B. The eIF5 protein promotes the GTPase activity of eIF2, producing an eIF2-GDP complex with reduced affinity for the initiator tRNA. The eIF5B protein is homologous to the bacterial IF2. It hydrolyzes its bound GTP and triggers dissociation of eIF2-GDP and other initiation factors, followed closely by association of the 60S subunit. This completes formation of the initiation complex.

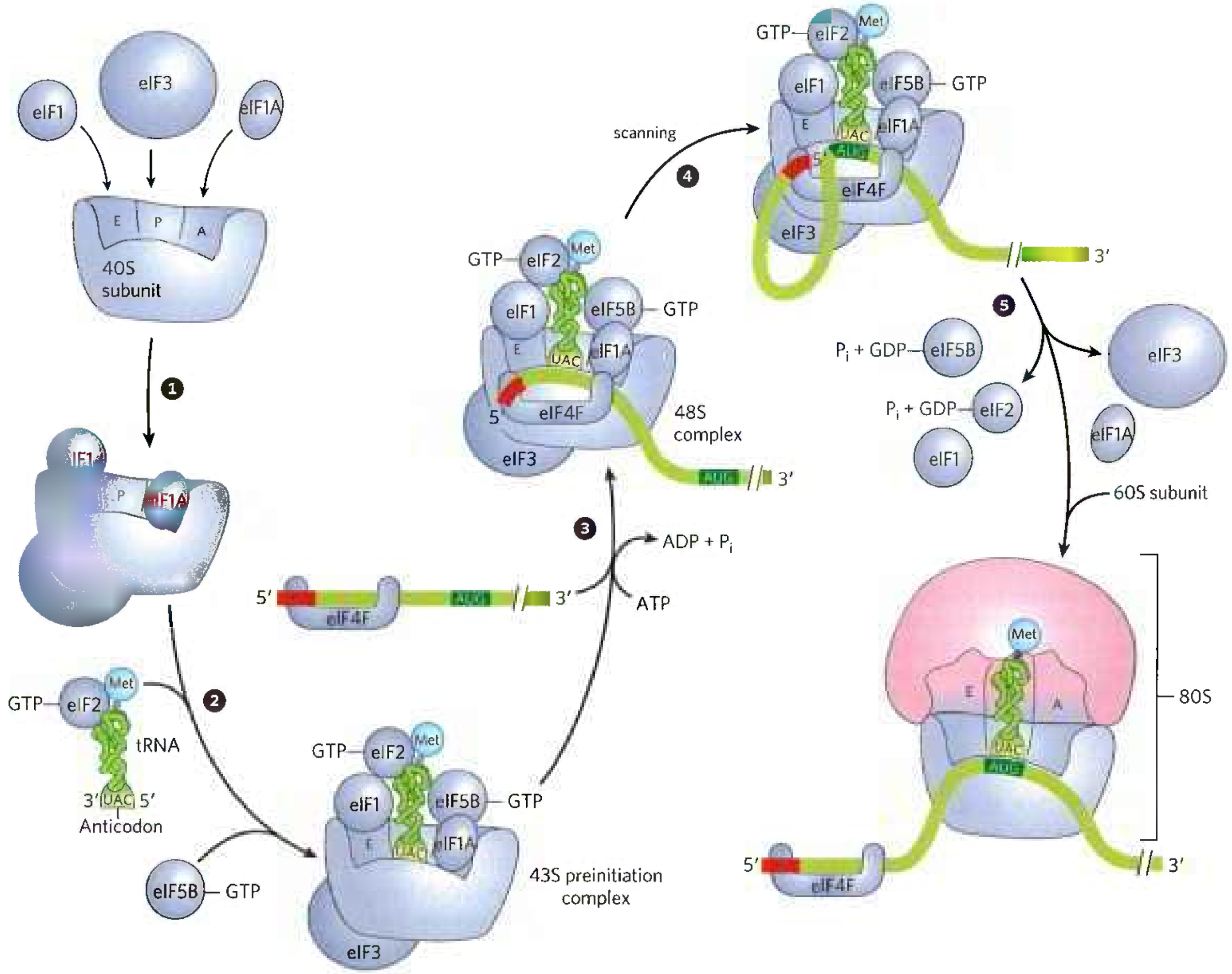

eIF1
eIF3
eIF1A
E
P
A
40S subunit
1
IF1A
P
eIF1A
5′
eIF4F
AUG
3′
2
GTP
eIF2
Met
tRNA
3′ UAC 5′
Anticodon
eIF5B
GTP
eIF1
eIF5B
GTP
E
UAC
eIF1A
eIF3
43S preinitiation complex
3
ADP + P$_i$
ATP
GTP
eIF2
Met
eIF1
eIF5B
GTP
E
UAC
eIF1A
5′
eIF4F
eIF3
48S complex
AUG
3′
4
scanning
GTP
eIF2
Met
eIF1
eIF5B
GTP
E
UAC
AUG
eIF1A
5′
eIF4F
eIF3
3′
5
P$_i$ + GDP
eIF5B
P$_i$ + GDP
eIF2
eIF1
eIF3
eIF1A
60S subunit
Met
E
A
UAC
AUG
80S
5′
eIF4F
3′

FIGURE 27-26 Initiation of protein synthesis in eukaryotes. The five steps are described in the text. Eukaryotic initiation factors mediate the association of, first, the charged initiator tRNA to form a 43S preinitiation complex, and then the mRNA (with the 5′ cap shown in red) to form a 48S complex. The final 80S initiation complex is formed as the 60S subunit associates, coupled with release of most of the initiation factors.

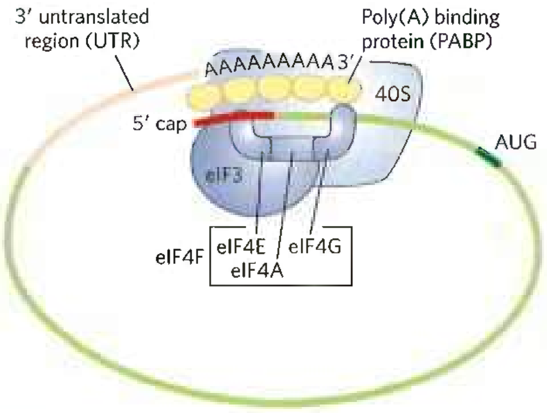

3′ untranslated region (UTR)
Poly(A) binding protein (PABP)
AAAAAAAAA 3′
40S
5′ cap
AUG
eIF3
eIF4F
eIF4E
eIF4G
eIF4A

FIGURE 27-27 Circularization of mRNA in the eukaryotic initiation complex. The 3′ and 5′ ends of eukaryotic mRNAs are linked by the eIF4F complex of proteins. The eIF4E subunit binds to the 5′ cap, and the eIF4G protein binds to the poly(A) binding protein (PABP) at the 3′ end of the mRNA. The eIF4G protein also binds to eIF3, linking the circularized mRNA to the 40S subunit of the ribosome.

The roles of the various bacterial and eukaryotic initiation factors in the overall process are summarized in Table 27-8. The mechanism by which these proteins act is an important area of investigation.

Stage 3: Peptide Bonds Are Formed in the Elongation Stage

The third stage of protein synthesis is **elongation**. Again, we begin with bacterial cells. Elongation requires (1) the initiation complex described above, (2) aminoacyl-tRNAs, (3) a set of three soluble cytosolic proteins called **elongation factors** (EF-Tu, EF-Ts, and EF-G in bacteria), and (4) GTP. Cells use three steps to add each amino acid residue, and the steps are repeated as many times as there are residues to be added.

Elongation Step 1: Binding of an Incoming Aminoacyl-tRNA In the first step of the elongation cycle (Fig. 27-28), the

TABLE 27-8 Protein Factors Required for Initiation of Translation in Bacterial and Eukaryotic Cells

Factor	Function
Bacterial	
IF1	Prevents premature binding of tRNAs to A site
IF2	Facilitates binding of fMet-tRNA$^{\text{fMet}}$ to 30S ribosomal subunit
IF3	Binds to 30S subunit; prevents premature association of 50S subunit; enhances specificity of P site for fMet-rRNA$^{\text{fMet}}$
Eukaryotic	
eIF1	Binds to the E site of the 40S subunit; facilitates interaction between eIF2-tRNA-GTP ternary complex and the 40S subunit
eIF1A	Homolog of bacterial IF1; prevents premature binding of tRNAs to A site
eIF2	GTPase; facilitates binding of initiating Met-tRNA$^{\text{Met}}$ to 40S ribosomal subunit
eIF2B[a], eIF3	First factors to bind 40S subunit; facilitate subsequent steps
eIF4F	Complex consisting of eIF4E, eIF4A, and eIF4G
eIF4A	RNA helicase activity; removes secondary structure in the mRNA to permit binding to 40S subunit; part of the eIF4F complex
eIF4B	Binds to mRNA; facilitates scanning of mRNA to locate the first AUG
eIF4E	Binds to the 5′ cap of mRNA; part of the eIF4F complex
eIF4G	Binds to eIF4E and to poly(A) binding protein (PABP); part of the eIF4F complex
eIF5[a]	Promotes dissociation of several other initiation factors from 40S subunit as a prelude to association of 60S subunit to form 80S initiation complex
eIF5b	GTPase homologous to bacterial IF2; promotes dissociation of initiation factors before final ribosome assembly

[a] Not shown in Figure 27-26.

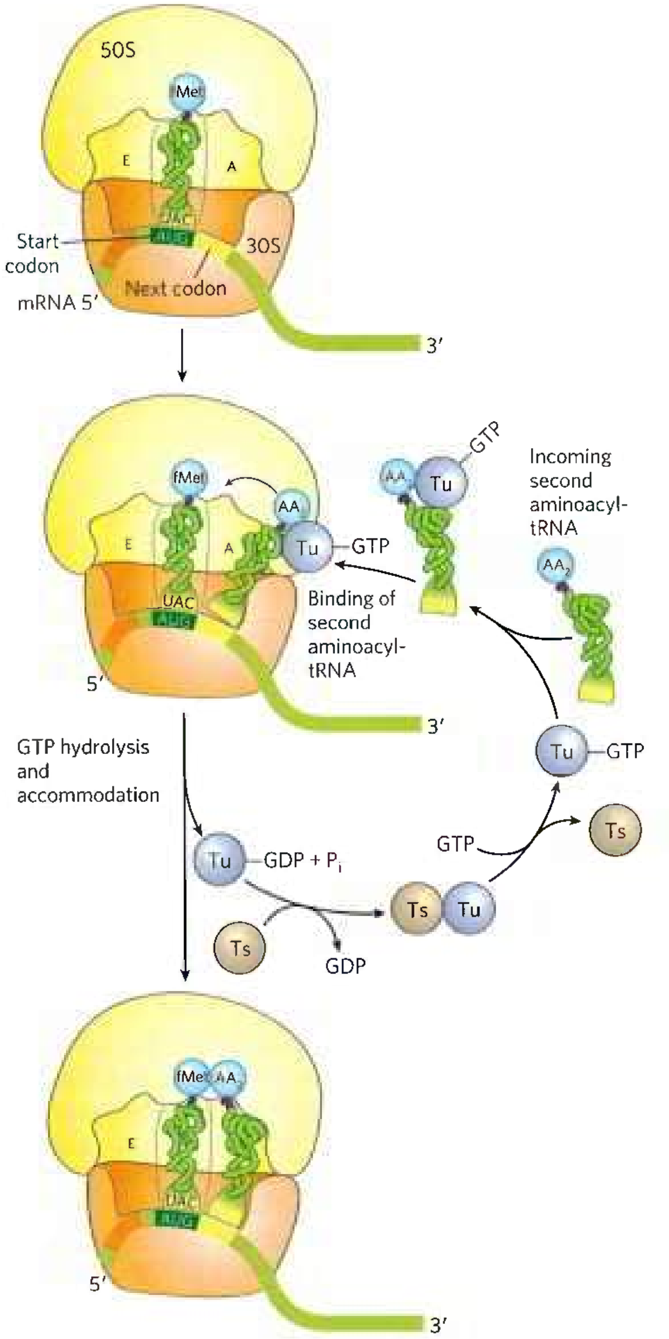


FIGURE 27-28 First elongation step in bacteria: binding of the second aminoacyl-tRNA. The second aminoacyl-tRNA (AA$_2$) enters the A site of the ribosome bound to GTP-bound EF-Tu (shown here as Tu). Binding of the second aminoacyl-tRNA to the A site is accompanied by hydrolysis of the GTP to GDP and P$_i$ and release of the EF-Tu–GDP complex from the ribosome. The EF-Tu–GTP complex is regenerated in a process requiring EF-Ts and GTP. "Accommodation" involves a change in the conformation of the second tRNA that pulls its aminoacyl end into the peptidyl transferase site.

appropriate incoming aminoacyl-tRNA binds to a complex of GTP-bound EF-Tu. The resulting aminoacyl-tRNA–EF-Tu–GTP complex binds to the A site of the 70S initiation complex. The GTP is hydrolyzed and an EF-Tu–GDP complex is released from the 70S ribosome. The bound GDP is released when the EF-Tu–GDP complex binds to EF-Ts, and EF-Ts is subsequently released when another molecule of GTP binds to EF-Tu, recycling it.

Elongation Step 2: Peptide Bond Formation A peptide bond is now formed between the two amino acids bound by their tRNAs to the A and P sites on the ribosome. This occurs by transfer of the initiating *N*-formylmethionyl group from its tRNA to the amino group of the second amino acid, now in the A site (**Fig. 27-29**). The α-amino group of the amino acid in the A site acts as a nucleophile, displacing the tRNA in the P site to form a peptide bond. The constrained structure of the proline side chain interferes with the alignment needed for peptide bonds to form properly. A special system binds to the ribosome to facilitate peptide bonds between two proline residues

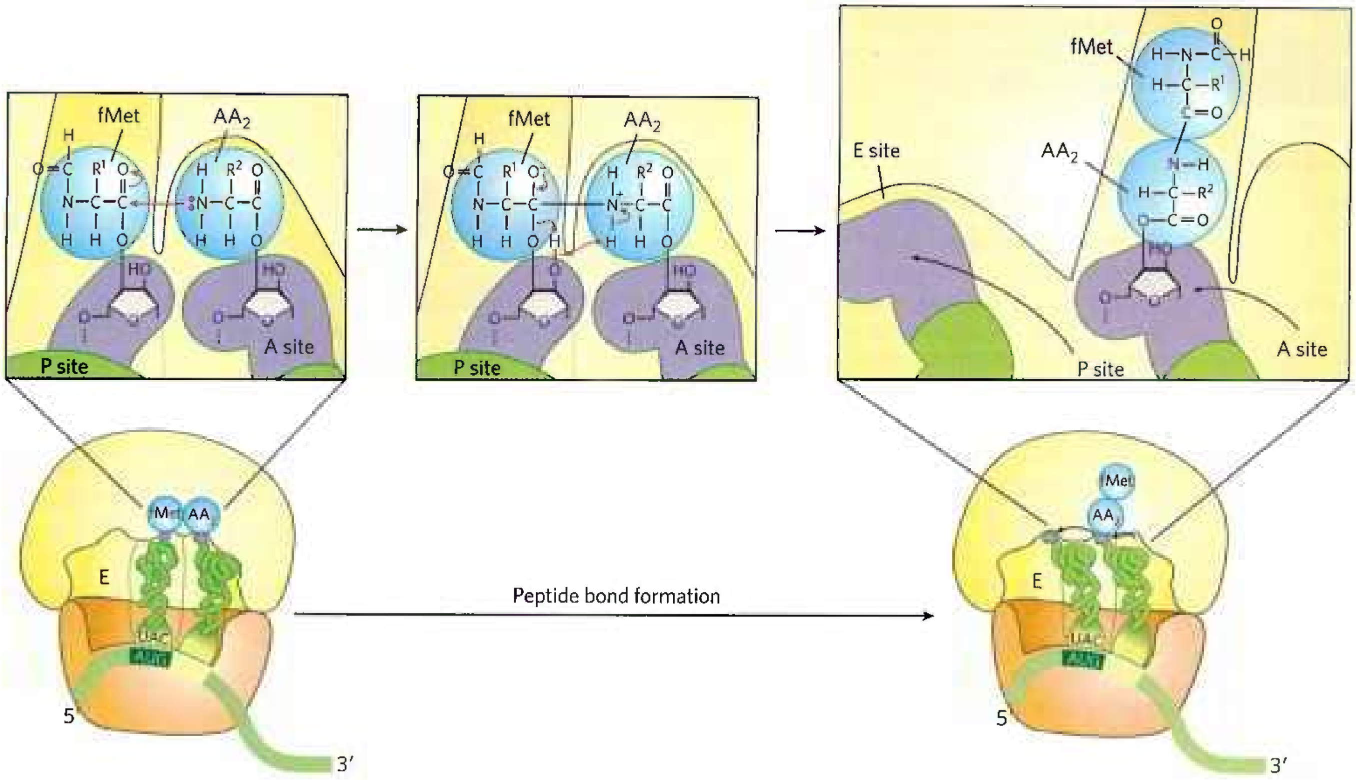


FIGURE 27-29 Second elongation step in bacteria: formation of the first peptide bond. The *N*-formylmethionyl group is transferred to the amino group of the second aminoacyl-tRNA in the A site, forming a dipeptidyl-tRNA. At this stage, both tRNAs bound to the ribosome shift position in the 50S subunit to take up a hybrid binding state. The uncharged tRNA shifts so that its 3′ and 5′ ends are in the E site. Similarly, the 3′ and 5′ ends of the peptidyl-tRNA shift to the P site. The anticodons remain in the P and A sites. Note the involvement of the 2′-hydroxyl group of the 3′-terminal adenosine as a general acid-base catalyst in this reaction.

whenever that is necessary (**Box 27-3**). The reaction produces a dipeptidyl-tRNA in the A site, and the now "uncharged" (deacylated) $tRNA^{fMet}$ remains bound to the P site. The tRNAs then shift to a hybrid binding state, with elements of each spanning two different sites on the ribosome, as shown in Figure 27-29.

P5 The **peptidyl transferase** activity that catalyzes peptide bond formation resides in the 23S rRNA rather than in any of the protein components of ribosomes. The ribosomal 23S rRNA catalyzes the reaction by binding and aligning the tRNAs in the A and P sites in the proper orientations for reaction. A highly conserved active site adenosine residue in the 23S rRNA (A2451 in *E. coli*) may facilitate the reaction by general base catalysis and transition state stabilization utilizing N-3 in the purine ring and/or the 2′-hydroxyl group. This addition to the known catalytic repertoire of ribozymes has interesting implications for the evolution of life (see Section 26.4).

Elongation Step 3: Translocation In the final step of the elongation cycle, **translocation**, the ribosome moves one codon toward the 3′ end of the mRNA (**Fig. 27-30a**). This movement shifts the anticodon of the dipeptidyl-tRNA, which is still attached to the second codon of the mRNA, from the A site to the P site, and shifts the deacylated tRNA from the P site to the E site, from where the tRNA is released into the cytosol. The third codon of the mRNA now lies in the A site and the second codon lies in the P site. Movement of the ribosome along the mRNA requires EF-G (also known as translocase) and the energy provided by hydrolysis of another molecule of GTP. Because the structure of EF-G mimics the structure of the EF-Tu–tRNA complex (Fig. 27-30b), EF-G can bind the A site and, presumably, displace the peptidyl-tRNA.

After translocation, the ribosome, with its attached dipeptidyl-tRNA and mRNA, is ready for the next elongation cycle and attachment of a third amino acid residue. This process occurs in the same way as addition of the second residue (as shown in Figs. 27-28, 27-29, and 27-30). For each amino acid residue correctly added to the growing polypeptide, two GTPs are hydrolyzed to GDP and P_i as the ribosome moves from codon to codon along the mRNA toward the 3′ end.

The polypeptide remains attached to the tRNA of the most recent amino acid to be inserted. This association maintains the functional connection between the information in the mRNA and its decoded polypeptide output. At the same time, the ester linkage between this tRNA and the carboxyl terminus of the growing

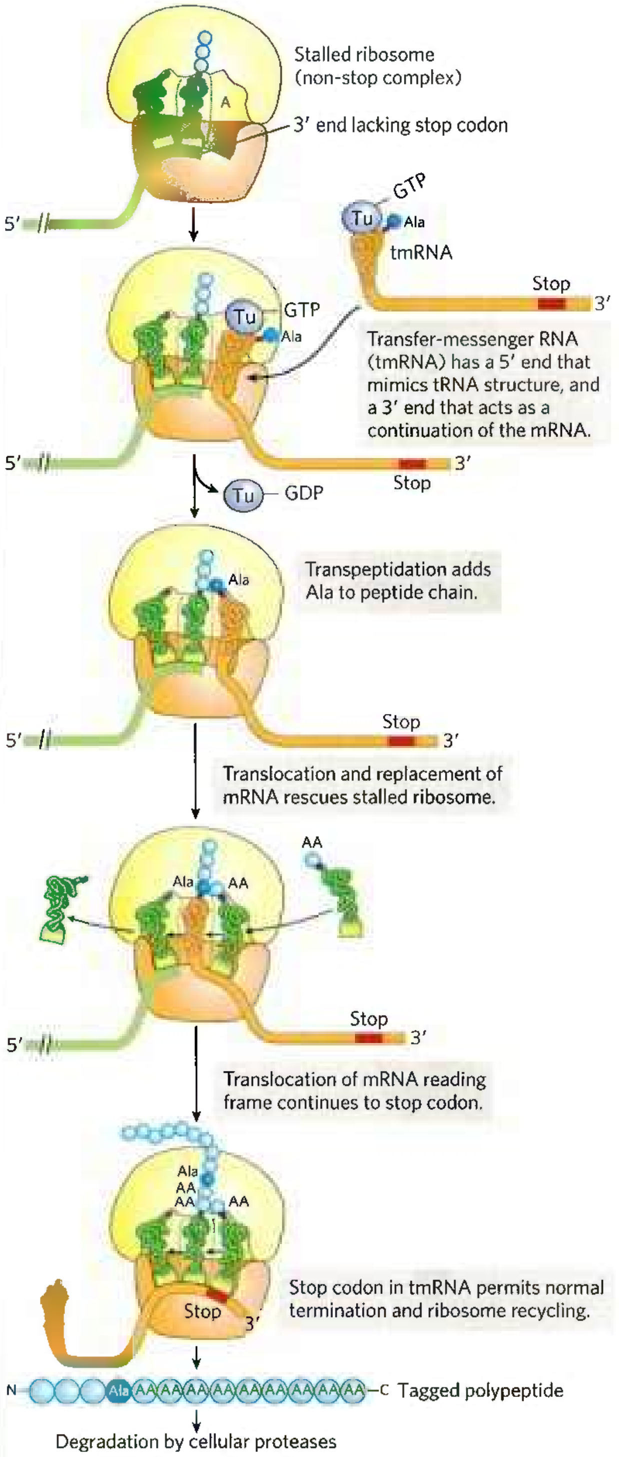
BOX 27-3

Ribosome Pausing, Arrest, and Rescue

Ribosomes may stall during protein biosynthesis, especially while translating an mRNA that is damaged or incomplete. If translation cannot proceed to the end of the gene, the termination factors cannot act, and the ribosome may become "stuck" and thus inactivated.

Translation can experience a variety of stalling events. One well-documented issue involves the addition of proline to the growing chain, particularly when two prolines must be added sequentially. Unlike the other amino acids, proline is a secondary amine and is not as good a nucleophile in the peptide bond formation step (Fig. 27-29). The constrained geometry of the proline side chain can also affect alignment of groups for reaction on the ribosome, an issue particularly acute when the peptide bond is to be between two prolines. Bacteria have an elongation factor P (EFP) that binds between the E and P sites on the ribosome next to the peptidyl-tRNA. EFP binding affects the positioning of adjacent bound tRNAs and facilitates an optimal alignment for peptide bond formation with proline. Ribosomes in cells lacking EFP stall regularly at locations where two or more proline codons must be read consecutively. In eukaryotes, a factor closely related to EFP, eIF5A, performs this same function.

When the ribosome encounters the end of an mRNA before encountering a stop codon, the translocation step leads to formation of a stable **non-stop complex**, in which the A site has no mRNA that can interact with a new charged tRNA. The non-stop complex cannot be recycled by the normal termination factors. Instead, the ribosome is rescued by **trans-translation** (Fig. 1). In virtually all bacteria, the rescue system consists of **transfer-messenger RNA (tmRNA)** and **small protein B (SmpB)**. These bind to the stalled complex in such a way that the tmRNA is positioned in the empty A site so that the ribosome can continue translation until it encounters a stop codon embedded in the tmRNA. The ribosome is then recycled, and both the defective mRNA and the polypeptide translated from it are degraded. Similar systems exist in eukaryotes.



Transfer-messenger RNA (tmRNA) has a 5′ end that mimics tRNA structure, and a 3′ end that acts as a continuation of the mRNA.

Transpeptidation adds Ala to peptide chain.

Translocation and replacement of mRNA rescues stalled ribosome.

Translocation of mRNA reading frame continues to stop codon.

Stop codon in tmRNA permits normal termination and ribosome recycling.

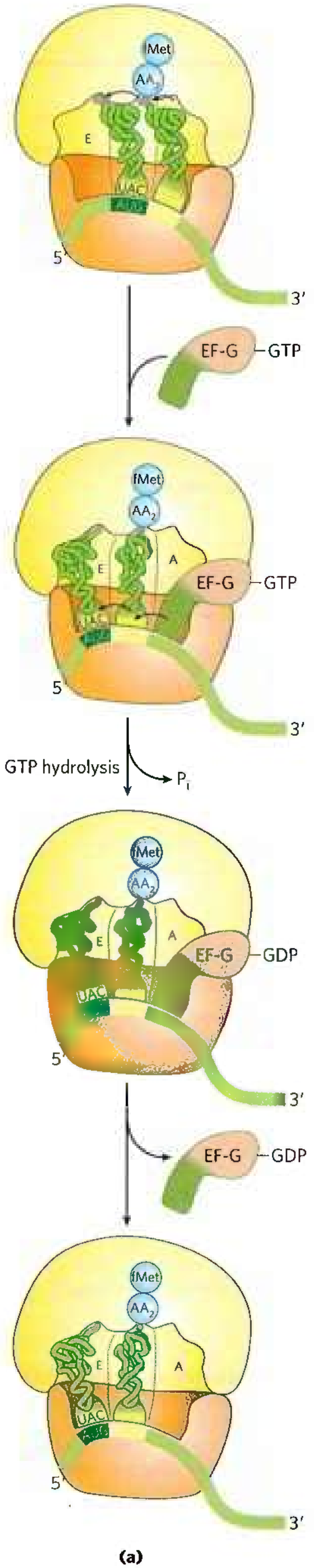
Degradation by cellular proteases

FIGURE 1 Rescue of stalled bacterial ribosomes by tmRNA. In bacteria, tmRNA rescues stalled ribosomes by mimicking both tRNA and mRNA. The multistep pathway permits release and degradation of the damaged mRNA and tags the truncated polypeptide with an abbreviated carboxyl-terminal amino acid sequence that ensures its rapid degradation.

polypeptide activates the terminal carboxyl group for nucleophilic attack by the incoming amino acid to form a new peptide bond (Fig. 27-29). As the existing ester linkage between the polypeptide and tRNA is broken during peptide bond formation, the linkage between the polypeptide and the information in the mRNA persists, because each newly added amino acid is still attached to its tRNA.

The elongation cycle in eukaryotes is similar to that in bacteria. Three eukaryotic elongation factors (eEF1α, eEF1$\beta\gamma$, and eEF2) have functions analogous to those of the bacterial elongation factors (EF-Tu, EF-Ts, and EF-G, respectively). When a new aminoacyl-tRNA binds to the A site, an allosteric interaction leads to ejection of the uncharged tRNA from the E site.

Proofreading on the Ribosome The GTPase activity of EF-Tu during the first step of elongation in bacterial cells (Fig. 27-28) makes an important contribution to the rate and fidelity of the overall biosynthetic process. Both the EF-Tu–GTP and EF-Tu–GDP complexes exist for a few milliseconds before they dissociate. These two intervals provide opportunities for the codon-anticodon interactions to be proofread. Incorrect aminoacyl-tRNAs normally dissociate from the A site during one of these periods. If the GTP analog guanosine



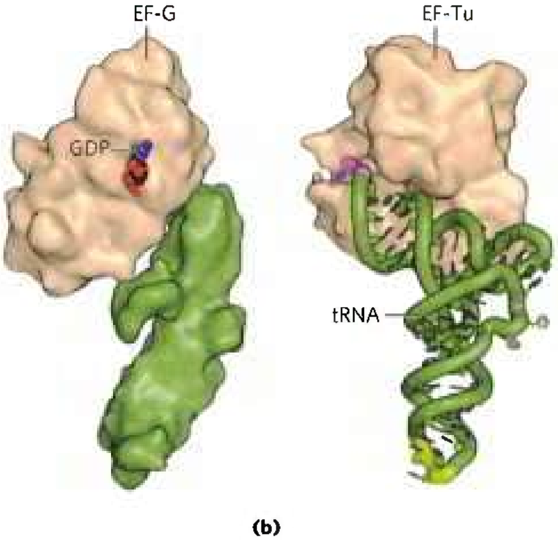
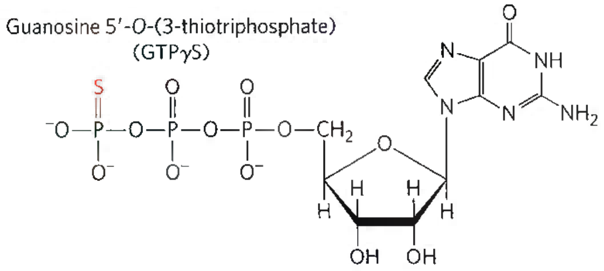


FIGURE 27-30 Third elongation step in bacteria: translocation. (a) The ribosome moves one codon toward the 3′ end of the mRNA, using energy provided by hydrolysis of GTP bound to EF-G (translocase). The dipeptidyl-tRNA is now entirely in the P site, leaving the A site open for an incoming (third) aminoacyl-tRNA. The uncharged tRNA later dissociates from the E site, and the elongation cycle begins again. (b) The structure of EF-G (left) mimics the structure of EF-Tu complexed with tRNA (right). The carboxyl-terminal part of EF-G mimics the structure of the anticodon loop of tRNA in both shape and charge distribution. [(b) Data from (left) PDB ID 1DAR, S. al-Karadaghi et al., *Structure* 4:555, 1996; (right) PDB ID 1B23, P. Nissen et al., *Structure* 7:143, 1999.]

5′-*O*-(3-thiotriphosphate) (GTPγS) is used in place of GTP, hydrolysis is slowed, improving the fidelity (by increasing the proofreading intervals) but reducing the rate of protein synthesis.


Guanosine 5′-*O*-(3-thiotriphosphate)
(GTPγS)

The process of protein synthesis (including the characteristics of codon-anticodon pairing already described) has clearly been optimized through evolution to balance the requirements for speed and fidelity. Improved fidelity might diminish speed, whereas increases in speed would probably compromise fidelity. And, recall that the proofreading mechanism on the ribosome establishes only that the proper codon-anticodon pairing has taken place, not that the correct amino acid is attached to the tRNA. If a tRNA is successfully aminoacylated with the wrong amino acid (as can be done experimentally), this incorrect amino acid is efficiently incorporated into a protein in response to whatever codon is normally recognized by the tRNA.

Stage 4: Termination of Polypeptide Synthesis Requires a Special Signal

Elongation continues until the ribosome adds the last amino acid coded by the mRNA. **Termination**, the fourth stage of polypeptide synthesis, is signaled by the presence of one of three termination codons in the mRNA (UAA, UAG, UGA), immediately following the final coded amino acid. Mutations in a tRNA anticodon that allow an amino acid to be inserted at a termination codon are generally deleterious to the cell. In bacteria, once a termination codon occupies the ribosomal A site, three **termination factors**, or **release factors**—the proteins RF1, RF2, and RF3—contribute to (1) hydrolysis of the terminal peptidyl-tRNA bond; (2) release of the free polypeptide and the last tRNA, now uncharged, from the P site; and (3) dissociation of the 70S ribosome into its 30S and 50S subunits, ready to start a new cycle of polypeptide synthesis (**Fig. 27-31**). RF1 recognizes the termination codons UAG and UAA, and RF2 recognizes UGA and UAA. Either RF1 or RF2 (depending on which codon is present) binds at a termination codon and induces peptidyl transferase to transfer the growing polypeptide to a water molecule rather than to another amino acid. The release factors have domains thought to mimic the structure of tRNA, as shown for the elongation factor EF-G in Figure 27-30b. The specific function of RF3 has not been firmly established, although it is thought to release the ribosomal subunit. In eukaryotes, a single release factor, eRF, recognizes all three termination codons.

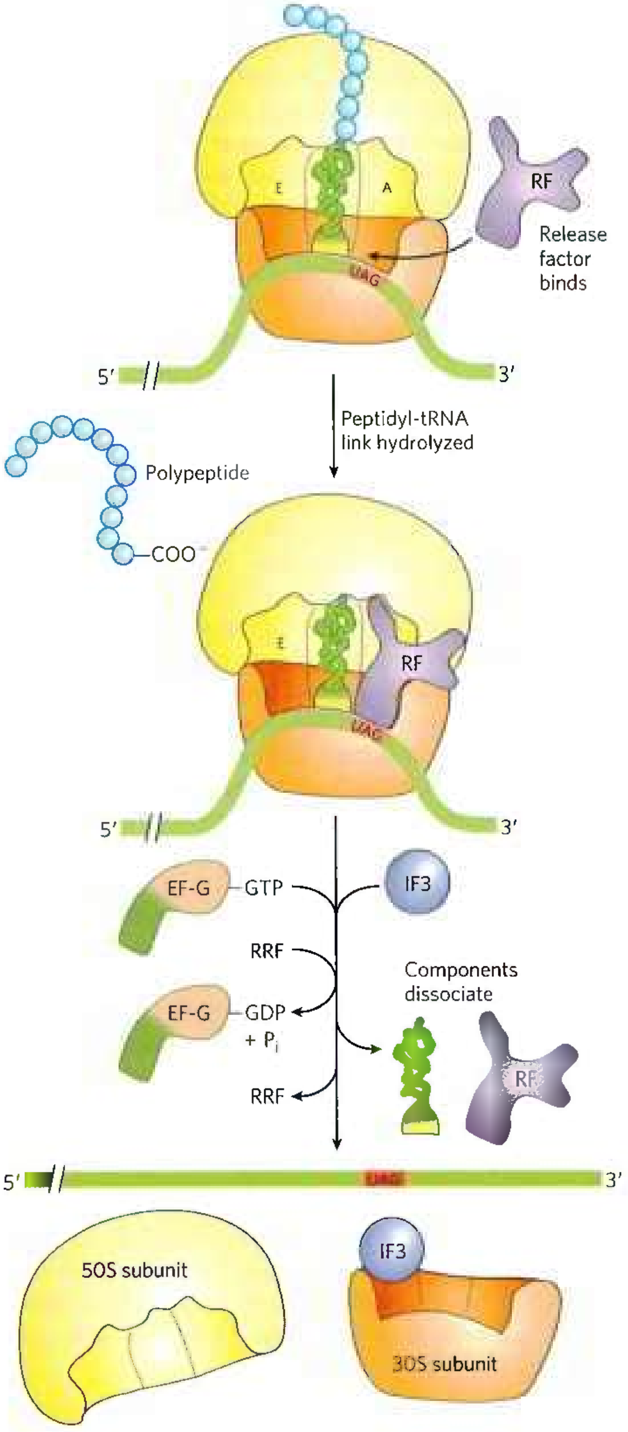


FIGURE 27-31 Termination of protein synthesis in bacteria. Synthesis is terminated in response to a termination codon in the A site. First, a release factor, RF (RF1 or RF2, depending on which termination codon is present), binds to the A site. This leads to hydrolysis of the ester linkage between the nascent polypeptide and the tRNA in the P site and release of the completed polypeptide. Finally, the mRNA, deacylated tRNA, and release factor leave the ribosome, which dissociates into its 30S and 50S subunits, aided by ribosome recycling factor (RRF), IF3, and energy provided by EF-G–mediated GTP hydrolysis. The 30S subunit complex with IF3 is ready to begin another cycle of translation.

Dissociation of the translation components leads to ribosome recycling. The release factors dissociate from the posttermination complex (with an uncharged tRNA in the P site) and are replaced by EF-G and a protein called **ribosome recycling factor (RRF**; M_r 20,300). Hydrolysis of GTP by EF-G leads to dissociation of the 50S subunit from the 30S–tRNA–mRNA complex. EF-G and RRF are replaced by IF3, which promotes dissociation of the tRNA. The mRNA is then released. The complex of IF3 and the 30S subunit is then ready to initiate another round of protein synthesis (Fig. 27-24).

Energy Cost of Fidelity in Protein Synthesis P1 Synthesis of a protein true to the information specified in its mRNA requires energy well beyond what would be required to synthesize peptide bonds linking a random sequence of amino acids. Formation of each aminoacyl-tRNA uses two high-energy phosphate groups. An additional ATP is consumed each time an incorrectly activated amino acid is hydrolyzed by the deacylation activity of an aminoacyl-tRNA synthetase as part of its proofreading activity. A GTP is cleaved to GDP and P_i during the first elongation step, and another during the translocation step. Thus, on average, the energy derived from the hydrolysis of more than four NTPs to NDPs is required for the formation of each peptide bond of a polypeptide.

This represents an exceedingly large thermodynamic "push" in the direction of synthesis: at least 4×30.5 kJ/mol = 122 kJ/mol of phosphodiester bond energy to generate a peptide bond, which has a standard free energy of hydrolysis of only about −21 kJ/mol. The net free-energy change during peptide bond synthesis is thus −101 kJ/mol. Proteins are information-containing polymers. The biochemical goal is not simply the formation of a peptide bond but the formation of a peptide bond between two *specified* amino acids. Each of the high-energy phosphate compounds expended in this process plays a critical role in maintaining proper alignment between each new codon in the mRNA and its associated amino acid at the growing end of the polypeptide. This energy permits very high fidelity in the biological translation of the genetic message of mRNA into the amino acid sequence of proteins.

Rapid Translation of a Single Message by Polysomes Large clusters of 10 to 100 ribosomes that are very active in protein synthesis can be isolated from both eukaryotic and bacterial cells. Electron micrographs show a fiber between adjacent ribosomes in the cluster, which is called a **polysome** (**Fig. 27-32a**). The connecting strand is a single molecule of mRNA that is being translated simultaneously by many closely spaced ribosomes, allowing the highly efficient use of the mRNA.

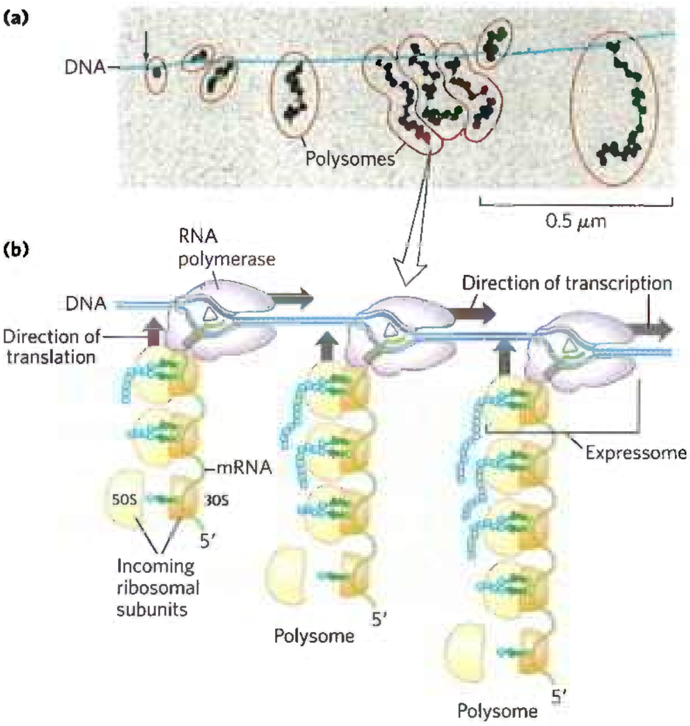


FIGURE 27-32 Coupling of transcription and translation in bacteria. (a) Electron micrograph of polysomes forming during transcription of a segment of DNA from *E. coli*. Each mRNA is being translated by many ribosomes simultaneously. The nascent polypeptide chains are difficult to see under the conditions used to prepare the sample shown here. The arrow marks the approximate beginning of the gene that is being transcribed. (b) Each mRNA is translated by ribosomes while it is still being transcribed from DNA by RNA polymerase. This is possible because the mRNA in bacteria does not have to be transported from a nucleus to the cytoplasm before encountering ribosomes. In this schematic diagram the ribosomes are depicted as smaller than the RNA polymerase. In reality, the ribosomes (M_r 2.7×10^6) are an order of magnitude larger than the RNA polymerase (M_r 3.9×10^5). [(a) O. L. Miller, Jr., et al. *Science* 169:392, 1970, Fig. 3. © 1970 American Association for the Advancement of Science.]

In bacteria, transcription and translation are tightly coupled. Messenger RNAs are synthesized and translated in the same 5′→3′ direction. As soon as the 5′ end of the mRNA appears, ribosomes and the RNA polymerase form a complex, the **expressome**, beginning translation long before transcription is complete (Fig. 27-32b). As the 5′ end of the mRNA exits one ribosome, additional ribosomes are loaded in succession to form a polysome. The situation is quite different in eukaryotic cells, where newly transcribed mRNAs must leave the nucleus before they can be translated (see Fig. 27-17).

Bacterial mRNAs generally exist for just a few minutes (p. 986) before they are degraded by nucleases. To maintain high rates of protein synthesis, the mRNA for a given protein or set of proteins must be made continuously and translated with maximum efficiency. The short lifetime of mRNAs in bacteria allows a rapid cessation of synthesis when the protein is no longer needed.

Stage 5: Newly Synthesized Polypeptide Chains Undergo Folding and Processing

In the final stage of protein synthesis, the nascent polypeptide chain is folded and processed into its biologically active form. During or after its synthesis, the polypeptide progressively assumes its native conformation. As introduced in Chapter 4, protein chaperones, chaperonins, and specific enzymes (e.g., protein disulfide isomerase and peptide prolyl cis-trans isomerase) play an important role in the correct folding of many proteins in all cells. Chaperones and chaperonins, exemplified by GroEL/GroES in bacteria (**Fig. 27-33**) and Hsp60 in eukaryotes, assist folding in part by restricting formation of unproductive aggregates and limiting the conformational space that a polypeptide may explore as it folds. **P4** ATP is hydrolyzed as part of this process. The GroEL/GroES system is required for the folding of about 10%–15% of the proteins in *E. coli*.

Some newly made proteins, bacterial, archaeal, and eukaryotic, do not attain their final biologically active conformation until they have been altered by one or more **posttranslational modifications**. Protein modifications of one type or another have been described in almost every chapter of this text, and some prominent examples are summarized here.

Amino-Terminal and Carboxyl-Terminal Modifications The first residue inserted in all polypeptides is *N*-formylmethionine (in bacteria) or methionine (in eukaryotes). However, the formyl group, the amino-terminal Met residue, and often additional amino-terminal (and, in some cases, carboxyl-terminal) residues may be removed enzymatically in formation of the final functional protein. In as many as 50% of eukaryotic proteins, the amino group of the amino-terminal residue is *N*-acetylated after translation. Carboxyl-terminal residues are also sometimes modified.

Loss of Signal Sequences As we shall see in Section 27.3, the 15 to 30 residues at the amino-terminal end of some proteins play a role in directing the protein to its ultimate destination in the cell. Such **signal sequences** are eventually removed by specific peptidases.

Modification of Individual Amino Acid Residues The hydroxyl groups of certain Ser, Thr, and Tyr residues of some proteins

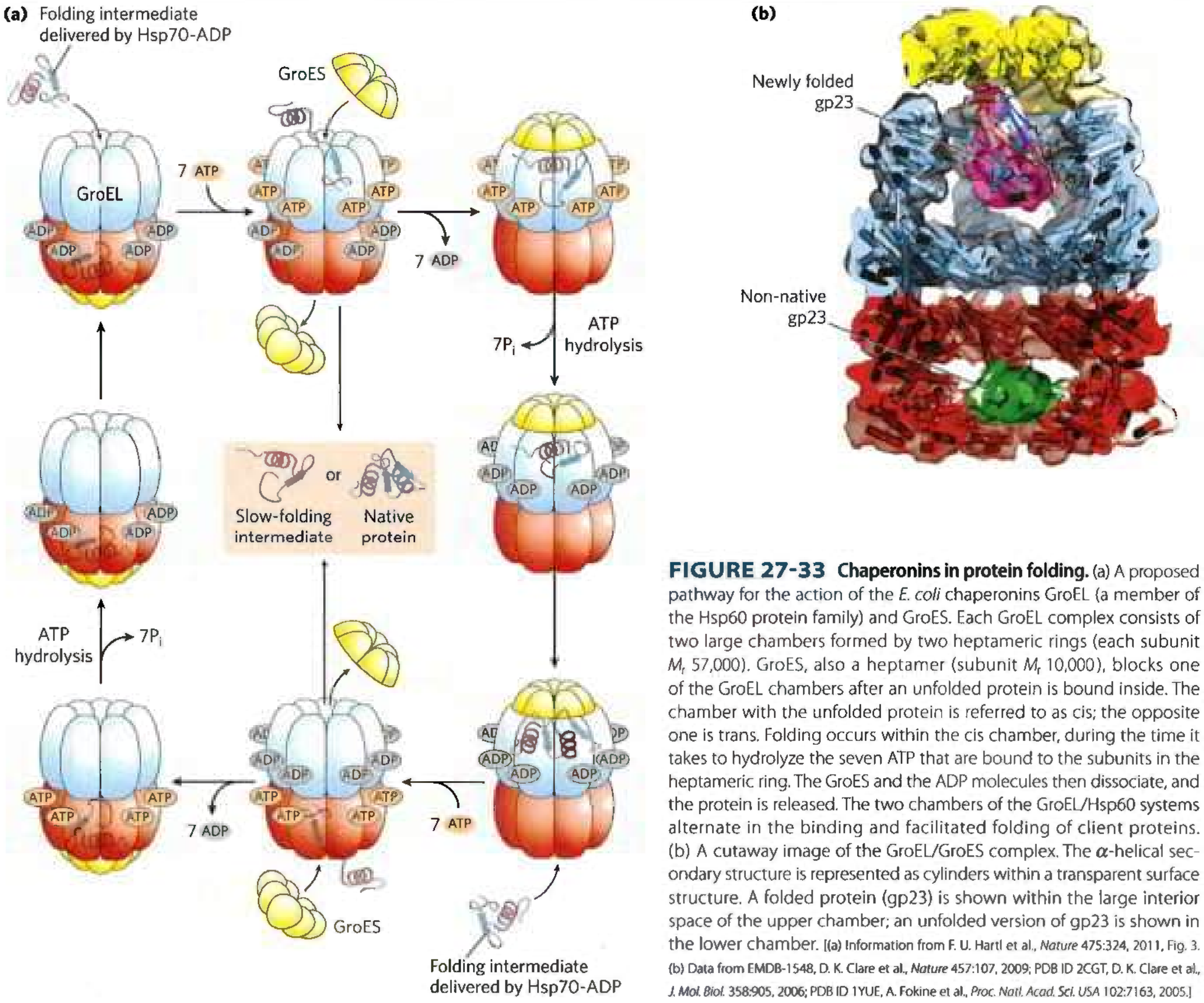

(a) Folding intermediate delivered by Hsp70-ADP
GroES
GroEL
7 ATP
ATP
ADP
7 ADP
ATP hydrolysis
$7P_i$
Slow-folding intermediate
or
Native protein
GroES
Folding intermediate delivered by Hsp70-ADP
(b)
Newly folded gp23
Non-native gp23

FIGURE 27-33 Chaperonins in protein folding. (a) A proposed pathway for the action of the *E. coli* chaperonins GroEL (a member of the Hsp60 protein family) and GroES. Each GroEL complex consists of two large chambers formed by two heptameric rings (each subunit M_r 57,000). GroES, also a heptamer (subunit M_r 10,000), blocks one of the GroEL chambers after an unfolded protein is bound inside. The chamber with the unfolded protein is referred to as cis; the opposite one is trans. Folding occurs within the cis chamber, during the time it takes to hydrolyze the seven ATP that are bound to the subunits in the heptameric ring. The GroES and the ADP molecules then dissociate, and the protein is released. The two chambers of the GroEL/Hsp60 systems alternate in the binding and facilitated folding of client proteins. (b) A cutaway image of the GroEL/GroES complex. The α-helical secondary structure is represented as cylinders within a transparent surface structure. A folded protein (gp23) is shown within the large interior space of the upper chamber; an unfolded version of gp23 is shown in the lower chamber. [(a) Information from F. U. Hartl et al., *Nature* 475:324, 2011, Fig. 3. (b) Data from EMDB-1548, D. K. Clare et al., *Nature* 457:107, 2009; PDB ID 2CGT, D. K. Clare et al., *J. Mol. Biol.* 358:905, 2006; PDB ID 1YUE, A. Fokine et al., *Proc. Natl. Acad. Sci. USA* 102:7163, 2005.]

are enzymatically phosphorylated by ATP (**Fig. 27-34a**); the phosphate groups add negative charges to these polypeptides. The functional significance of this modification varies from one protein to the next. For example, the milk protein casein has many phosphoserine groups that bind Ca^{2+}. Calcium, phosphate, and amino acids are all valuable to suckling young, so casein efficiently provides three essential nutrients. And as we have seen in numerous instances, phosphorylation-dephosphorylation cycles regulate the activity of many enzymes and regulatory proteins.

Extra carboxyl groups may be added to Glu residues of some proteins. For example, the blood-clotting protein prothrombin contains γ-carboxyglutamate residues (Fig. 27-34b) in its amino-terminal region; the γ-carboxyl groups are introduced by an enzyme that requires vitamin K. These carboxyl groups bind Ca^{2+}, which is required to initiate the clotting mechanism.

Monomethyl- and dimethyllysine residues (Fig. 27-34c) occur in some muscle proteins and in cytochrome *c*. The calmodulin of most species contains one trimethyllysine residue at a specific position. In other proteins, the carboxyl groups of some Glu residues undergo methylation, removing their negative charge.

Attachment of Carbohydrate Side Chains The carbohydrate side chains of glycoproteins are attached covalently during or after synthesis of the polypeptide. In some glycoproteins, the carbohydrate side chain is attached enzymatically to Asn residues (*N*-linked oligosaccharides), in others to Ser or Thr residues (*O*-linked oligosaccharides; see Fig. 7-27). Many proteins that function extracellularly, as well as the lubricating proteoglycans that coat mucous membranes, contain oligosaccharide side chains (see Fig. 7-25).

Addition of Isoprenyl Groups Some eukaryotic proteins are modified by the addition of groups derived from isoprene (isoprenyl groups). A thioether bond is formed between the isoprenyl group and a Cys residue of the protein (see Fig. 11-16). The isoprenyl groups are derived from pyrophosphorylated intermediates of the cholesterol biosynthetic pathway (see Fig. 21-36), such as farnesyl pyrophosphate (**Fig. 27-35**). Proteins modified in this way include the Ras proteins (small G proteins), which are products of the *ras* oncogenes and proto-oncogenes, and the trimeric G proteins (both discussed in Chapter 12), as well as lamins, proteins found in the nuclear matrix. The isoprenyl group helps to anchor the protein in a membrane. The transforming (carcinogenic) activity of the *ras* oncogene is lost when isoprenylation of the Ras protein is blocked, a finding that has stimulated interest in identifying inhibitors of this posttranslational modification pathway for use in cancer chemotherapy.

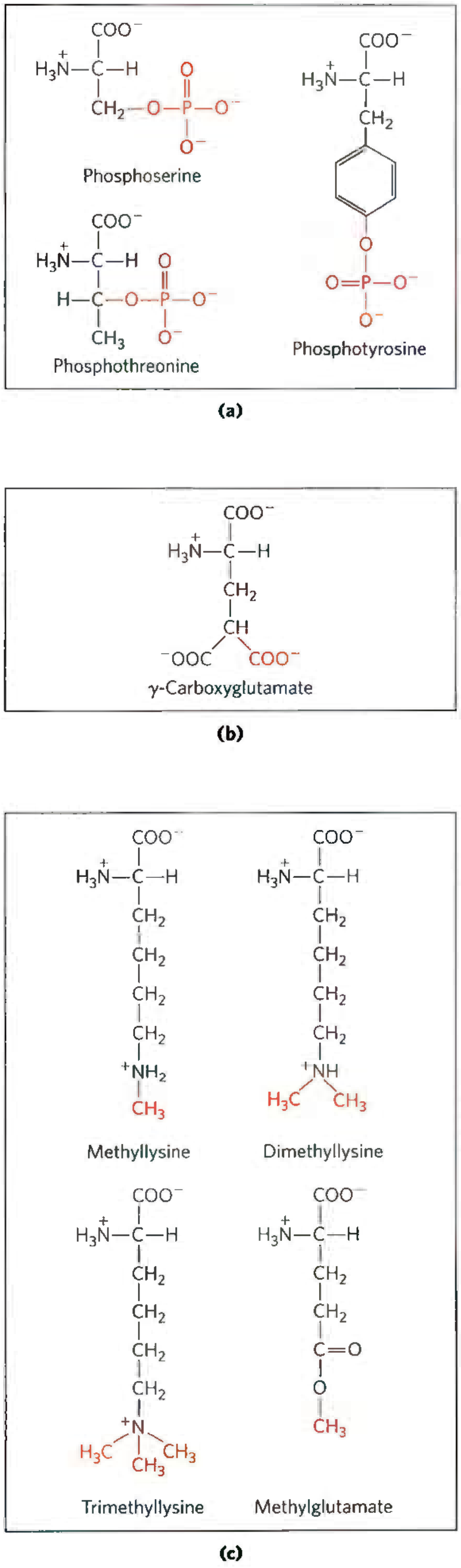


FIGURE 27-34 **Some modified amino acid residues.** (a) Phosphorylated amino acids. (b) A carboxylated amino acid. (c) Some methylated amino acids.

Addition of Prosthetic Groups Many proteins require for their activity covalently bound prosthetic groups. Two examples are the biotin molecule of acetyl-CoA carboxylase and the heme group of hemoglobin or cytochrome *c*.

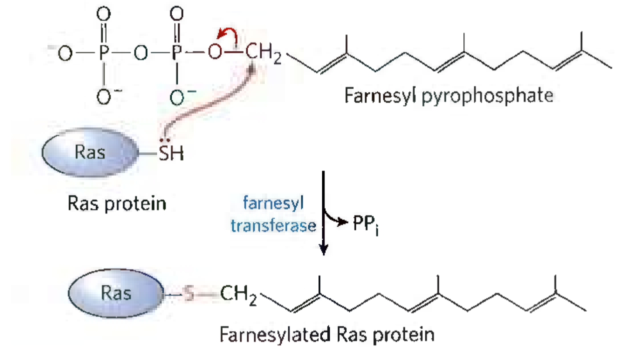

O
O
−O—P—O—P—O—CH2
O−
O−
Farnesyl pyrophosphate
Ras
SH
Ras protein
farnesyl transferase
PPi
Ras
S
CH2
Farnesylated Ras protein

FIGURE 27-35 Farnesylation of a Cys residue. The thioether linkage is shown in red. The Ras protein is the product of the *ras* oncogene.

Proteolytic Processing Many proteins are initially synthesized as large, inactive precursor polypeptides that are proteolytically trimmed to form their smaller, active forms. Examples include proinsulin (Fig. 23-4), some viral proteins (Fig. 26-30), and proteases such as chymotrypsinogen and trypsinogen (Fig. 6-42).

Formation of Disulfide Cross-Links After folding into their native conformations, some proteins form intrachain or interchain disulfide bridges between Cys residues. In eukaryotes, disulfide bonds are common in proteins to be exported from cells. The cross-links formed in this way help to protect the native conformation of the protein molecule from denaturation in the extracellular environment, which can differ greatly from intracellular conditions and is generally oxidizing.

Protein Synthesis Is Inhibited by Many Antibiotics and Toxins

Protein synthesis is a central function in cellular physiology and is the primary target of many naturally occurring antibiotics and toxins. Except as noted otherwise, these antibiotics inhibit protein synthesis in bacteria. The differences between bacterial and eukaryotic protein synthesis, though in some cases subtle, are such that most of the compounds discussed below are relatively harmless to eukaryotic cells. Natural selection has favored the evolution of compounds that exploit minor differences in order to affect bacterial systems selectively, so that these biochemical weapons are synthesized by some microorganisms and are extremely toxic to others. Because nearly every step in protein synthesis can be specifically inhibited by one antibiotic or another, antibiotics have become valuable tools in the study of protein biosynthesis.

Puromycin, made by the mold *Streptomyces alboniger*, is one of the best-understood inhibitory antibiotics. Its structure is very similar to the 3′ end of an aminoacyl-tRNA, enabling it to bind to the ribosomal A site and participate in peptide bond formation, producing peptidylpuromycin (**Fig. 27-36**). However, because

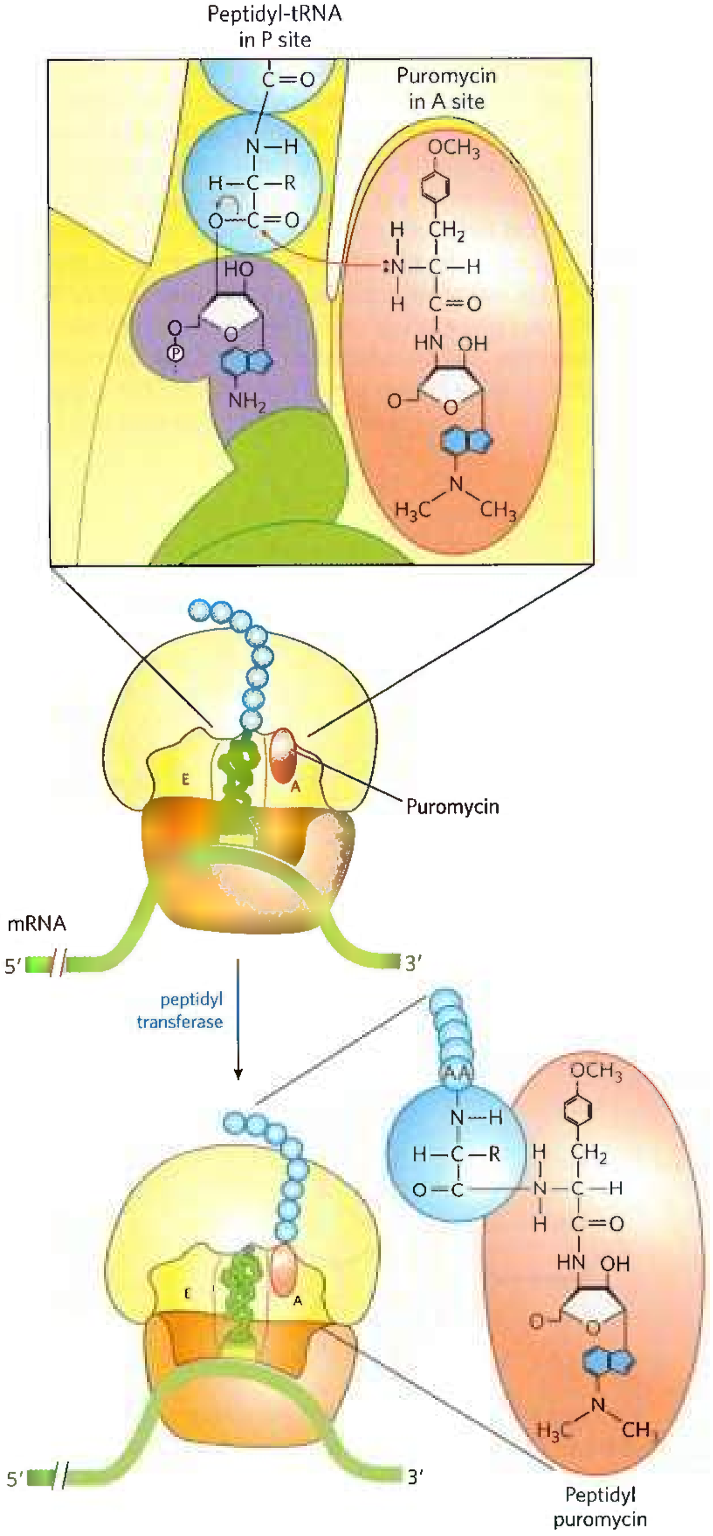
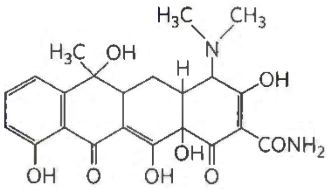
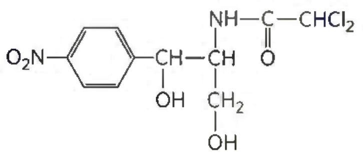

Peptidyl-tRNA in P site
Puromycin in A site
C=O
N—H
H—C—R
O—C=O
HO
O
P
NH2
OCH3
H CH2
N—C—H
H C=O
HN OH
O
N
H3C CH3
E
A
Puromycin
mRNA
5′
3′
peptidyl transferase
AA
N—H
H—C—R
O=C
H CH2
N—C—H
H C=O
HN OH
O
N
H3C CH3
E
A
5′
3′
Peptidyl puromycin

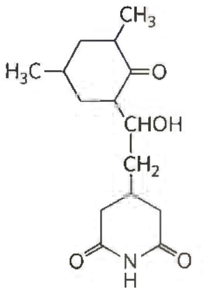
FIGURE 27-36 Disruption of peptide bond formation by puromycin. The antibiotic puromycin resembles the aminoacyl end of a charged tRNA, and it can bind to the ribosomal A site and participate in peptide bond formation. The product of this reaction, peptidyl puromycin, is not translocated to the P site. Instead, it dissociates from the ribosome, causing premature chain termination.

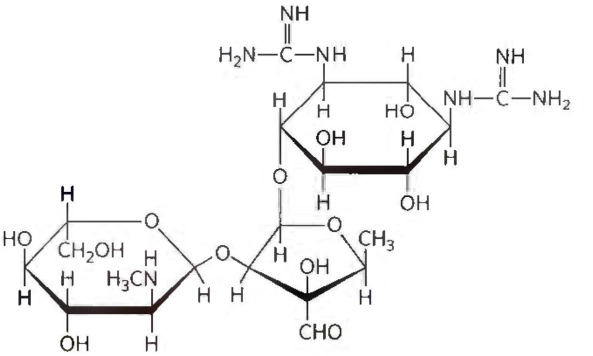
puromycin resembles only the 3′ end of the tRNA, it does not engage in translocation and dissociates from the ribosome shortly after it is linked to the carboxyl terminus of the peptide. This prematurely terminates polypeptide synthesis.

Tetracyclines inhibit protein synthesis in bacteria by blocking the A site on the ribosome, preventing the binding of aminoacyl-tRNAs. **Chloramphenicol** inhibits protein synthesis by bacterial (and mitochondrial and chloroplast) ribosomes by blocking peptidyl transfer; it does not affect cytosolic protein synthesis in eukaryotes. Conversely, **cycloheximide** blocks the peptidyl transferase of 80S eukaryotic ribosomes but not that of 70S bacterial (and mitochondrial and chloroplast) ribosomes. **Streptomycin**, a basic trisaccharide, causes misreading of the genetic code (in bacteria) at relatively low concentrations and inhibits initiation at higher concentrations.


Tetracycline


Chloramphenicol


Cycloheximide


Streptomycin

Several other inhibitors of protein synthesis are notable because of their toxicity to humans and other mammals. Diphtheria is a serious bacterial illness that causes sore throat, swollen glands, breathing difficulties, and often death. Although it has been largely eradicated in the developed world, a few thousand cases still occur each year in countries where vaccination is limited. The bacterium *Corynebacterium diphtheriae* releases the **diphtheria toxin** (M_r 58,330), which catalyzes the ADP-ribosylation of a diphthamide (a modified histidine) residue of eukaryotic elongation factor eEF2, thereby inactivating it. The resulting dead cells form a thick, gray membrane that covers the throat and tonsils, creating a putrid odor that is one hallmark of the disease. **Ricin** (M_r 29,895), an extremely toxic protein of the castor bean, inactivates the 60S subunit of eukaryotic ribosomes by depurinating a specific adenosine residue in 28S rRNA. Ricin was used in the infamous 1978 murder of BBC journalist and Bulgarian dissident Georgi Markov, presumably by the Bulgarian secret police. Using a syringe hidden at the end of an umbrella, a member of the secret police injected Markov in the leg with a ricin-infused pellet. He died four days later. ■

SUMMARY 27.2 *Protein Synthesis*

- Protein synthesis occurs on the ribosomes, which consist of protein and rRNA. Bacteria have 70S ribosomes, with a large (50S) and a small (30S) subunit. Eukaryotic ribosomes are significantly larger (80S) and contain more proteins. The growth of polypeptides on ribosomes begins with the amino-terminal amino acid and proceeds by successive additions of new residues to the carboxyl-terminal end.
- Transfer RNAs have 73 to 93 nucleotide residues, some of which have modified bases. Each tRNA has an amino acid arm with the terminal sequence CCA(3′) to which an amino acid is esterified, an anticodon arm, a TψC arm, and a D arm; some tRNAs have a fifth arm. The anticodon is responsible for the specificity of interaction between the aminoacyl-tRNA and the complementary mRNA codon.
- In stage 1 of the five stages of protein synthesis, amino acids are activated by specific aminoacyl-tRNA synthetases in the cytosol. These enzymes catalyze the formation of aminoacyl-tRNAs, with simultaneous cleavage of ATP to AMP and PP_i. The fidelity of protein synthesis depends on the accuracy of this reaction, and some of these enzymes carry out proofreading steps at separate active sites.
- Stage 2 is initiation. In bacteria, the initiating aminoacyl-tRNA in all proteins is *N*-formylmethionyl-$\text{tRNA}^{\text{fMet}}$. Initiation of protein synthesis involves formation of a complex between the 30S ribosomal subunit, mRNA, GTP, fMet-$\text{tRNA}^{\text{fMet}}$, three initiation factors, and the 50S subunit; GTP is hydrolyzed to GDP and P_i.
- Stage 3 is elongation. In the elongation steps, GTP and elongation factors are required for binding the incoming aminoacyl-tRNA to the A site on the ribosome. In the first peptidyl transfer reaction, the fMet residue is transferred to the amino group of the incoming aminoacyl-tRNA. Movement of the ribosome along the mRNA then translocates the dipeptidyl-tRNA from the A site to the P site, a process requiring hydrolysis of GTP. Deacylated tRNAs dissociate from the ribosomal E site.
- Stage 4 is termination. After many such elongation cycles, synthesis of the polypeptide is terminated with

the aid of release factors. At least four high-energy phosphate equivalents (from ATP and GTP) are required to generate each peptide bond, an energy investment required to guarantee fidelity of translation.

- Stage 5 is protein processing. Polypeptides fold into their active, three-dimensional forms. Many proteins are further processed by posttranslational modification reactions.
- Many well-studied antibiotics and toxins inhibit some aspect of protein synthesis.

27.3 Protein Targeting and Degradation

The eukaryotic cell is made up of many structures, compartments, and organelles, each with specific functions that require distinct sets of proteins and enzymes. These proteins (with the exception of those produced in mitochondria and plastids) are synthesized on ribosomes in the cytosol, so how are they directed to their final cellular destinations?

We are now beginning to understand this complex and fascinating process. Proteins destined for secretion, integration in the plasma membrane, or inclusion in lysosomes generally share the first few steps of a pathway that begins in the endoplasmic reticulum. Proteins destined for mitochondria, chloroplasts, or the nucleus use three separate mechanisms. And proteins destined for the cytosol simply remain where they are synthesized. **P1** The thermodynamic cost of protein synthesis is magnified by the processes used by cells to transport proteins to their correct cellular locations.

The most important element in many of these targeting pathways is a short sequence of amino acids called a **signal sequence**, whose function was first postulated by Günter Blobel and colleagues in 1970. The signal sequence directs a protein to its appropriate location in the cell and, for many proteins, is removed during transport or after the protein has reached its final destination. In proteins slated for transport into mitochondria, chloroplasts, or the ER, the signal sequence is at the amino terminus of a newly synthesized polypeptide. In many cases, the targeting capacity of particular signal sequences has been confirmed by fusing the signal sequence from one protein to a second protein and showing that the signal directs the second protein to the location where the first protein is normally found. The selective degradation of proteins no longer needed by the cell also relies largely on a set of molecular signals embedded in each protein's structure.

In this concluding section we examine protein targeting and degradation, emphasizing the underlying signals and molecular regulation that are so crucial to cellular metabolism. Except where noted, the focus is now on eukaryotic cells.

Posttranslational Modification of Many Eukaryotic Proteins Begins in the Endoplasmic Reticulum

Perhaps the best-characterized targeting system begins in the ER. Most lysosomal, membrane, or secreted proteins have an amino-terminal signal sequence (**Fig. 27-37**) that marks them for translocation into the lumen of the ER; hundreds of such signal sequences have been determined. The carboxyl terminus of the signal sequence is defined by a cleavage site, where protease action removes the sequence after the protein is imported into the ER. Signal sequences vary in length from 13 to 36 amino acid residues, but all have the following features: (1) about 10 to 15 hydrophobic amino acid residues; (2) one or more positively charged residues, usually near the amino terminus, preceding the hydrophobic sequence; and (3) a short sequence at the carboxyl terminus (near the cleavage site) that is relatively polar, typically having amino acid residues with short side chains (especially Ala) at the positions closest to the cleavage site.

As originally demonstrated by the cell biologist George Palade, proteins with these signal sequences are synthesized on ribosomes attached to the ER. The signal sequence itself helps to direct the ribosome to the ER, as illustrated in **Figure 27-38**. The targeting pathway begins in step ①, with initiation of protein synthesis on

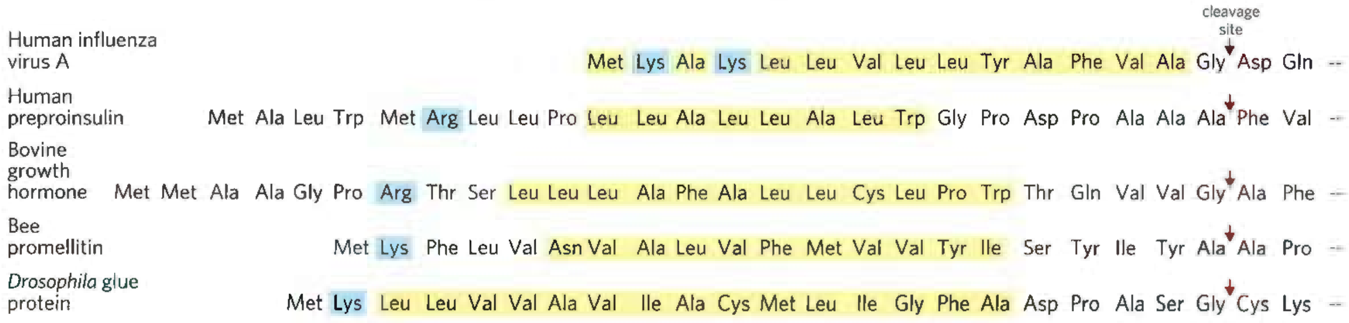

cleavage site
Human influenza virus A
Met Lys Ala Lys Leu Leu Val Leu Leu Tyr Ala Phe Val Ala Gly Asp Gln --
Human preproinsulin
Met Ala Leu Trp Met Arg Leu Leu Pro Leu Leu Ala Leu Leu Ala Leu Trp Gly Pro Asp Pro Ala Ala Ala Phe Val --
Bovine growth hormone
Met Met Ala Ala Gly Pro Arg Thr Ser Leu Leu Leu Ala Phe Ala Leu Leu Cys Leu Pro Trp Thr Gln Val Val Gly Ala Phe --
Bee promellitin
Met Lys Phe Leu Val Asn Val Ala Leu Val Phe Met Val Val Tyr Ile Ser Tyr Ile Tyr Ala Ala Pro --
Drosophila glue protein
Met Lys Leu Leu Val Val Ala Val Ile Ala Cys Met Leu Ile Gly Phe Ala Asp Pro Ala Ser Gly Cys Lys --

FIGURE 27-37 Amino-terminal signal sequences of some eukaryotic proteins that direct their translocation into the ER. The hydrophobic core (yellow) is preceded by one or more basic residues (blue). Polar and short-side-chain residues immediately precede (to the left, as shown here) the cleavage sites (indicated by red arrows).

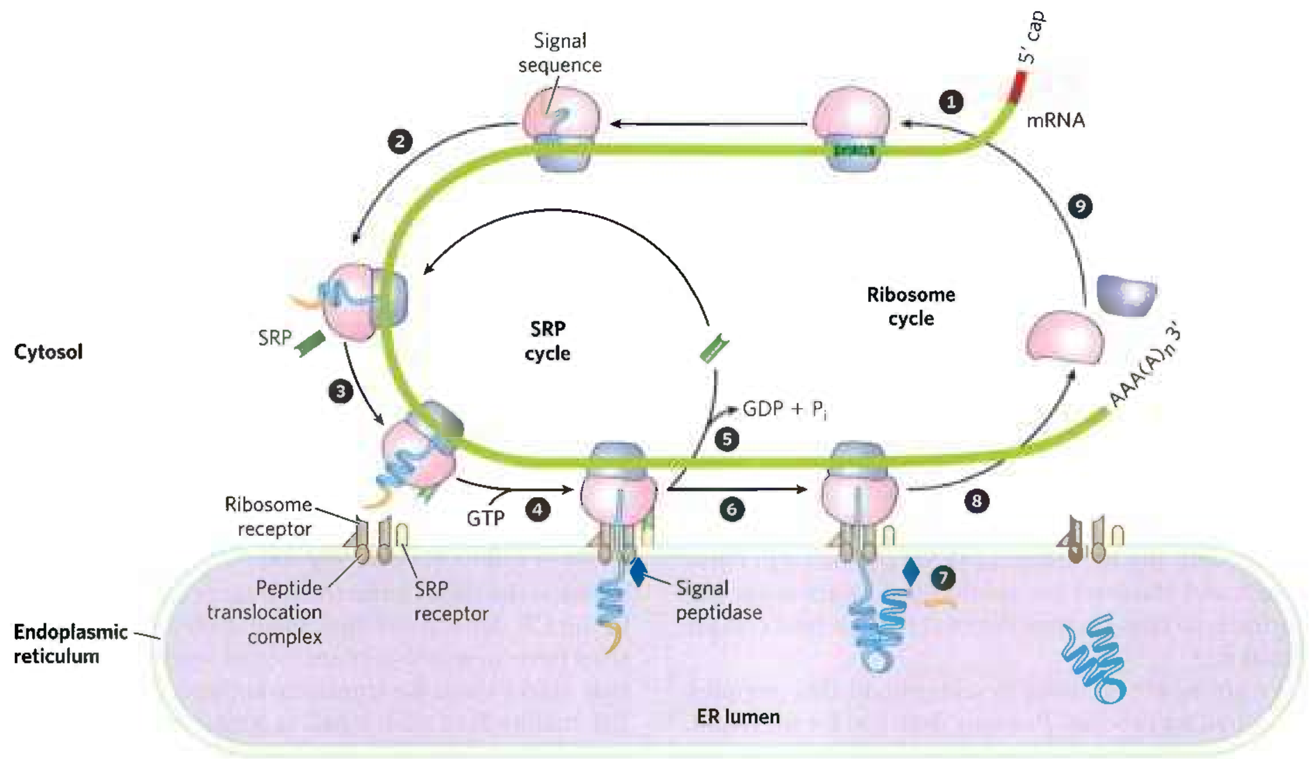


FIGURE 27-38 Directing eukaryotic proteins with the appropriate signals to the endoplasmic reticulum. This process involves the SRP cycle and the translocation and cleavage of the nascent polypeptide. One protein subunit of SRP binds directly to the signal sequence, obstructing elongation by sterically blocking the entry of aminoacyl-tRNAs and inhibiting peptidyl transferase. Another protein subunit binds and hydrolyzes GTP.

free ribosomes. The signal sequence appears early in the synthetic process (step ②), because it is at the amino terminus, which, as we have seen, is synthesized first. As it emerges from the ribosome (step ③), the signal sequence—and the ribosome itself—is bound by the large **signal recognition particle (SRP)**. The SRP is a rod-shaped complex containing a 300 nucleotide RNA (7SL-RNA) and six different proteins (combined M_r 325,000). The SRP then binds GTP and halts elongation of the polypeptide when it is about 70 amino acids long and the signal sequence has completely emerged from the ribosome. In step ④, the GTP-bound SRP directs the ribosome (still bound to the mRNA) and the incomplete polypeptide to GTP-bound SRP receptors in the cytosolic face of the ER; the nascent polypeptide is delivered to a **peptide translocation complex** in the ER, which interacts directly with the ribosome. In step ⑤, the SRP dissociates from the ribosome, accompanied by hydrolysis of GTP in both the SRP and the SRP receptor. The SRP receptor is a heterodimer of α (M_r 69,000) and β (M_r 30,000) subunits, both of which bind and hydrolyze multiple GTP molecules during this process.

Elongation of the polypeptide now resumes (step ⑥), with the ATP-driven translocation complex feeding the growing polypeptide into the ER lumen until the complete protein has been synthesized. In step ⑦, the signal sequence is removed by a signal peptidase within the ER lumen. The ribosome dissociates (step ⑧) and is recycled (step ⑨).

Glycosylation Plays a Key Role in Protein Targeting

In the ER lumen, newly synthesized proteins are further modified in several ways. Following the removal of signal sequences, polypeptides are folded, disulfide bonds are formed, and many proteins are glycosylated to form glycoproteins. In many glycoproteins, the linkage to their oligosaccharides is through Asn residues. These *N*-linked oligosaccharides are diverse (Chapter 7), but the pathways by which they form have a common first step. A 14 residue core oligosaccharide is built up stepwise, first on the cytosolic face of the membrane and then on the lumenal face. Once completed, it is transferred from a dolichol phosphate donor molecule to certain Asn residues in the protein (Fig. 27-39). The transferase is on the lumenal face of the ER and thus cannot catalyze glycosylation of cytosolic proteins. After transfer, the core oligosaccharide is trimmed and elaborated in different ways on different proteins, but all *N*-linked oligosaccharides retain a pentasaccharide core derived from the original 14 residue oligosaccharide.

Several antibiotics act by interfering with one or more steps in this process and have aided in elucidating the steps of protein glycosylation. The best characterized is **tunicamycin**, which mimics the structure of UDP-*N*-acetylglucosamine and blocks the first step of the process (Fig. 27-39, step ①). A few proteins are *O*-glycosylated in the ER, but most *O*-glycosylation occurs in the Golgi complex or in the cytosol (for proteins that do not enter the ER).

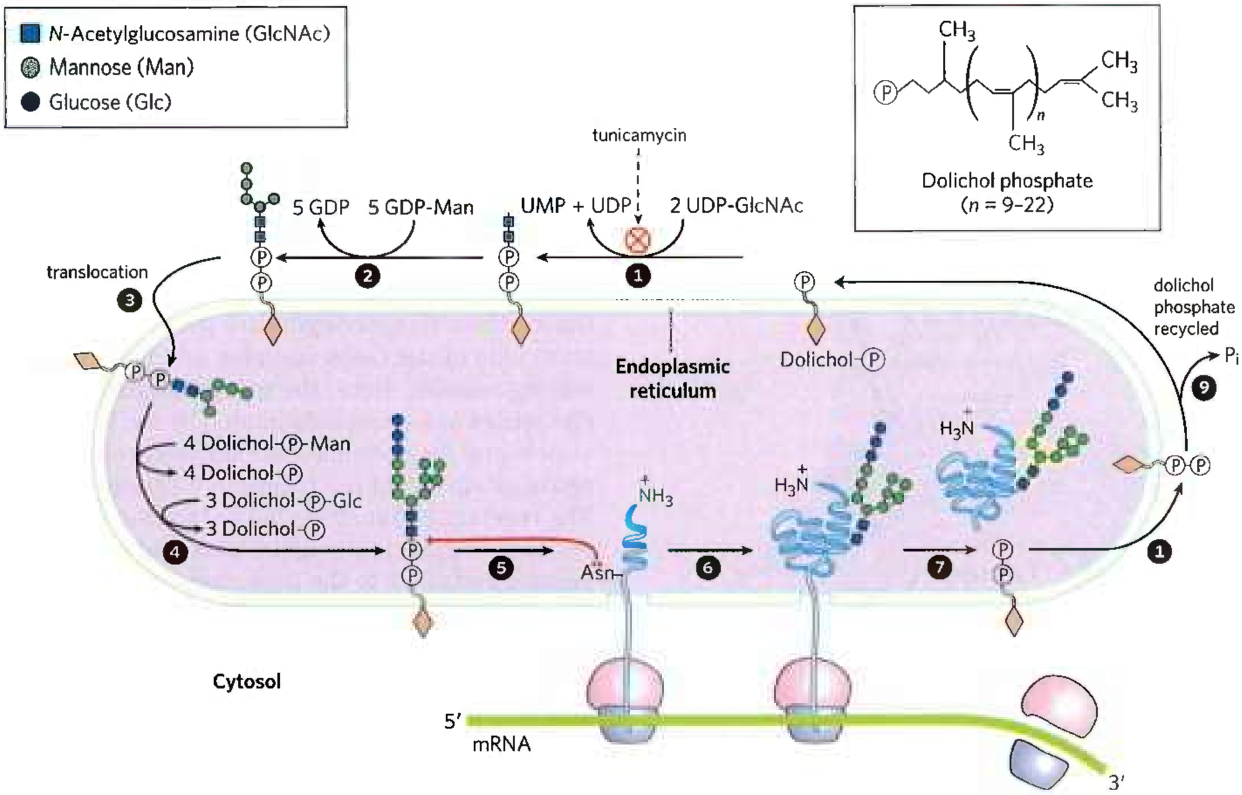
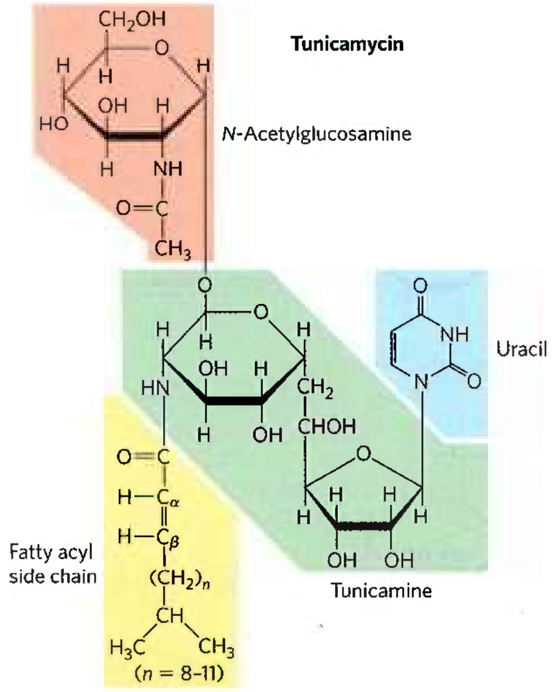

■ N-Acetylglucosamine (GlcNAc)
● Mannose (Man)
● Glucose (Glc)
CH_3
CH_3
CH_3
n
CH_3
Ⓟ
Dolichol phosphate
(n = 9–22)
tunicamycin
5 GDP
5 GDP-Man
UMP + UDP
2 UDP-GlcNAc
translocation
③
②
①
Ⓟ
Ⓟ
Endoplasmic reticulum
Dolichol-Ⓟ
dolichol phosphate recycled
P_i
⑨
4 Dolichol-Ⓟ-Man
4 Dolichol-Ⓟ
3 Dolichol-Ⓟ-Glc
3 Dolichol-Ⓟ
④
⑤
⑥
⑦
①
$\overset{+}{N}H_3$
$H_3\overset{+}{N}$
$H_3\overset{+}{N}$
Asn
Cytosol
5′
mRNA
3′

FIGURE 27-39 Synthesis of the core oligosaccharide of glycoproteins. The core oligosaccharide is built up by the successive addition of monosaccharide units. ①, ② The first steps occur on the cytosolic face of the ER. ③ Translocation moves the incomplete oligosaccharide across the membrane (mechanism not shown), and ④ completion of the core oligosaccharide occurs within the lumen of the ER. The precursors that contribute additional mannose and glucose residues to the growing oligosaccharide in the lumen are dolichol phosphate derivatives. ⑤ In the first step in construction of the *N*-linked oligosaccharide moiety of a glycoprotein, the core oligosaccharide is transferred from dolichol phosphate to an Asn residue of the protein, and ⑥ protein synthesis continues. The core oligosaccharide is then further modified in the ER and the Golgi complex in pathways that differ for different proteins. The five sugar residues shown surrounded by a beige screen, after step ⑦, are retained in the final structure of all *N*-linked oligosaccharides. ⑧ The released dolichol pyrophosphate is again translocated so that the pyrophosphate is on the cytosolic face of the ER, then ⑨ a phosphate is hydrolytically removed to regenerate dolichol phosphate.


Tunicamycin
N-Acetylglucosamine
Uracil
Tunicamine
Fatty acyl side chain
CH_2OH
O
H
H
H
OH
H
HO
H
NH
O=C
CH_3
O
O
H
H
H
HN
OH
H
CH_2
H
OH
CHOH
O=C
$H-C_\alpha$
$H-C_\beta$
$(CH_2)_n$
CH
H_3C
CH_3
(n = 8-11)
O
NH
N
O
O
H
H
H
H
OH
OH

Suitably modified proteins can now be moved to a variety of intracellular destinations. Proteins travel from the ER to the Golgi complex in transport vesicles (**Fig. 27-40**). In the Golgi complex, oligosaccharides are *O*-linked to some proteins, and *N*-linked oligosaccharides are further modified. By mechanisms not yet fully understood, the Golgi complex also sorts proteins and sends them to their final destinations. The processes that segregate proteins targeted for secretion from those targeted for the plasma membrane or lysosomes must distinguish among these proteins on the basis of structural features other than signal sequences, which were removed in the ER lumen.

This sorting process is best understood in the case of hydrolases destined for transport to lysosomes. On arrival of a hydrolase (a glycoprotein) in the Golgi complex, an as yet undetermined feature (sometimes called a signal patch) of the three-dimensional structure of the hydrolase is recognized by a phosphotransferase, which phosphorylates terminal mannose residues in the oligosaccharides (**Fig. 27-41**). The presence of one or more mannose 6-phosphate residues in its *N*-linked

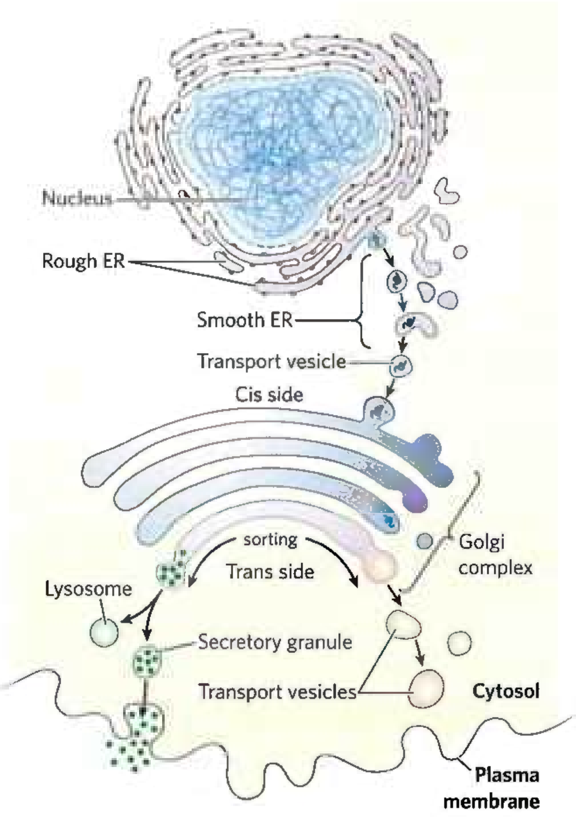

Nucleus
Rough ER
Smooth ER
Transport vesicle
Cis side
sorting
Trans side
Golgi complex
Lysosome
Secretory granule
Transport vesicles
Cytosol
Plasma membrane

FIGURE 27-40 **Pathway taken by proteins destined for lysosomes, the plasma membrane, or secretion.** Proteins are moved from the ER to the cis side of the Golgi complex in transport vesicles. Sorting occurs primarily in the trans side of the Golgi complex.

oligosaccharide is the structural signal that targets a protein to lysosomes. A receptor protein in the membrane of the Golgi complex recognizes the mannose 6-phosphate signal and binds the hydrolase so marked. Vesicles containing these receptor-hydrolase complexes bud from the trans side of the Golgi complex and make their way to sorting vesicles. Here, the receptor-hydrolase complex dissociates in a process facilitated by the lower pH in the vesicle and by phosphatase-catalyzed removal of phosphate groups from the mannose 6-phosphate residues. The receptor is then recycled to the Golgi complex, and vesicles containing the hydrolases bud from the sorting vesicles and move to the lysosomes. In cells treated with tunicamycin (Fig. 27-39, step ①), hydrolases that should be targeted to lysosomes are instead secreted, confirming that the *N*-linked oligosaccharide plays a key role in targeting these enzymes to lysosomes.

The pathways that target proteins to mitochondria and chloroplasts also rely on amino-terminal signal sequences. Although mitochondria and chloroplasts contain DNA,

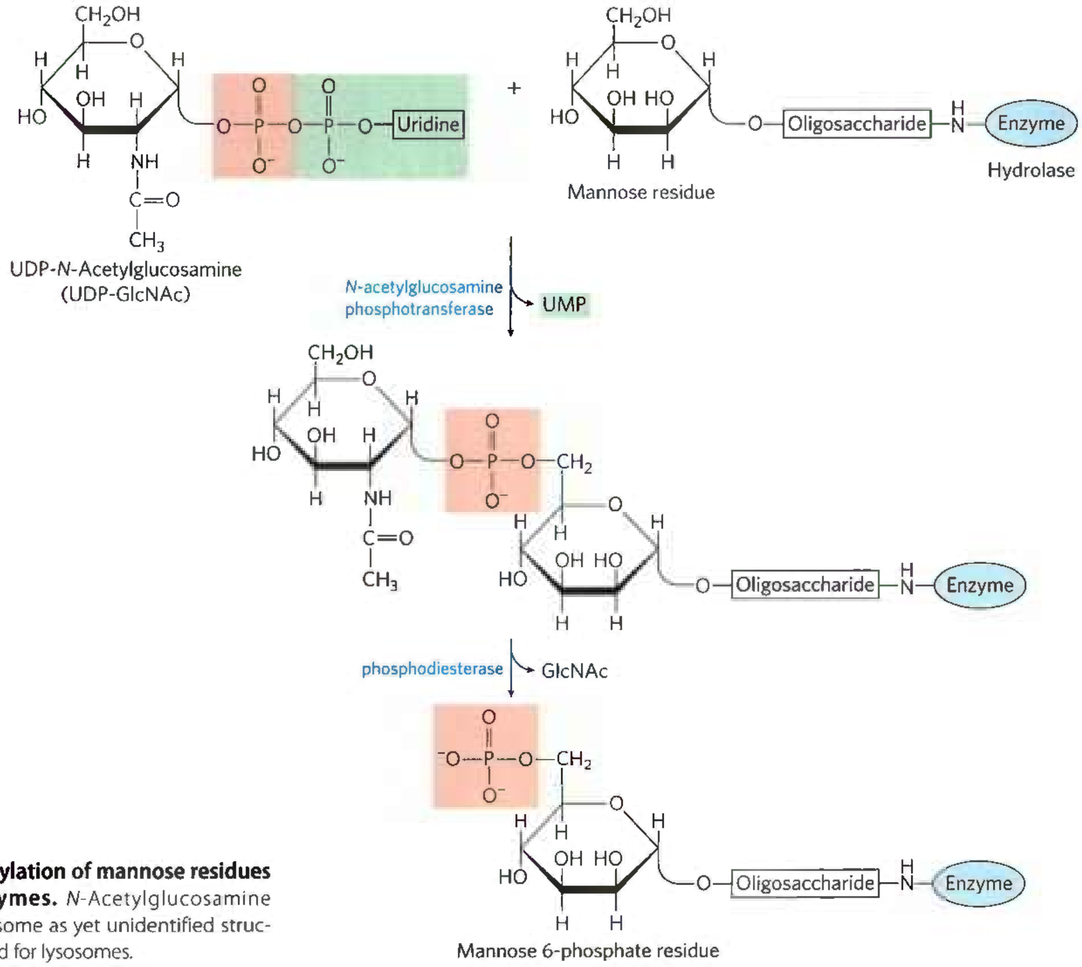

UDP-*N*-Acetylglucosamine (UDP-GlcNAc)
Uridine
Mannose residue
Oligosaccharide
Enzyme
Hydrolase
N-acetylglucosamine phosphotransferase
UMP
phosphodiesterase
GlcNAc
Mannose 6-phosphate residue

FIGURE 27-41 **Phosphorylation of mannose residues on lysosome-targeted enzymes.** *N*-Acetylglucosamine phosphotransferase recognizes some as yet unidentified structural feature of hydrolases destined for lysosomes.

most of their proteins are encoded by nuclear DNA and must be targeted to the appropriate organelle. Unlike other targeting pathways, however, the mitochondrial and chloroplast pathways begin only *after* a precursor protein has been completely synthesized and released from the ribosome. Precursor proteins destined for mitochondria or chloroplasts are bound by cytosolic chaperone proteins and delivered to receptors on the exterior surface of the target organelle. Specialized translocation mechanisms then transport the protein to its final destination in the organelle, after which the signal sequence is removed.

Signal Sequences for Nuclear Transport Are Not Cleaved

Molecular communication between the nucleus and the cytosol requires the movement of macromolecules through nuclear pores. RNA molecules synthesized in the nucleus are exported to the cytosol. Ribosomal proteins synthesized on cytosolic ribosomes are imported into the nucleus and assembled into 60S and 40S ribosomal subunits in the nucleolus; completed subunits are then exported back to the cytosol (Fig. 27-17). A variety of nuclear proteins (RNA and DNA polymerases, histones, topoisomerases, proteins that regulate gene expression, and so forth) are synthesized in the cytosol and imported into the nucleus. This traffic is modulated by a complex system of molecular signals and transport proteins that is gradually being elucidated.

In most multicellular eukaryotes, the nuclear envelope breaks down at each cell division, and once division is completed and the nuclear envelope reestablished, the dispersed nuclear proteins must be reimported. To allow this repeated nuclear importation, the signal sequence that targets a protein to the nucleus—the **nuclear localization sequence (NLS)**—is not removed after the protein arrives at its destination. An NLS, unlike other signal sequences, may be located almost anywhere along the primary sequence of the protein. NLSs can vary considerably in structure, but many consist of four to eight amino acid residues and include several consecutive basic (Arg or Lys) residues.

Nuclear importation is mediated by several proteins that cycle between the cytosol and the nucleus (Fig. 27-42),

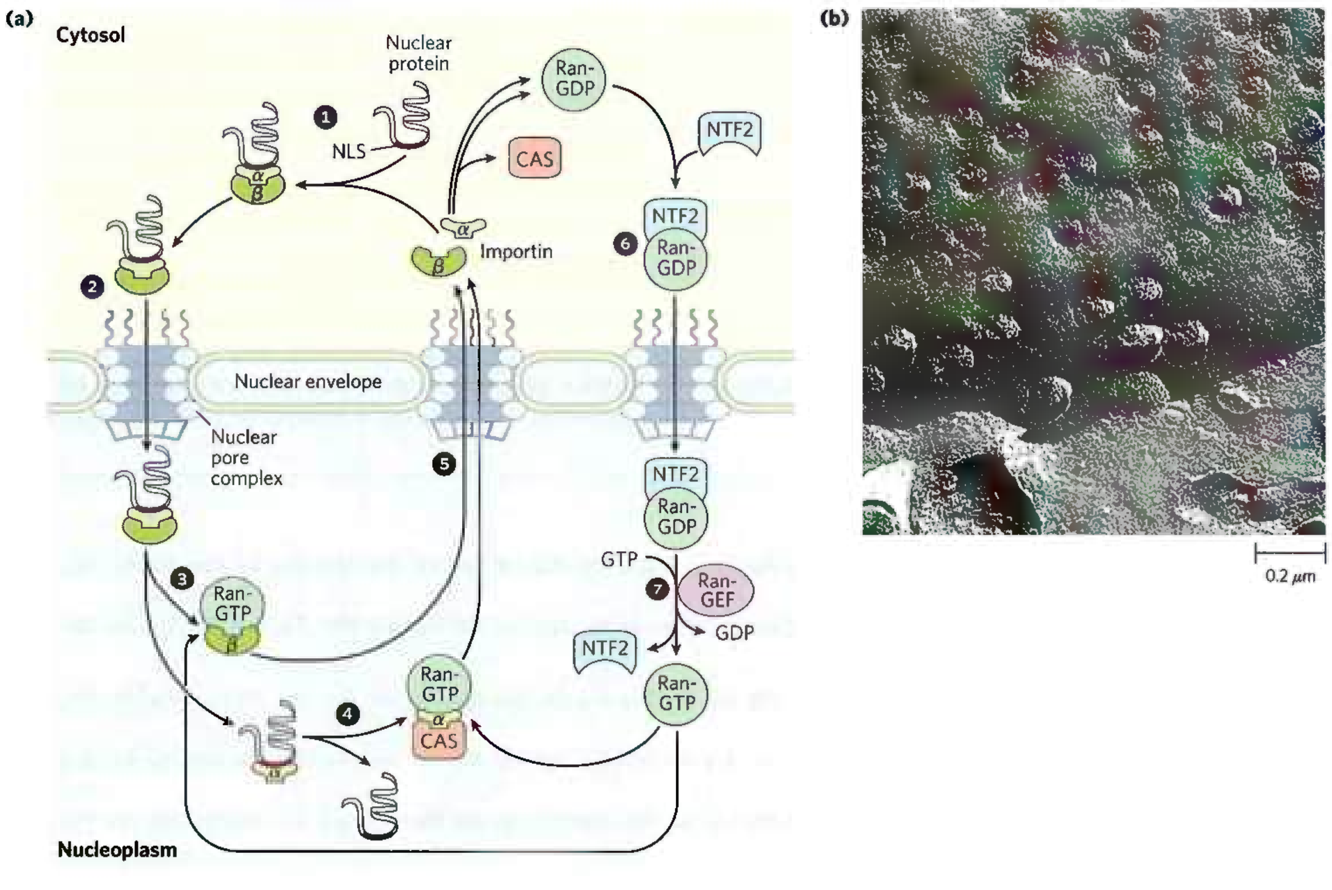


FIGURE 27-42 Targeting of nuclear proteins. (a) ① A protein with an appropriate nuclear localization signal (NLS) is bound by a complex of importins α and β. ② The resulting complex binds to a nuclear pore and translocates. ③ Inside the nucleus, dissociation of importin β is promoted by the binding of Ran-GTP. ④ Importin α binds to Ran-GTP and CAS (cellular apoptosis susceptibility protein), releasing the nuclear protein. ⑤ Importins α and β and CAS are transported out of the nucleus and recycled. They are released in the cytosol when Ran hydrolyzes its bound GTP. ⑥ Ran-GDP is bound by NTF2, and transported back into the nucleus. ⑦ Ran-GEF promotes the exchange of GDP for GTP in the nucleus, and Ran-GTP is ready to process another NLS-bearing protein-importin complex. (b) Transmission electron micrograph of a freeze-fractured nucleus, showing numerous nuclear pores. The nuclear pore complex is one of the largest molecular aggregates in the cell (M_r ~5×10^7). It is made up of multiple copies of more than 30 different proteins. [(a) Information from C. Strambio-De-Castillia et al., *Nat. Rev. Mol. Cell Biol.* 11:490, 2010, Fig. 1. (b) Don W. Fawcett/Science Source.]

including importin α and β and a small GTPase known as Ran (*Ra*s-related *n*uclear protein). A heterodimer of importin α and β functions as a soluble receptor for proteins targeted to the nucleus, with the α subunit binding NLS-bearing proteins in the cytosol. The complex of the NLS-bearing protein and the importin docks at a nuclear pore and is translocated through the pore by an energy-dependent mechanism. In the nucleus, the importin β is bound by Ran GTPase, releasing importin β from the imported protein. Importin β is bound by Ran and by CAS (*c*ellular *a*poptosis *s*usceptibility protein) and separated from the NLS-bearing protein. Importin α and β, in their complexes with Ran and CAS, are then exported from the nucleus. Ran hydrolyzes GTP in the cytosol to release the importins, which are then free to begin another importation cycle. Ran itself is also cycled back into the nucleus by the binding of Ran-GDP to nuclear transport factor 2 (NTF2). Inside the nucleus, the GDP bound to Ran is replaced with GTP through the action of Ran guanosine nucleotide–exchange factor (Ran-GEF).

During mitosis, when the nuclear envelope transiently breaks down, the Ran GTPase and the importins play additional roles. The Ran GTPase–importin β complex helps to position the spindle microtubules on the cell perimeter to facilitate chromosome segregation as the cell divides, and this complex also regulates microtubule interaction with other cellular structures.

Bacteria Also Use Signal Sequences for Protein Targeting

Bacteria can target proteins to their inner or outer membranes, to the periplasmic space between these membranes, or to the extracellular medium. They use signal sequences at the amino terminus of the proteins (**Fig. 27-43**), much like those on eukaryotic proteins targeted to the ER, mitochondria, and chloroplasts.

Most proteins exported from *E. coli* make use of the pathway shown in **Figure 27-44**. Following translation, a protein to be exported may fold only slowly, the amino-terminal signal sequence impeding the folding. The soluble chaperone protein SecB binds to the protein's signal sequence or other features of its incompletely folded structure. The bound protein is then delivered to SecA, a protein associated with the inner surface of the plasma membrane. SecA acts as both a receptor and a translocating ATPase. Released from SecB and bound to SecA, the protein is delivered to a translocation complex in the membrane, made up of SecY, E, and G, and is translocated stepwise through the membrane at the SecYEG complex in lengths of about 20 amino acid residues. Each step requires the hydrolysis of ATP, catalyzed by SecA.

Although most exported bacterial proteins use this pathway, some follow an alternative pathway that uses signal recognition and receptor proteins homologous to components of the eukaryotic SRP and the SRP receptor (see Fig. 27-38).

Cells Import Proteins by Receptor-Mediated Endocytosis

Some proteins are imported into eukaryotic cells from the surrounding medium; examples include low-density lipoprotein (LDL), the iron-carrying protein transferrin, peptide hormones, and circulating proteins destined for degradation. There are several importation pathways (**Fig. 27-45**). In one path, proteins bind to receptors in invaginations of the membrane called **coated pits**, which concentrate endocytic receptors in preference to other cell-surface proteins. The pits are coated on their cytosolic side with a lattice of the protein **clathrin**, which forms closed polyhedral structures (**Fig. 27-46**). The clathrin lattice grows as more receptors are occupied by target proteins. Eventually, a complete membrane-bounded

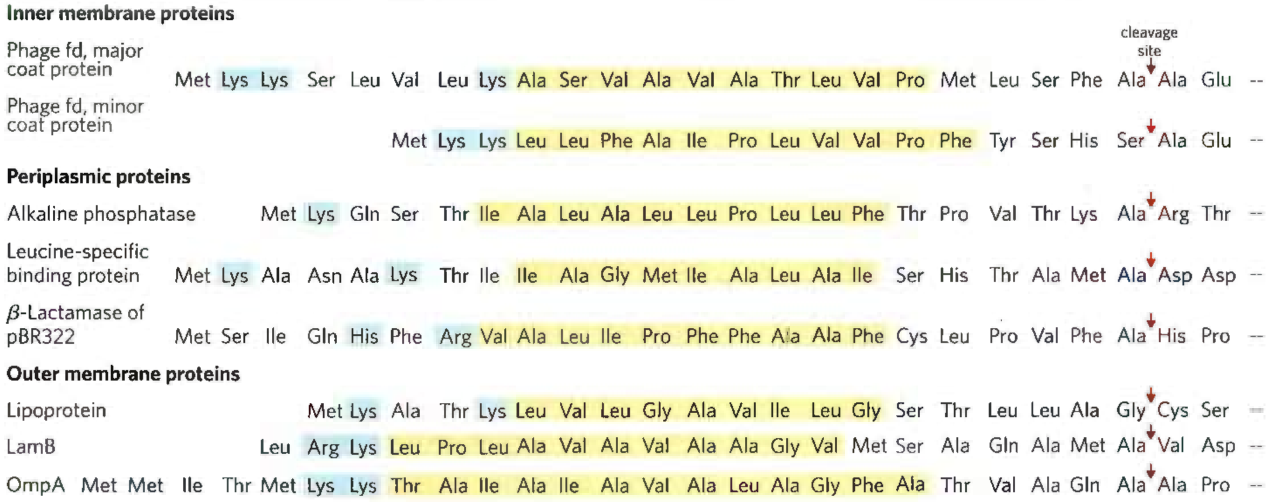

Inner membrane proteins
cleavage site
Phage fd, major coat protein Met Lys Lys Ser Leu Val Leu Lys Ala Ser Val Ala Val Ala Thr Leu Val Pro Met Leu Ser Phe Ala Ala Glu --
Phage fd, minor coat protein Met Lys Lys Leu Leu Phe Ala Ile Pro Leu Val Val Pro Phe Tyr Ser His Ser Ala Glu --
Periplasmic proteins
Alkaline phosphatase Met Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys Ala Arg Thr --
Leucine-specific binding protein Met Lys Ala Asn Ala Lys Thr Ile Ile Ala Gly Met Ile Ala Leu Ala Ile Ser His Thr Ala Met Ala Asp Asp --
β-Lactamase of pBR322 Met Ser Ile Gln His Phe Arg Val Ala Leu Ile Pro Phe Phe Ala Ala Phe Cys Leu Pro Val Phe Ala His Pro --
Outer membrane proteins
Lipoprotein Met Lys Ala Thr Lys Leu Val Leu Gly Ala Val Ile Leu Gly Ser Thr Leu Leu Ala Gly Cys Ser --
LamB Leu Arg Lys Leu Pro Leu Ala Val Ala Val Ala Ala Gly Val Met Ser Ala Gln Ala Met Ala Val Asp --
OmpA Met Met Ile Thr Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala Thr Val Ala Gln Ala Ala Pro --

FIGURE 27-43 Signal sequences that target proteins to different locations in bacteria. Basic amino acids near the amino terminus are highlighted in blue, hydrophobic core amino acids in yellow. Cleavage sites marking the ends of the signal sequences are indicated by red arrows. Note that the inner bacterial cell membrane is where phage fd coat proteins and DNA are assembled into phage particles. OmpA is outer membrane protein A; LamB is a cell surface receptor protein for λ phage.

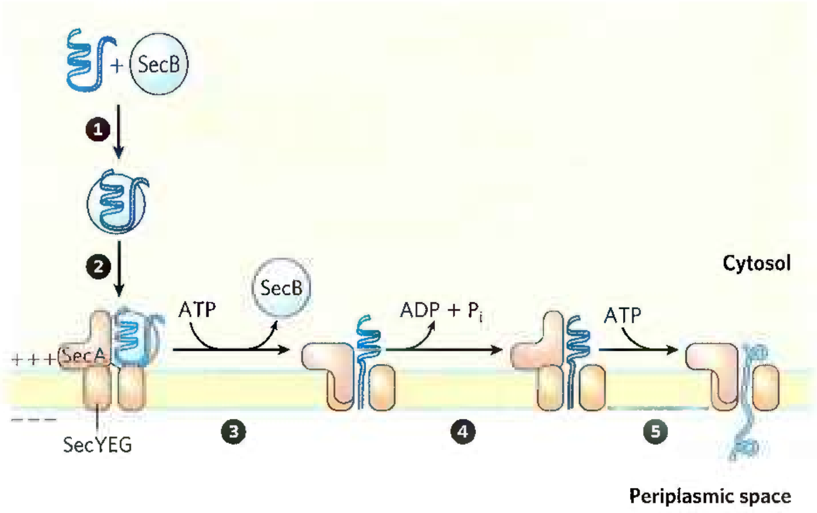


FIGURE 27-44 Model for protein export in bacteria. ① A newly translated polypeptide binds to the cytosolic chaperone protein SecB, which ② delivers it to SecA, a protein associated with the translocation complex (SecYEG) in the bacterial cell membrane. ③ SecB is released, and SecA inserts itself into the membrane, forcing about 20 amino acid residues of the protein to be exported through the translocation complex. ④ Hydrolysis of an ATP by SecA provides the energy for a conformational change that causes SecA to withdraw from the membrane, releasing the polypeptide. ⑤ SecA binds another ATP, and the next stretch of 20 amino acid residues is pushed across the membrane through the translocation complex. Steps ④ and ⑤ are repeated until the entire protein has passed through and is released to the periplasm. The electrochemical potential across the membrane (denoted by + and −) also provides some of the driving force required for protein translocation.

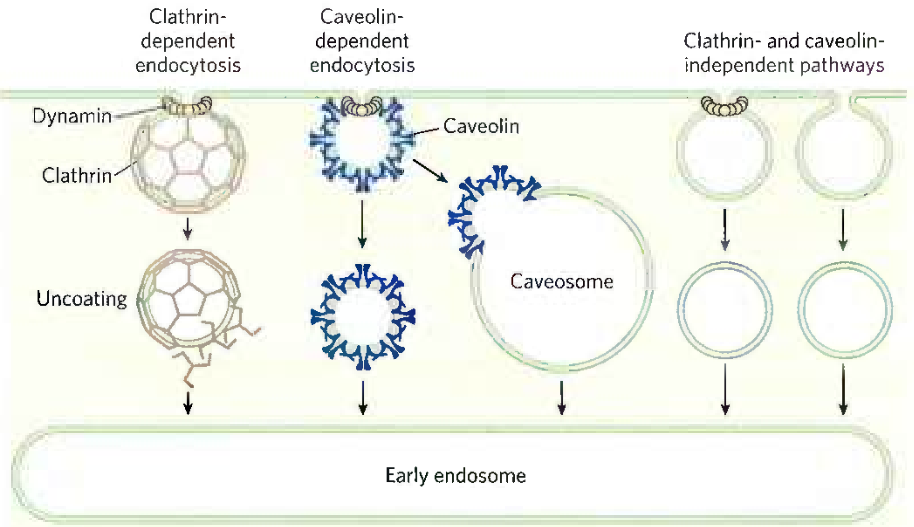
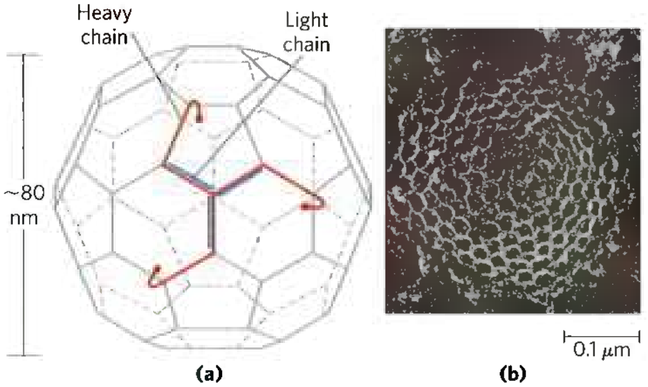

Clathrin-dependent endocytosis
Caveolin-dependent endocytosis
Clathrin- and caveolin-independent pathways
Dynamin
Clathrin
Caveolin
Caveosome
Uncoating
Early endosome

FIGURE 27-45 Summary of endocytosis pathways in eukaryotic cells. Pathways dependent on clathrin or caveolin make use of the GTPase dynamin to pinch vesicles from the plasma membrane. Some pathways do not use clathrin or caveolin; some of these make use of dynamin and some do not.



(a) (b)

FIGURE 27-46 Clathrin. (a) The protein has three light (L) chains (M_r 35,000) and three heavy (H) chains (M_r 180,000) of the $(HL)_3$ clathrin unit, organized as a three-legged structure called a triskelion. Triskelions tend to assemble into polyhedral lattices. (b) Electron micrograph of a coated pit on the cytosolic face of the plasma membrane of a fibroblast. [(a) Information from S. Mayor and R. E. Pagano, *Nat. Rev. Mol. Cell Biol.* 8:603, 2007. (b) ©1980 Heuser. The Rockefeller University Press. J. Heuser, *J. Cell Biol.* 84:560, 1980.]

endocytic vesicle is pinched off the plasma membrane with the aid of the large GTPase **dynamin**, and it enters the cytoplasm. The clathrin is quickly removed by uncoating enzymes, and the vesicle fuses with an endosome. ATPase activity in the endosomal membranes reduces the pH therein, facilitating dissociation of receptors from their target proteins. In a related pathway, caveolin causes invagination of patches of membrane containing lipid rafts associated with certain types of receptors (see Fig. 11-23). These endocytic vesicles then fuse with caveolin-containing internal structures called caveosomes, where the internalized molecules are sorted and redirected to other parts of the cell and the caveolins are prepared for recycling to the membrane surface. There are also clathrin- and caveolin-independent pathways; some make use of dynamin and others do not.

The imported proteins and receptors then go their separate ways, their fates varying with the cell and protein type. Transferrin and its receptor are eventually recycled. Some hormones, growth factors, and immune complexes, after eliciting the appropriate cellular response, are degraded along with their receptors. LDL is degraded after the associated cholesterol has been delivered to its destination, but the LDL receptor is recycled (see Fig. 21-42).

Receptor-mediated endocytosis is exploited by some toxins and viruses to gain entry to cells. Influenza virus, diphtheria toxin, SARS-CoV-2 (the virus that causes COVID-19), and cholera toxin all enter cells in this way.

Protein Degradation Is Mediated by Specialized Systems in All Cells

Protein degradation is critical to overall cellular proteostasis, preventing the buildup of abnormal or unwanted proteins and permitting the recycling of amino acids. The half-lives of eukaryotic proteins vary from 30 seconds to many days. Most proteins turn over rapidly relative to the lifetime of a cell, although a few (such as hemoglobin) can last for the life of the cell (about 110 days for an erythrocyte). Rapidly degraded proteins include those that are defective because of incorrectly inserted amino acids or because of damage accumulated during normal functioning. And enzymes that act at key regulatory points in metabolic pathways often turn over rapidly.

Defective proteins and those with characteristically short half-lives are generally degraded in both bacterial and eukaryotic cells by selective ATP-dependent cytosolic systems. A second system in vertebrates, operating in lysosomes, recycles the amino acids of membrane proteins, extracellular proteins, and proteins with characteristically long half-lives.

In *E. coli*, many proteins are degraded by one of several proteolytic systems that contain AAA+ ATPases (see Chapter 25), including Lon (the name refers to the "long form" of proteins, observed only when this protease is absent), ClpXP, ClpAP, ClpCP, ClpYQ, and FtsH. Each system targets particular proteins distinguished by their structure or subcellular location or both. Typically, ATP hydrolysis is used to maneuver a target protein through a pore into a proteolytic chamber, unfolding the protein in the process. Proteins are cleaved within the chamber. Once a protein has been reduced to small, inactive peptides, other ATP-independent proteases complete their degradation.

The ATP-dependent pathway in eukaryotic cells is quite different, involving the protein **ubiquitin**, which, as its name suggests, occurs throughout the eukaryotic kingdoms. One of the most highly conserved proteins known, ubiquitin (76 amino acid residues) is essentially identical in organisms as different as yeasts and humans and is key to proteostasis (see Figs. 4-23 and 13-29) and cell cycle regulation (see Fig. 12-38). Ubiquitin is covalently linked to proteins slated for destruction via an ATP-dependent pathway that includes three separate types of enzymes: E1 activating enzymes, E2 conjugating enzymes, and E3 ligases (**Fig. 27-47**).

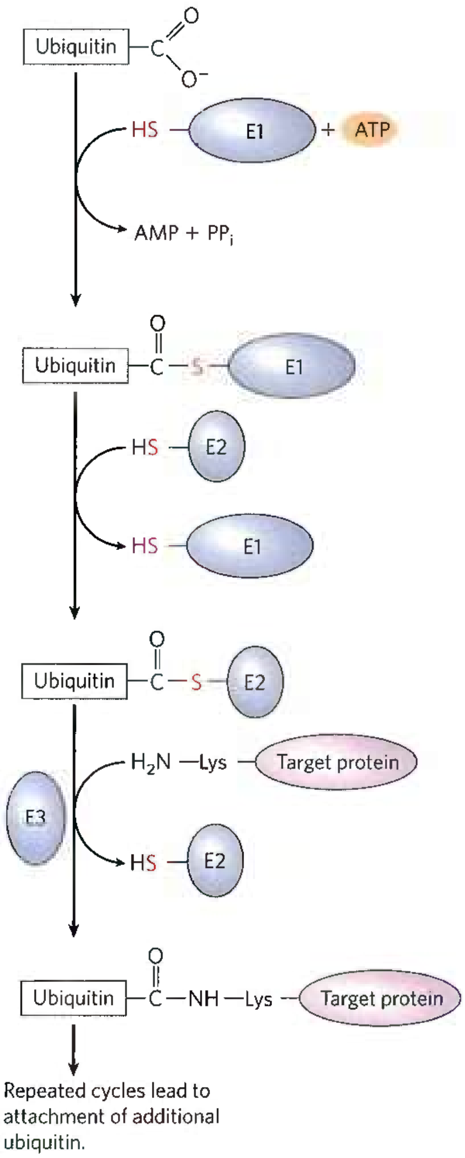


FIGURE 27-47 Three-step pathway by which ubiquitin is attached to a protein. The pathway includes two different enzyme-ubiquitin intermediates. First, the free carboxyl group of ubiquitin's carboxyl-terminal Gly residue becomes linked to an E1-class activating enzyme through a thioester. The ubiquitin is then transferred to an E2 conjugating enzyme. An E3 ligase ultimately catalyzes transfer of the ubiquitin from E2 to the target, linking ubiquitin through an amide (isopeptide) bond to the ε-amino group of a Lys residue in the target protein. Additional cycles produce polyubiquitin, a covalent polymer of ubiquitin subunits that targets the attached protein for destruction in eukaryotes. Multiple pathways of this sort, with different protein targets, are present in most eukaryotic cells.

Ubiquitinated proteins are degraded by a large complex known as the **26S proteasome** (M_r 2.5×10^6) (**Fig. 27-48**). The eukaryotic proteasome consists of two copies each of at least 32 different subunits, most of which are highly conserved from yeasts to humans. The proteasome contains two main types of subcomplexes: a barrel-like core particle and regulatory particles at each end of the barrel. The 19S regulatory particle on each end of the core particle contains approximately 18 subunits,

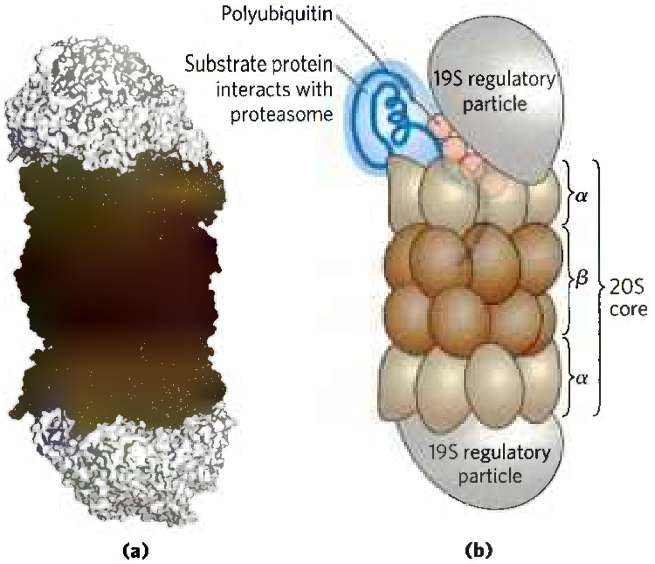


FIGURE 27-48 Three-dimensional structure of the eukaryotic proteasome. The 20S core particle and the 19S regulatory particle, or cap, are shown (a) as a molecular structure and (b) in schematic form. The core particle consists of four rings arranged to form a barrel-like structure. The outer rings are formed from seven α subunits, and the inner rings from seven β subunits. Three of the β subunits have protease activities, each with different substrate specificity. A regulatory particle forms a cap on each end of the core particle. The regulatory particle binds ubiquitinated proteins, unfolds them, and translocates them into the core particle, where they are degraded to peptides of 3 to 25 amino acid residues. [(a) Data from PDB ID 3L5Q, K. Sadre-Bazzaz et al., *Mol. Cell* 37:728, 2010.]

TABLE 27-9 Relationship between Protein Half-Life and Amino-Terminal Amino Acid Residue

Amino-terminal residue	Half-life[a]
Stabilizing	
Ala, Gly, Met, Ser, Thr, Val	>20 h
Destabilizing	
Gln, Ile	~30 min
Glu, Tyr	~10 min
Pro	~7 min
Asp, Leu, Lys, Phe	~3 min
Arg	~2 min

Information from A. Bachmair et al., *Science* 234:179, 1986.

[a] Half-lives were measured in yeast for the β-galactosidase protein modified so that in each experiment it had a different amino-terminal residue. Half-lives may vary for different proteins and in different organisms, but this general pattern appears to hold for all organisms.

including some that recognize and bind to ubiquitinated proteins. Six of the subunits are AAA+ ATPases that probably function in unfolding the ubiquitinated proteins and translocating the unfolded polypeptide into the core particle for degradation. The 19S particle also deubiquitinates the proteins as they are degraded in the proteasome. Most cells have additional regulatory complexes that can replace the 19S particle. These alternative regulators do not hydrolyze ATP and do not bind to ubiquitin, but they are important for the degradation of particular cellular proteins. The 26S proteasome can be effectively "accessorized" with regulatory complexes that change with changing cellular conditions.

Although we do not yet understand all the signals that trigger ubiquitination, one simple signal has been found. For many proteins, the identity of the first residue that remains after removal of the amino-terminal Met residue, and any other posttranslational proteolytic processing of the amino-terminal end, has a profound influence on half-life (**Table 27-9**). These amino-terminal signals have been conserved over billions of years of evolution and are the same in bacterial protein degradation systems and in the human ubiquitination pathway. More complex signals, such as the destruction box discussed in Chapter 12 (see Fig. 12-38), are also being identified.

Ubiquitin-dependent proteolysis is as important for the regulation of cellular processes as it is for the elimination of defective proteins. Many proteins required at only one stage of the eukaryotic cell cycle are rapidly degraded by the ubiquitin-dependent pathway after fulfilling their function. Ubiquitin-dependent destruction of cyclin is critical to cell-cycle regulation. The E1, E2, and E3 components of the ubiquitination pathway (Fig. 27-47) are large families of proteins. Different E1, E2, and E3 enzymes exhibit different specificities for target proteins and thus regulate different cellular processes. Some of these enzymes are highly localized in certain cellular compartments, reflecting a specialized function.

Not surprisingly, defects in the ubiquitination pathway have been implicated in a wide range of disease states. An inability to degrade certain proteins that activate cell division (the products of oncogenes) can lead to tumor formation, and a too-rapid degradation of proteins that act as tumor suppressors can have the same effect. The ineffective or overly rapid degradation of cellular proteins also seems to play a role in a range of other conditions, including renal diseases, asthma, and neurodegenerative disorders such as Alzheimer and Parkinson diseases that are associated with the formation of characteristic proteinaceous structures in neurons. Cystic fibrosis is caused in some cases by a too-rapid degradation of a chloride ion channel, with resultant loss of function (see Box 11-2). Liddle syndrome, in which a sodium channel in the kidney is not degraded, leads to excessive Na^+ absorption and early-onset hypertension. Drugs designed to inhibit proteasome function are being developed as potential treatments for some of these conditions. Many bacterial pathogens have found ways to hijack the eukaryotic ubiquitination system, evolving enzymes that ubiquitinate and thus eliminate host proteins as required to facilitate infection. In a changing metabolic environment, protein degradation is as important to a cell's survival as is protein synthesis, and much remains to be learned about these interesting pathways. ■

SUMMARY 27.3 *Protein Targeting and Degradation*

- After synthesis, many proteins are directed to particular locations in the cell, a process mediated by signal sequences embedded in the polypeptide chain. In eukaryotic cells, one class of signal sequences is recognized by the signal recognition particle (SRP), which binds the signal sequence as soon as it appears on the ribosome and transfers the entire ribosome and incomplete polypeptide to the endoplasmic reticulum. Polypeptides with these signal sequences are moved into the endoplasmic reticulum lumen as they are synthesized.
- Once in the lumen of the ER, many proteins are glycosylated. So modified, they are moved to the Golgi complex, then sorted and sent to lysosomes, the plasma membrane, or transport vesicles.
- Proteins targeted to the nucleus have an internal signal sequence that, unlike other signal sequences, is not cleaved once the protein is successfully targeted.
- Proteins targeted to mitochondria and chloroplasts in eukaryotic cells, and those destined for export in bacteria, also make use of an amino-terminal signal sequence.
- Some eukaryotic cells import proteins by receptor-mediated endocytosis.
- All cells eventually degrade proteins, using specialized proteolytic systems. Defective proteins and those slated for rapid turnover are generally degraded by an ATP-dependent system. In eukaryotic cells, the proteins are first tagged by linkage to ubiquitin, a highly conserved protein. Ubiquitin-dependent proteolysis, critical to the regulation of many cellular processes, is carried out by proteasomes, which also are highly conserved.

KEY TERMS

Terms in bold are defined in the glossary.

proteostasis	1005
translation	1006
aminoacyl-tRNA	1006
aminoacyl-tRNA synthetases	1006
codon	1007
reading frame	1007
initiation codon	1009
termination codons	1009
open reading frame (ORF)	1009
degenerate code	1009
anticodon	1010
wobble	1012
translational frameshifting	1013
RNA editing	1013
initiation	1028
Shine-Dalgarno sequence	1028
aminoacyl (A) site	1028
peptidyl (P) site	1028
exit (E) site	1028
initiation complex	1028
elongation	1030
elongation factors	1030
peptidyl transferase	1032
translocation	1032
termination	1035
termination factors	1035
release factors	1035
polysome	1036
posttranslational modification	1037
puromycin	1039
tetracycline	1040
chloramphenicol	1040
cycloheximide	1040
streptomycin	1040
diphtheria toxin	1040
ricin	1040
signal sequence	1041
signal recognition particle (SRP)	1042
peptide translocation complex	1042
tunicamycin	1042
nuclear localization sequence (NLS)	1045
coated pits	1046
clathrin	1046
dynamin	1047
ubiquitin	1048
proteasome	1048

PROBLEMS

1. **Messenger RNA Translation** Predict the amino acid sequences of peptides formed by ribosomes in response to each mRNA sequence, assuming that the reading frame begins with the first three bases in each sequence.

(a) GGUCAGUCGCUCCUGAUU
(b) UUGGAUGCGCCAUAAUUUGCU
(c) CAUGAUGCCUGUUGCUAC
(d) AUGGACGAA

2. **How Many Different mRNA Sequences Can Specify One Amino Acid Sequence?** Write all the possible mRNA sequences that can code for the simple tripeptide segment Leu–Met–Tyr. Your answer will give you some idea of the number of possible mRNAs that can code for one polypeptide.

3. **Can the Base Sequence of an mRNA Be Predicted from the Amino Acid Sequence of Its Polypeptide Product?** A given sequence of bases in an mRNA will code for one and only one sequence of amino acids in a polypeptide, if the reading frame is specified. From a given sequence of amino acid residues in a protein such as cytochrome *c*, can we predict the base sequence of the unique mRNA that encoded it? Give reasons for your answer.

4. **Coding of a Polypeptide by Duplex DNA** The template strand of a segment of double-helical DNA contains the sequence

(5′)CTTAACACCCCTGACTTCGCGCCGTCG(3′)

(a) What is the base sequence of the mRNA that can be transcribed from this strand?
(b) What amino acid sequence could be coded by the mRNA in (a), starting from the 5′ end?
(c) If the complementary (nontemplate) strand of this DNA were transcribed and translated, would the resulting amino acid sequence be the same as in (b)? Explain the biological significance of your answer.

5. **The Genetic Code and Mutation** A mutation occasionally arises that converts a codon specifying an amino acid to a stop or nonsense codon. When this occurs in the middle of a gene, the resulting protein is truncated and often inactive. If the protein is essential, cell death can result. Which of these secondary mutations might restore some or all of the protein function so that the cell can survive (there may be more than one correct answer)?

(a) A mutation restoring the codon to one encoding the original amino acid
(b) A mutation changing the nonsense codon to one encoding a different but similar amino acid

(c) A mutation in the anticodon of a tRNA such that the tRNA now recognizes the nonsense codon

(d) A mutation in which an additional nucleotide inserts just upstream of the nonsense codon, changing the reading frame so the nonsense codon is no longer read as "stop"

6. The Direction of Protein Synthesis In 1961, Howard Dintzis established that protein synthesis on ribosomes begins at the amino terminus and proceeds toward the carboxyl terminus. He used immature red blood cells that were still synthesizing hemoglobin. He added radioactively labeled leucine (chosen because it occurs frequently in both the α and β subunits) for various lengths of time, rapidly isolated only the full-length (completed) α subunits, and then determined where in the peptide the labeled amino acids were located. After the labeled leucine and extract had been incubated together for one hour, the protein was labeled uniformly along its length. However, after much shorter incubation times, the labeled amino acids were clustered at one end. At which end, amino or carboxyl terminus, did Dintzis find the labeled residues after the short exposure to labeled leucine?

7. Methionine Has Only One Codon Methionine is one of two amino acids with only one codon. How does the single codon for methionine specify both the initiating residue and the interior Met residues of polypeptides synthesized by *E. coli*?

8. The Genetic Code in Action Translate the mRNA shown, starting at the first 5′ nucleotide, assuming that translation occurs in an *E. coli* cell. If all tRNAs make maximum use of wobble rules but do not contain inosine, how many distinct tRNAs are required to translate this RNA?

(5′)AUGGGUCGUGAGUCAUCGUUAAUUGUAGCUGGAGGGGA GGAAUGA(3′)

9. Synthetic mRNAs The genetic code was elucidated through the use of polyribonucleotides synthesized either enzymatically or chemically in the laboratory. Given what we now know about the genetic code, how would you make a polyribonucleotide that could serve as an mRNA coding predominantly for many Phe residues and for a small number of Leu and Ser residues? What other amino acid(s) would be encoded by this polyribonucleotide, but in smaller amounts?

10. Energy Cost of Protein Biosynthesis Determine the minimum energy cost, in terms of ATP equivalents expended, for the biosynthesis of the β-globin chain of hemoglobin (146 residues), starting from a pool including all necessary amino acids, ATP, and GTP. Compare your answer with the direct energy cost of the biosynthesis of a linear glycogen chain of 146 glucose residues in ($\alpha 1 \rightarrow 4$) linkage, starting from a pool including glucose, UTP, and ATP. From your data, what is the *extra* energy cost of making a protein, in which all the residues are ordered in a specific sequence, compared with the cost of making a polysaccharide containing the same number of residues but lacking the informational content of the protein?

In addition to the direct energy cost for the synthesis of a protein, there are indirect energy costs—those required for the cell to make the necessary enzymes for protein synthesis. Compare the magnitude of the indirect costs to a eukaryotic cell of the biosynthesis of linear ($\alpha 1 \rightarrow 4$) glycogen chains and the biosynthesis of polypeptides, in terms of the enzymatic machinery involved.

11. Predicting Anticodons from Codons Most amino acids have more than one codon and attach to more than one tRNA, each with a different anticodon. Write all possible anticodons for the four codons of glycine: (5′)GGU, GGC, GGA, and GGG.

(a) From your answer, which of the positions in the anticodons are primary determinants of their codon specificity in the case of glycine?

(b) Which of these anticodon-codon pairings has/have a wobbly base pair?

(c) In which of the anticodon-codon pairings do all three positions exhibit strong Watson-Crick hydrogen bonding?

12. Effect of Single-Base Changes on Amino Acid Sequence Much important confirmatory evidence on the genetic code has come from assessing changes in the amino acid sequence of mutant proteins after a single base has been changed in the gene that encodes the protein. Which of the listed amino acid replacements would be consistent with the genetic code if the replacements were caused by a single base change? Which cannot be the result of a single-base mutation? Why?

(a) Phe → Leu
(b) Lys → Ala
(c) Ala → Thr
(d) Phe → Lys
(e) Ile → Leu
(f) His → Glu
(g) Pro → Ser

13. Resistance of the Genetic Code to Mutation The RNA sequence shown represents the beginning of an open reading frame (ORF). What changes (if any) can occur at each position without generating a change in the encoded amino acid residue?

(5′)AUGAUAUUGCUAUCUUGGACU

14. Basis of the Sickle Cell Mutation Sickle cell hemoglobin has a Val residue at position 6 of the β-globin chain instead of the Glu residue found in normal hemoglobin A. Can you predict what change took place in the DNA codon for glutamate to account for replacement of the Glu residue by Val?

15. Proofreading by Aminoacyl-tRNA Synthetases The isoleucyl-tRNA synthetase has a proofreading function that ensures the fidelity of the aminoacylation reaction, but the histidyl-tRNA synthetase lacks such a proofreading function. Explain.

16. Importance of the "Second Genetic Code" Some aminoacyl-tRNA synthetases do not recognize and bind the anticodon of their cognate tRNAs but instead use other structural features of the tRNAs to impart binding specificity. The tRNAs for alanine apparently fall into this category.

(a) What features of tRNA^{Ala} does Ala-tRNA synthetase recognize?

(b) Describe the consequences of a C → G mutation in the third position of the anticodon of tRNA^{Ala}.

(c) What other kinds of mutations might have similar effects?

(d) Mutations of these types are never found in natural populations of organisms. Why? (Hint: Consider what might happen both to individual proteins and to the organism as a whole.)

17. Rate of Protein Synthesis A bacterial ribosome can synthesize about 20 peptide bonds per minute. If the average bacterial protein is approximately 260 amino acid residues long, how many proteins can the ribosomes in an *E. coli* cell synthesize in 20 minutes if all ribosomes are functioning at maximum rates?

18. The Role of Translation Factors A researcher isolates mutant variants of the bacterial translation factors IF2, EF-Tu, and EF-G. In each case, the mutation allows proper folding of the protein and the binding of GTP but does not allow GTP hydrolysis. At what stage would translation be blocked by each mutant protein?

19. Maintaining the Fidelity of Protein Synthesis The chemical mechanisms used to avoid errors in protein synthesis are different from those used during DNA replication. DNA polymerases use a $3' \rightarrow 5'$ exonuclease proofreading activity to remove mispaired nucleotides incorrectly inserted into a growing DNA strand. There is no analogous proofreading function on ribosomes, and, in fact, the identity of an amino acid attached to an incoming tRNA and added to the growing polypeptide is never checked. A proofreading step that hydrolyzed the previously formed peptide bond after insertion of an incorrect amino acid into a growing polypeptide (analogous to the proofreading step of DNA polymerases) would be impractical. Why? (Hint: Consider how the link between the growing polypeptide and the mRNA is maintained during elongation; see Figs. 27-28 and 27-29.)

20. Bacterial Protein Export Bacteria mostly use the system shown in Fig. 27-44 to export proteins out of the cell. SecB, one of the chaperone proteins found only in gram-negative bacteria, delivers a newly translated polypeptide to the SecA ATPase on the interior side of the membrane. SecA pushes the exported protein through a membrane pore formed by the SecYEG complex. The SecYEG complex is homologous to the Sec61 complex in eukaryotes. Which component of this bacterial protein export system would be the most attractive target for antibiotic development? Explain.

21. Predicting the Cellular Location of a Protein You alter the gene for a eukaryotic polypeptide 300 amino acid residues long so that a signal sequence recognized by the SRP occurs at the polypeptide's amino terminus and a nuclear localization signal (NLS) occurs internally, beginning at residue 150. Where would you likely find the protein in the cell?

22. Requirements for Protein Translocation across a Membrane The secreted bacterial protein OmpA has a precursor, ProOmpA, which has the amino-terminal signal sequence required for secretion. If you denature purified ProOmpA with 8 M urea and then remove the urea (such as by running the protein solution rapidly through a gel filtration column), the protein can translocate across isolated bacterial inner membranes in vitro. However, translocation becomes impossible if you first incubate ProOmpA for a few hours in the absence of urea. Furthermore, ProOmpA maintains its capacity for translocation for an extended period if you first incubate it in the presence of another bacterial protein called trigger factor. Describe the probable function of trigger factor.

23. Protein-Coding Capacity of a Viral DNA The 5,386 bp genome of bacteriophage ϕX174 includes genes for 10 proteins, designated A to K (omitting "I"), with sizes given in the table. How much DNA would be required to encode these 10 proteins? How can you reconcile the size of the ϕX174 genome with its protein-coding capacity?

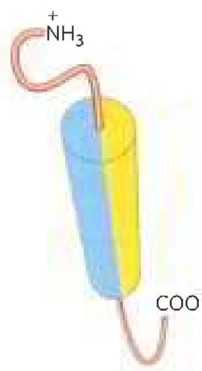
Protein	Number of amino acid residues	Protein	Number of amino acid residues
A	455	F	427
B	120	G	175
C	86	H	328
D	152	J	38
E	91	K	56

DATA ANALYSIS PROBLEM

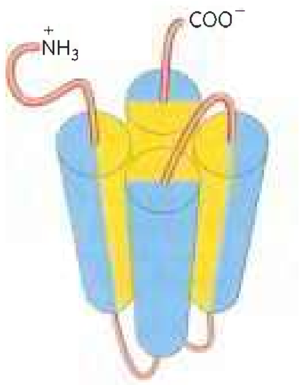
24. Designing Proteins by Using Randomly Generated Genes Studies of the amino acid sequence and corresponding three-dimensional structure of wild-type or mutant proteins have led to significant insights into the principles that govern protein folding. An important test of this understanding would be to *design* a protein based on these principles and see whether it folds as expected.

Kamtekar and colleagues (1993) used aspects of the genetic code to generate random protein sequences with defined patterns of hydrophilic and hydrophobic residues. Their clever approach combined knowledge about protein structure, amino acid properties, and the genetic code to explore the factors that influence protein structure.

The researchers set out to generate a set of proteins with the simple four-helix bundle structure shown below, with α helices (shown as cylinders) connected by segments of random coil (light red).



An amphipathic α helix



Four-helix bundle

Each α helix is amphipathic—the R groups on one side of the helix are exclusively hydrophobic (yellow), and those on the other side are exclusively hydrophilic (blue). A protein consisting of four of these helices separated by short segments of random coil would be expected to fold so that the hydrophilic sides of the helices face the solvent.

(a) What forces or interactions hold the four α helices together in this bundled structure?

Figure 4-3a shows a segment of α helix consisting of 10 amino acid residues. With the gray central rod as a divider, four of the R groups (purple spheres) extend from the left side of the helix, and six extend from the right.

(b) Number the R groups in Figure 4-3a, from top (amino terminus; 1) to bottom (carboxyl terminus; 10). Which R groups extend from the left side, and which extend from the right?

(c) Suppose you wanted to design this 10 amino acid segment to be an amphipathic helix, with the left side hydrophilic and the right side hydrophobic. Give a sequence of 10 amino acids that could potentially fold into such a structure. There are many possible correct answers.

(d) Give one possible double-stranded DNA sequence that could encode the amino acid sequence you chose for (c). (It is an internal portion of a protein, so you do not need to include start or stop codons.)

Rather than designing proteins with specific sequences, Kamtekar and colleagues designed proteins with partially random sequences, with hydrophilic and hydrophobic amino acid residues placed in a controlled pattern. They did this by taking advantage of some interesting features of the genetic code to construct a library of synthetic DNA molecules with partially random sequences arranged in a particular pattern.

To design a DNA sequence that would encode random hydrophobic amino acid sequences, the researchers began with the degenerate codon NTN, where N can be A, G, C, or T. They filled each N position by including an equimolar mixture of A, G, C, and T in the DNA synthesis reaction to generate a mixture of DNA molecules with different nucleotides at that position (see Fig. 8-32). Similarly, to encode random polar amino acid sequences, they began with the degenerate codon NAN and used an equimolar mixture of A, G, and C (but in this case, no T) to fill the N positions.

(e) Which amino acids can be encoded by the NTN triplet? Are all amino acids in this set hydrophobic? Does the set include *all* the hydrophobic amino acids?

(f) Which amino acids can be encoded by the NAN triplet? Are all of these polar? Does the set include *all* the polar amino acids?

(g) In creating the NAN codons, why was it necessary to leave T out of the reaction mixture?

Kamtekar and coworkers cloned this library of random DNA sequences into plasmids, selected 48 that produced the correct patterning of hydrophilic and hydrophobic amino acids, and expressed these in *E. coli*. The next challenge was to determine whether the proteins folded as expected. It would be very time-consuming to express each protein, crystallize it, and determine its complete three-dimensional structure. Instead, the investigators used the *E. coli* protein-processing machinery to screen out sequences that led to highly defective proteins. In this initial screening, they kept only those clones that resulted in a band of protein with the expected molecular weight on SDS polyacrylamide gel electrophoresis (see Fig. 3-18).

(h) Why would a grossly misfolded protein fail to produce a band of the expected molecular weight on electrophoresis?

Several proteins passed this initial test, and further exploration showed that they had the expected four-helix structure.

(i) Why didn't all of the random-sequence proteins that passed the initial screening test produce four-helix structures?

Reference

Kamtekar, S., J.M. Schiffer, H. Xiong, J.M. Babik, and M.H. Hecht. 1993. Protein design by binary patterning of polar and nonpolar amino acids. *Science* 262:1680–1685.

Chapter 28

REGULATION OF GENE EXPRESSION

28.1 The Proteins and RNAs of Gene Regulation *1055*

28.2 Regulation of Gene Expression in Bacteria *1065*

28.3 Regulation of Gene Expression in Eukaryotes *1075*

Biological information is expensive. We have discussed the extraordinary energetic cost of replication, transcription, and translation in previous chapters. The ATP requirements of converting information from a gene in a chromosome to protein imposes a need for efficiency, explaining the pervasive and often complex regulation of the expression of every gene.

Of the 4,000 or so genes in the typical bacterial genome, or the 20,000 genes in the human genome, only a fraction are expressed in a cell at any given time. Some gene products are present in very large amounts: the elongation factors required for protein synthesis, for example, are among the most abundant proteins in bacteria, and ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) of plants and photosynthetic bacteria is one of the most abundant enzymes in the biosphere. Other gene products occur in much smaller amounts; for instance, a cell may contain only a few molecules of the enzymes that repair rare DNA lesions. Requirements for some gene products change over time. The need for enzymes in certain metabolic pathways may wax and wane as food sources change or are depleted. During development of a multicellular organism, some proteins that influence cellular differentiation are present for just a brief time in only a few cells. Specialization of cellular function can greatly affect the need for various gene products; an example is the uniquely high concentration of a single protein—hemoglobin—in erythrocytes. It is clear from these examples that the appearance of gene products must be regulated. Our exploration of the regulation of gene expression is once again guided by multiple principles:

P1 **The cellular concentration of a protein is determined by a delicate balance of at least seven processes, each having several potential points of regulation.** These processes include synthesis of the primary RNA transcript (transcription); posttranscriptional modification of mRNA; degradation of mRNA; protein synthesis (translation); posttranslational modification of proteins; protein targeting and transport; and protein degradation.

P2 **Regulation is achieved by specialized proteins and RNAs.** The proteins are usually ligand-binding proteins with no other function. They bind to specific sequences in DNA or RNA. They respond to molecular signals that can be any kind of biological molecule. The RNAs either interact with other RNAs or serve as protein cofactors.

P3 **Regulated gene expression may bring about increases or decreases in the amount of a gene product.** Gene products that increase in concentration under particular molecular circumstances are referred to as **inducible**; the process of increasing their expression is **induction**. Conversely, gene products that decrease in concentration in response to a molecular signal are referred to as **repressible**, and the process is called **repression**.

P4 **The default transcriptional state of a gene, on or off, is dictated in part by the size and complexity of the genome.** In bacteria, where genomes are relatively small and DNA is readily accessible, the default state of genes is generally "on." Transcription of each gene or gene cluster is usually limited by a specific protein repressor. In eukaryotes, where genomes are larger and genes are encapsulated in chromatin, the default state of most genes is "off." Gene transcription requires chromatin modification followed by the action of transcription activators.

P5 **Regulation is expensive.** For many genes, especially in eukaryotes, the regulatory processes can require a considerable investment of chemical energy. That expenditure is nevertheless small when compared to the cost of RNA and protein synthesis when the gene is expressed.

P1 The steps required to generate and then remove an active protein or RNA, all of which may be regulated, are summarized in **Figure 28-1**. We have examined several of the relevant regulatory mechanisms in previous chapters. Posttranscriptional modification of mRNA, by processes such as alternative splicing patterns (see Fig. 26-20) or RNA editing (see Figs 27-10 and 27-12), can affect which proteins are produced from an mRNA transcript and in what amounts. A variety of nucleotide sequences in an mRNA can affect the rate of its degradation (p. 986). Many factors affect the rate at which an mRNA is translated into a protein, as well as the posttranslational modification, targeting, and eventual degradation of that protein (Chapter 27).

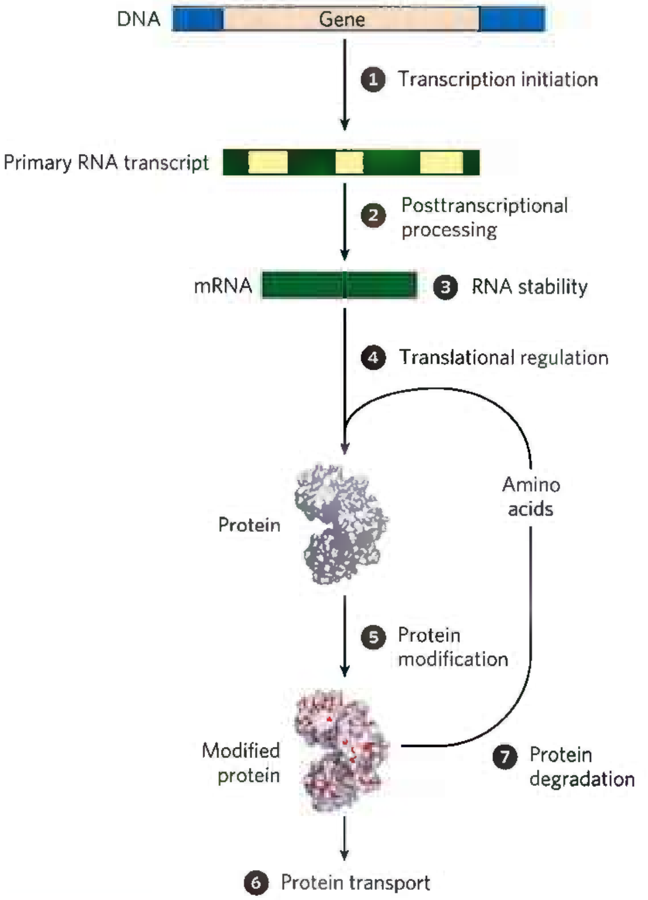


FIGURE 28-1 Seven processes that affect the steady-state concentration of a protein. Each process has several potential points of regulation.

Of the regulatory processes illustrated in Figure 28-1, those operating at the level of transcription initiation are particularly well-documented. These processes are a major focus of this chapter, although we also consider other mechanisms. As noted in earlier chapters, the complexity of an organism is not reflected in the number of its protein-coding genes. Instead, as complexity increases from bacteria to mammals, mechanisms of gene regulation become more elaborate, and posttranscriptional and translational regulation play greater roles.

Control of transcription initiation permits the synchronized regulation of multiple genes encoding products with interdependent activities. For example, when their DNA is heavily damaged, bacterial cells require a coordinated increase in the levels of the many DNA repair enzymes. And perhaps the most sophisticated form of coordination occurs in the complex regulatory circuits that guide the development of multicellular eukaryotes, which can include many types of regulatory mechanisms.

We begin by examining the interactions between proteins and DNA that are the key to transcriptional regulation. We next discuss the specific proteins that influence the expression of specific genes, first in bacterial and then in eukaryotic cells. Information about posttranscriptional and translational controls is included in the discussion, where relevant, to provide a more complete overview of the rich complexity of cellular regulation.

28.1 The Proteins and RNAs of Gene Regulation

Transcription is mediated and regulated by protein-DNA interactions, especially those involving the protein components of RNA polymerase (Chapter 26). We first consider how the activity of RNA polymerase is regulated. Next, we proceed to a general description of the proteins participating in this regulation. We then examine the molecular basis for the recognition of specific DNA sequences by DNA-binding proteins. Regulatory RNAs are encountered most often in the regulation of mRNA translation in eukaryotes, but also play a role in the life of some bacterial mRNAs. We consider them briefly in this section, and in more detail in Section 28.3.

RNA Polymerase Binds to DNA at Promoters

RNA polymerases bind to DNA and initiate transcription at promoters (see Fig. 26-5), sites generally found near points at which RNA synthesis begins on the DNA template. The regulation of transcription initiation often entails changes in how RNA polymerase interacts with a promoter.

The nucleotide sequences of promoters vary considerably, affecting the binding affinity of RNA polymerases

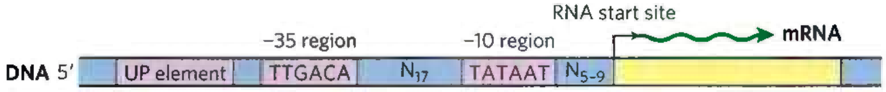


FIGURE 28-2 Consensus sequence for many *E. coli* promoters. Most base substitutions in the −10 and −35 regions have a negative effect on promoter function. Some promoters also include the UP (upstream promoter) element.

and thus the frequency of transcription initiation. Some *Escherichia coli* genes are transcribed once per second, others less than once per cell generation. Much of this variation is due to differences in promoter sequence. In the absence of regulatory proteins, differences in promoter sequence may affect the frequency of transcription initiation by a factor of 1,000 or more. Most *E. coli* promoters have a sequence close to a consensus (**Fig. 28-2**). Mutations that result in a shift away from the consensus sequence usually decrease the function of bacterial promoters; conversely, mutations toward consensus usually enhance promoter function.

KEY CONVENTION By convention, DNA sequences are shown as they exist in the nontemplate strand, with the 5′ terminus on the left. Nucleotides are numbered from the transcription start site, with positive numbers to the right (in the direction of transcription) and negative numbers to the left. N indicates any nucleotide. ■

Genes for products that are required at all times, such as those for the enzymes of central metabolic pathways, are expressed continuously, with little variation in virtually every cell of a species or organism. Such genes are often referred to as **housekeeping genes**. Expression of a gene at approximately constant levels is called **constitutive gene expression**. Although housekeeping genes are expressed constitutively, the cellular concentrations of the proteins they encode vary widely. For these genes, the RNA polymerase–promoter interaction strongly influences the rate of transcription initiation. Differences in promoter sequence may be the only level of regulation for a housekeeping gene, allowing the cell to synthesize the appropriate level of each housekeeping gene product.

The basal rate of transcription initiation at the promoters of nonhousekeeping genes is also determined by the promoter sequence, but expression of these genes is further modulated by regulatory proteins. Many of these proteins work by enhancing or interfering with the interaction between RNA polymerase and the promoter.

The sequences of eukaryotic promoters are much more variable than their bacterial counterparts. The three eukaryotic RNA polymerases usually require an array of general transcription factors in order to bind to a promoter, and these can heavily influence basal transcription rates. Yet, as with bacterial gene expression, the basal level of transcription is determined in part by the effect of promoter sequences on the function of RNA polymerase and its associated transcription factors.

Transcription Initiation Is Regulated by Proteins and RNAs

P2 At least three types of regulatory proteins regulate transcription initiation by RNA polymerase: **specificity factors** alter the specificity of RNA polymerase for a given promoter or set of promoters, **repressors** impede access of RNA polymerase to the promoter, and **activators** enhance the RNA polymerase–promoter interaction.

As our understanding of the roles of protein regulators slowly matures, many new roles for gene regulation by **noncoding RNAs (ncRNAs)** are also beginning to emerge. Among these are the **long noncoding RNAs (lncRNAs)**, generally defined as noncoding RNAs more than 200 nucleotides long that lack an open reading frame (ORF) that encodes a protein—thus distinguishing them from the small, functional ncRNAs (miRNA, snoRNA, snRNA, etc.) described in Chapter 26. The lncRNAs are found in all types of organisms, with tens of thousands expressed in mammalian cells. Known functions of lncRNAs include regulation of nucleosome positioning and chromatin structure, control of DNA methylation and posttranscriptional histone modifications, transcriptional gene silencing, multiple roles in transcriptional activation and repression, and much more.

We introduced bacterial specificity factors in Chapter 26, although we did not refer to these proteins by that name. The σ subunit of the *E. coli* RNA polymerase holoenzyme is a specificity factor that mediates promoter recognition and binding. Most *E. coli* promoters are recognized by a single σ subunit (M_r 70,000), σ^{70} (see Fig. 26-5). Under some conditions, some of the σ^{70} subunits are replaced by one of six other specificity factors. One notable case arises when bacteria are subjected to heat stress, leading to the replacement of σ^{70} by σ^{32} (M_r 32,000). When bound to σ^{32}, RNA polymerase is directed to a specialized set of promoters with a different consensus sequence (**Fig. 28-3**). These promoters control the

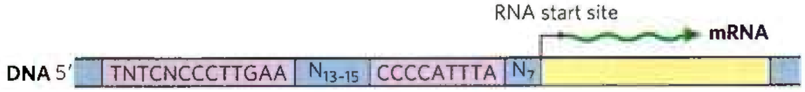


FIGURE 28-3 Consensus sequence for promoters that regulate expression of the *E. coli* heat shock genes. This system responds to temperature increases as well as some other environmental stresses, resulting in the induction of a set of proteins. Binding of RNA polymerase to heat shock promoters is mediated by a specialized σ subunit of the polymerase, σ^{32}, which replaces σ^{70} in the RNA polymerase initiation complex.

expression of a set of genes that encode proteins, including some protein chaperones (p. 132), that are part of a stress-induced system called the heat shock response. Thus, through changes in the binding affinity of the polymerase that direct the enzyme to different promoters, a set of genes involved in related processes is coordinately regulated. In eukaryotic cells, some of the general transcription factors, in particular the TATA-binding protein (TBP; see Fig. 26-9), may be considered specificity factors.

Repressors bind to specific sites on the DNA. In bacterial cells, such binding sites, called **operators**, are generally near a promoter. RNA polymerase binding, or its movement along the DNA after binding, is blocked when the repressor is present. **P3** Regulation by means of a repressor protein that blocks transcription is referred to as **negative regulation**. Repressor binding to DNA is regulated by a molecular signal, or **effector**, usually a small molecule or a protein that binds to the repressor and causes a conformational change. The interaction between repressor and signal molecule either increases or decreases transcription. In some cases, the conformational change results in dissociation of a DNA-bound repressor from the operator (**Fig. 28-4a**). Transcription initiation can then proceed unhindered. In other cases, interaction between an inactive repressor and the signal molecule causes the repressor to bind to the operator (Fig. 28-4b). **P4** In eukaryotic cells, gene regulation by a repressor is less common. Where it does occur (more often in lower eukaryotes such as yeast), the binding site for a repressor may be some distance from the promoter. Binding of these repressors to their binding sites has the same effect as in bacterial cells: inhibiting the assembly or activity of a transcription complex at the promoter.

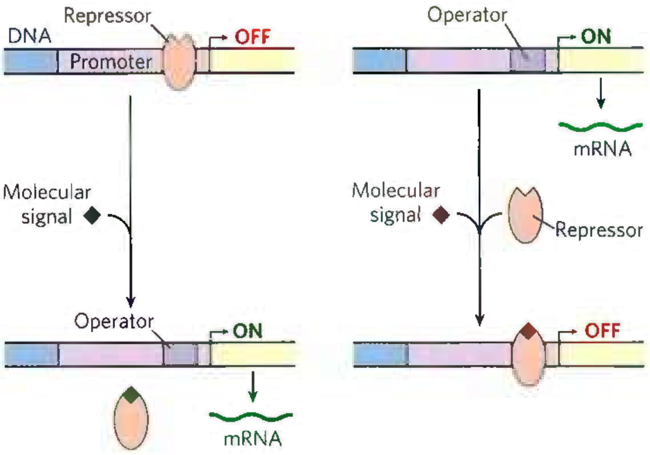
P3 Activators provide a molecular counterpoint to repressors; they bind to DNA and *enhance* the activity of RNA polymerase at a promoter; this is **positive regulation**. In bacteria, activator-binding sites are often adjacent to promoters that are bound weakly or not at all by RNA polymerase alone, such that little transcription occurs in the absence of the activator. Some activators are usually bound to DNA, enhancing transcription until dissociation of the activator is triggered by the binding of a signal molecule (Fig. 28-4c). In other cases the activator binds to DNA only after interaction with a signal molecule (Fig. 28-4d). Signal molecules can therefore increase or decrease transcription, depending on how they affect the activator.

P4 Positive regulation by activators is particularly common in eukaryotes. Many eukaryotic activators bind to DNA sites, called enhancers, that are distant from the promoter, affecting the rate of transcription at a promoter that may be located thousands of base pairs away.

The distance between a promoter and the binding site of an activator or repressor is bridged by looping out of the DNA between the two sites (**Fig. 28-5**). The looping is facilitated in some cases by proteins called **architectural regulators** that bind to intervening sites. Interaction between activators and the RNA polymerase at the promoter is often mediated by intermediary proteins called coactivators. In some instances, protein repressors may take the place of coactivators, binding to the activators and preventing the activating interaction.

(a) Negative regulation
Molecular signal causes dissociation of repressor from DNA, inducing transcription.

(b) Negative regulation
Molecular signal causes binding of repressor to DNA, inhibiting transcription.



(c) Positive regulation
Molecular signal causes dissociation of activator from DNA, inhibiting transcription.

(d) Positive regulation
Molecular signal causes binding of activator to DNA, inducing transcription.

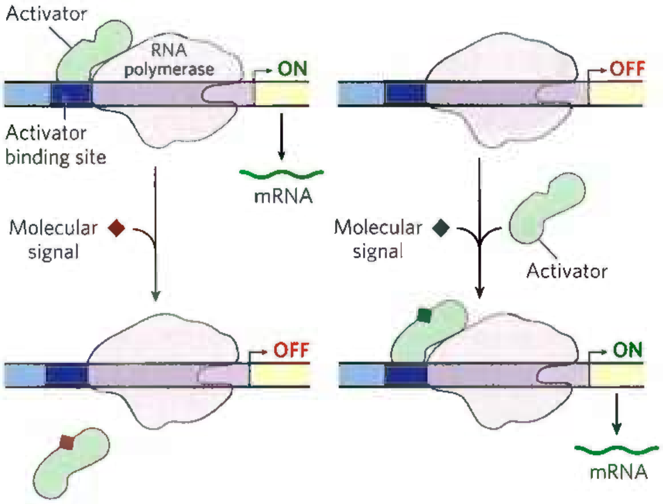


FIGURE 28-4 Common patterns of regulation of transcription initiation. Two types of negative regulation are illustrated. (a) Repressor binds to the operator in the absence of the molecular signal; the external signal causes dissociation of the repressor to permit transcription. (b) Repressor binds in the presence of the signal; the repressor dissociates, and transcription ensues when the signal is removed. Positive regulation is mediated by gene activators. Again, two types are shown. (c) Activator binds in the absence of the molecular signal and transcription proceeds; when the signal is added, the activator dissociates and transcription is inhibited. (d) Activator binds in the presence of the signal; it dissociates only when the signal is removed. Note that "positive" regulation and "negative" regulation refer to the type of regulatory protein involved: the bound protein either facilitates or inhibits transcription. In either case, addition of the molecular signal may increase or decrease transcription, depending on its effect on the regulatory protein.

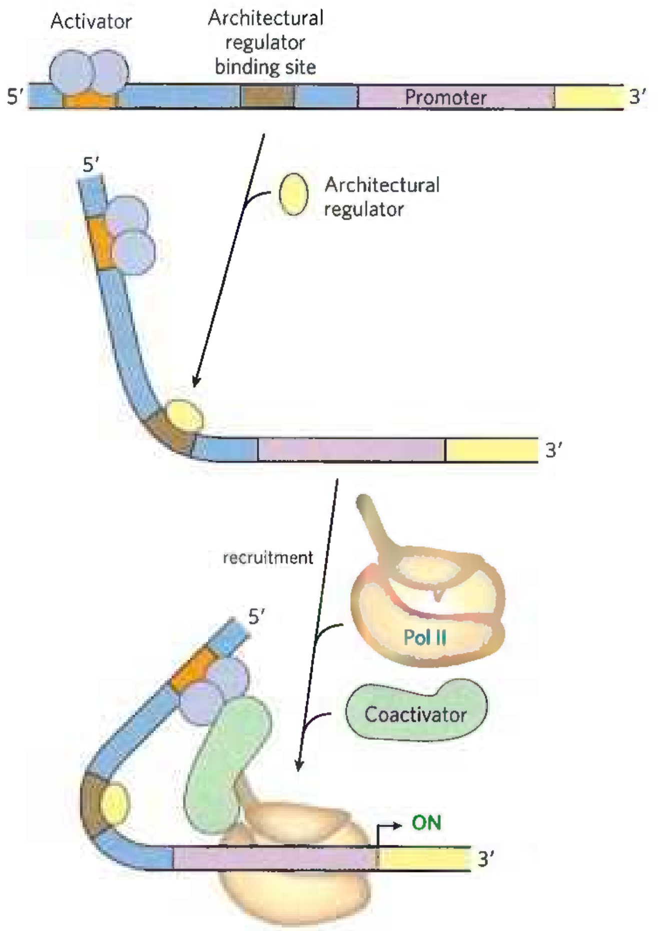


FIGURE 28-5 Interaction between activators/repressors and RNA polymerase in eukaryotes. Eukaryotic activators and repressors frequently bind sites thousands of base pairs distant from the promoters they regulate. DNA looping, often facilitated by architectural regulators, brings the sites together. The interaction between activators and RNA polymerase may be mediated by coactivators, as shown. Repression is sometimes mediated by repressors (described later) that bind to activators, thereby preventing the activating interaction with RNA polymerase.

Many Bacterial Genes Are Clustered and Regulated in Operons

Bacteria have a simple general mechanism for coordinating the regulation of multiple genes: these genes are clustered on the chromosome and are transcribed together. Many bacterial mRNAs are polycistronic—multiple genes on a single transcript—and the single promoter that initiates transcription of the cluster is the site of regulation for expression of all the genes in the cluster. The gene cluster and promoter, plus additional sequences that function together in regulation, are called an **operon** (**Fig. 28-6**). Operons that include two to six genes transcribed as a unit are common; some operons contain 20 or more genes. The identity and order of the genes in an operon are not random. In many cases, genes in the same operon encode subunits of a larger protein complex, and cotranslation directly enables assembly of the complex. Some operons organize genes involved in related processes that require coordinated regulation. In other cases, the genes may seem to be unrelated, but they encode products required by the cell under similar conditions.

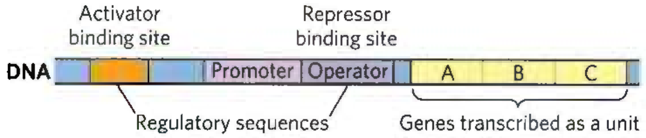


FIGURE 28-6 Representative bacterial operon. Genes A, B, and C are transcribed on one polycistronic mRNA. Typical regulatory sequences include binding sites for proteins that either activate or repress transcription from the promoter.

Many of the principles of bacterial gene expression were first defined by studies of lactose metabolism in *E. coli*, which can use lactose as its sole carbon source. In 1960, François Jacob and Jacques Monod published a short paper in the *Proceedings of the French Academy of Sciences* that described how two adjacent genes involved in lactose metabolism were coordinately regulated by a genetic element located at one end of the gene cluster. The genes were those for β-galactosidase, which cleaves lactose to galactose and glucose, and for galactoside permease, which transports lactose into the cell (**Fig. 28-7**). The terms "operon" and "operator" were first introduced in this paper. With the operon model, gene regulation could, for the first time, be considered in molecular terms.

The *lac* Operon Is Subject to Negative Regulation

The lactose (*lac*) operon (**Fig. 28-8a**) includes the genes for β-galactosidase (Z), galactoside permease (Y), and thiogalactoside transacetylase (A). The last of these enzymes seems to modify toxic galactosides to facilitate their removal from the cell. Each of the three genes is preceded by a ribosome-binding site (not shown in Fig. 28-8) that independently directs the translation of that gene (Chapter 27). Regulation of the *lac* operon by the *lac* repressor protein (Lac) follows the pattern outlined in Figure 28-4a.

The study of *lac* operon mutants has revealed some details of the workings of the operon's regulatory system. In the absence of lactose, the *lac* operon genes are repressed. Mutations in the operator or in another gene, the *I* gene, result in constitutive synthesis of the gene products. When the *I* gene is defective, repression can be restored by introducing a functional *I* gene into the cell on another DNA molecule, demonstrating that the *I* gene encodes a diffusible molecule that causes gene repression. This molecule proved to be a protein, now called the Lac repressor, a tetramer of identical monomers. The operator to which it binds most tightly (O_1) abuts the transcription start site (Fig. 28-8a). The *I* gene is transcribed from its own promoter (P_I) independent of the *lac* operon genes. The *lac* operon has two secondary

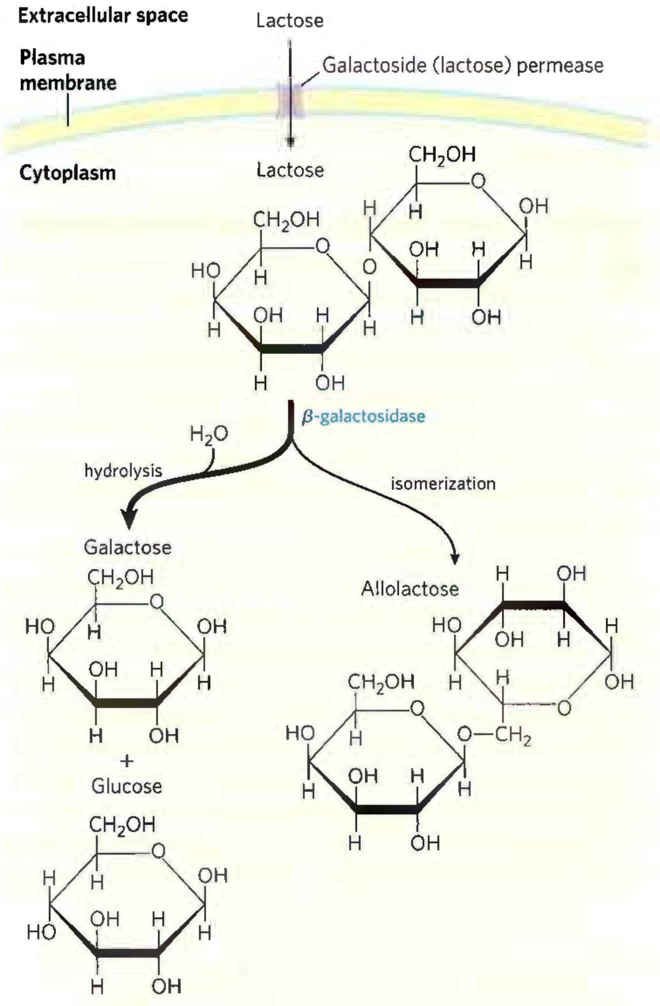


FIGURE 28-7 Lactose metabolism in *E. coli*. Uptake and metabolism of lactose require the activities of galactoside (lactose) permease and β-galactosidase. Conversion of lactose to allolactose by transglycosylation is a minor reaction also catalyzed by β-galactosidase.

binding sites for the Lac repressor: O_2 and O_3. O_2 is centered near position +410, within the gene encoding β-galactosidase (*Z*); O_3 is near position –90, within the *I* gene. To repress the operon, the Lac repressor seems to bind to both the main operator and one of the two secondary sites, with the intervening DNA looped out (Fig. 28-8b, c). Either binding arrangement blocks transcription initiation.

Despite this elaborate binding complex, repression is not absolute. Binding of the Lac repressor reduces the rate of transcription initiation by a factor of 10^3. If the O_2 and O_3 sites are eliminated by deletion or mutation, the binding of repressor to O_1 alone reduces transcription by a factor of about 10^2. Even in the repressed state, each cell has a few molecules of β-galactosidase and galactoside permease, presumably synthesized on the rare occasions when the repressor transiently dissociates from the operators. This basal level of transcription is essential to operon regulation.

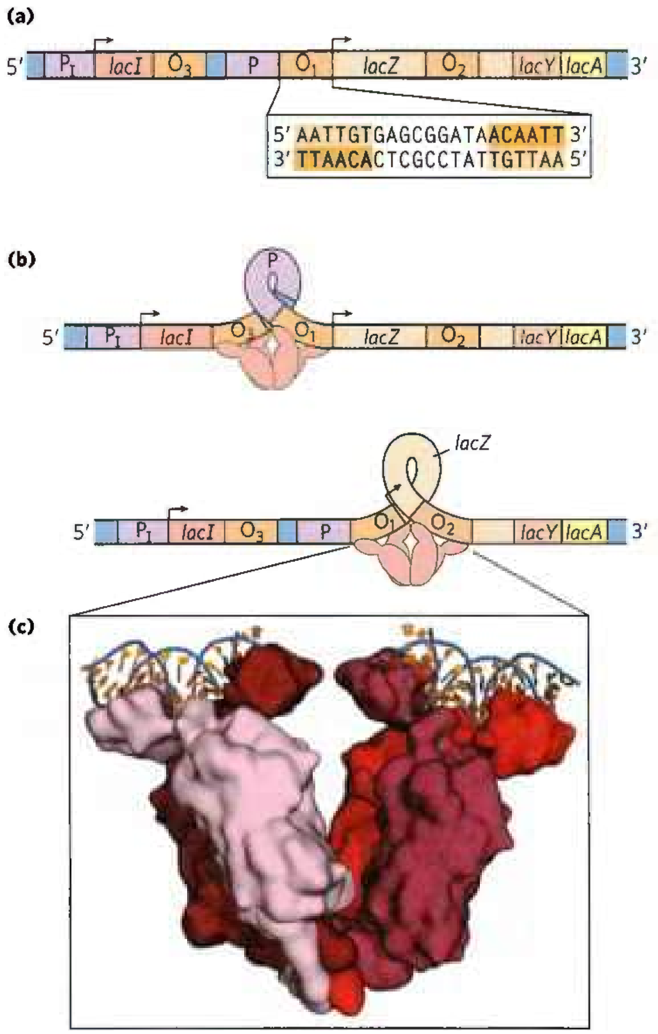
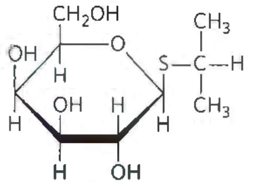


FIGURE 28-8 The *lac* operon. (a) In the *lac* operon, the *lacI* gene encodes the Lac repressor. The *lac Z*, *Y*, and *A* genes encode β-galactosidase, galactoside permease, and thiogalactoside transacetylase, respectively. P is the promoter for the *lac* genes, and P_I is the promoter for the *I* gene. O_1 is the main operator for the *lac* operon; O_2 and O_3 are secondary operator sites of lesser affinity for the Lac repressor. The inverted repeat to which the Lac repressor binds in O_1 is shown. (b) The Lac repressor binds to the main operator and O_2 or O_3, and it seems to form a loop in the DNA. (c) Lac repressor (shades of red) is shown bound to short, discontinuous segments of DNA (blue and orange). [(c) Data from PDB ID 2PE5, R. Daber et al., *J. Mol. Biol.* 370:609, 2007.]

When cells are provided with lactose, the *lac* operon is induced. An inducer (signal) molecule binds to a specific site on the Lac repressor, causing a conformational change that results in dissociation of the repressor from the operator. The inducer in the *lac* operon system is not lactose itself but allolactose, an isomer of lactose (Fig. 28-7). After entry into the *E. coli* cell (via the few preexisting molecules of lactose permease), lactose is converted to allolactose by one of the few preexisting β-galactosidase molecules. Release of the operator by Lac repressor, triggered as the repressor binds to allolactose, allows expression of the *lac* operon genes and leads to a 10^3-fold increase in the concentration of β-galactosidase.

Several β-galactosides structurally related to allolactose are inducers of the *lac* operon but are not substrates for β-galactosidase; others are substrates but not inducers. One particularly effective and nonmetabolizable inducer of the *lac* operon that is often used experimentally is isopropylthiogalactoside (IPTG).



Isopropyl-β-D-thiogalactoside (IPTG)

An inducer that cannot be metabolized allows researchers to explore the physiological function of lactose as a carbon source for growth, separate from its function in the regulation of gene expression.

In addition to the multitude of operons now known in bacteria, a few polycistronic operons have been found in the cells of lower eukaryotes. In the cells of higher eukaryotes, however, almost all protein-coding genes are transcribed separately.

The mechanisms by which operons are regulated can vary significantly from the simple model presented in Figure 28-8. Even the *lac* operon is more complex than indicated here, with an activator also contributing to the overall scheme, as we shall see in Section 28.2. Before any further discussion of the layers of regulation of gene expression, however, we examine the critical molecular interactions between DNA-binding proteins (such as repressors and activators) and the DNA sequences to which they bind.

Regulatory Proteins Have Discrete DNA-Binding Domains

P2 Regulatory proteins generally bind to specific DNA sequences. Their affinity for these target sequences is roughly 10^4 to 10^6 times higher than their affinity for any other DNA sequence. Most regulatory proteins have discrete DNA-binding domains containing substructures that interact closely and specifically with the DNA. These binding domains usually include one or more of a relatively small group of recognizable and characteristic structural motifs.

To bind specifically to DNA sequences, regulatory proteins must recognize surface features on the DNA (see Fig. 8-13). Most of the chemical groups that differ among the four bases and thus permit discrimination between base pairs are hydrogen-bond donor and acceptor groups exposed in the major groove of DNA (**Fig. 28-9**), and most of the protein-DNA contacts that impart specificity are hydrogen bonds. A notable exception is the nonpolar surface near C-5 of pyrimidines, where thymine is readily distinguished from cytosine by its protruding methyl group. Protein-DNA contacts are also possible in the

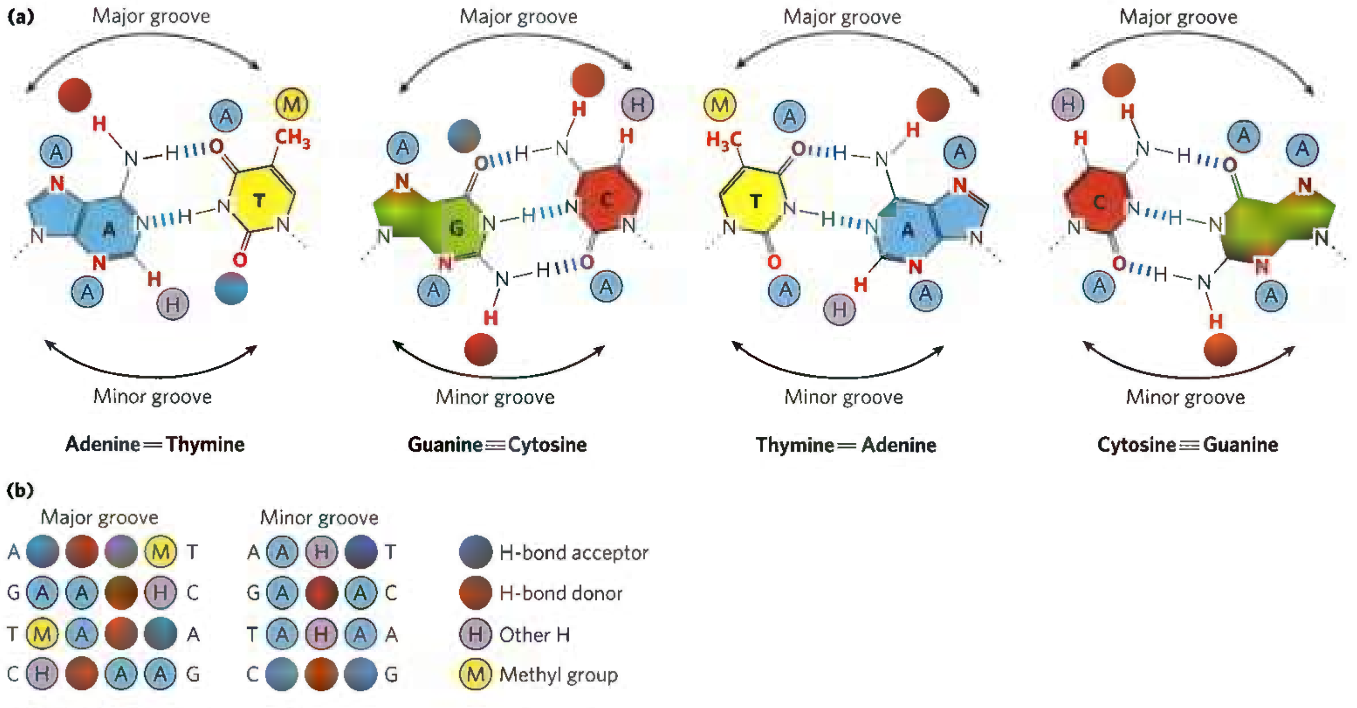


FIGURE 28-9 Groups in DNA available for protein binding. (a) Shown here are functional groups on all four base pairs that are displayed in the major and minor grooves of DNA. Hydrogen-bond acceptor (A) and donor (D) atoms are marked by blue and red disks, respectively. Other hydrogen atoms (H) are marked with purple disks, and methyl groups (M) with yellow disks. (b) Recognition patterns for each base pair (from left to right). The much greater variation in the patterns for the major groove gives rise to a much greater discriminatory power in the major groove relative to the minor groove. [Information from J. L. Huret, *Atlas Genet. Cytogenet. Oncol. Haematol.* 2006, http://atlasgeneticsoncology.org/Educ/DNAEngID30001ES.html.]

minor groove of the DNA, but the hydrogen-bonding patterns there generally do not allow ready discrimination between base pairs.

Within regulatory proteins, the amino acid side chains most often hydrogen-bonding to bases in the DNA are those of Asn, Gln, Glu, Lys, and Arg residues. Is there a simple recognition code in which a particular amino acid always pairs with a particular base? The two hydrogen bonds that can form between Gln or Asn and the N^6 and N-7 positions of adenine cannot form with any other base. And an Arg residue can form two hydrogen bonds with N-7 and O^6 of guanine (**Fig. 28-10**). Examination of the structure of many DNA-binding proteins, however, has shown that a protein can recognize each base pair in more than one way, leading to the conclusion that there is no simple amino acid–base code. For some proteins, the Gln-adenine interaction can specify A═T base pairs, but in others a van der Waals pocket for the methyl group of thymine can recognize A═T base pairs. Researchers cannot yet examine the structure of a DNA-binding protein and infer the DNA sequence to which it binds.

To interact with bases in the major groove of DNA, a protein requires a relatively small substructure that can stably protrude from the protein surface. The DNA-binding domains of regulatory proteins tend to be small (60 to 90 amino acid residues), and the structural motifs within these domains that are actually in contact with the DNA are smaller still. Many small proteins are unstable because of their limited capacity to form layers of structure to bury hydrophobic groups. The DNA-binding motifs provide either a very compact stable structure or a way of allowing a segment of protein to protrude from the protein surface.

P2 The DNA-binding sites for regulatory proteins are often inverted repeats of a short DNA sequence (a palindrome) at which multiple (usually two) subunits of a regulatory protein bind cooperatively. The Lac repressor is unusual in that it functions as a tetramer, with two dimers tethered together at the end distant from the DNA-binding sites (Fig. 28-8b). An *E. coli* cell usually contains about 20 tetramers of the Lac repressor. Each of the tethered dimers separately binds to a palindromic operator sequence, in contact with 17 bp of a 22 bp region in the *lac* operon. And each of the tethered dimers can independently bind to an operator sequence, with one generally binding to O_1 and the other to O_2 or O_3 (as in Fig. 28-8b). The symmetry of the O_1 operator sequence corresponds to the twofold axis of symmetry of two paired Lac repressor subunits. The tetrameric Lac repressor binds to its operator sequences in vivo with an estimated dissociation constant of 10^{-10} M. The repressor discriminates between the operators and other sequences by a factor of about 10^6, so binding to these few base pairs among the 4.6 million or so of the *E. coli* chromosome is highly specific.

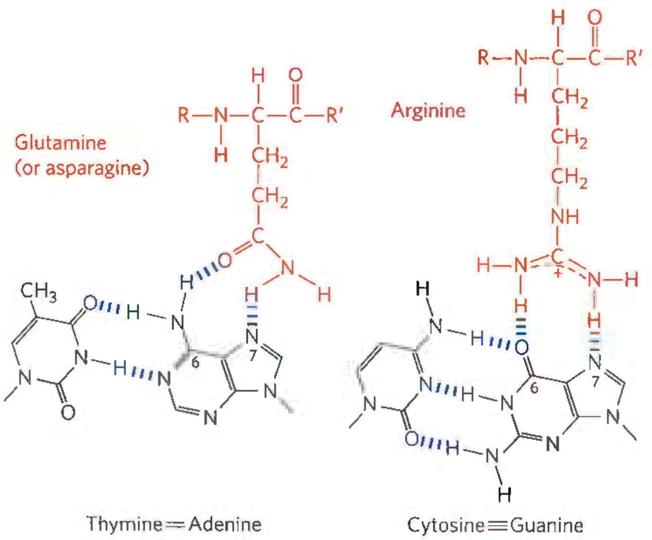


FIGURE 28-10 Specific amino acid residue–base pair interactions. The two examples shown have been observed in DNA-protein binding.

Several DNA-binding motifs have been described, but here we focus on two that play prominent roles in the binding of DNA by regulatory proteins from all domains of life: the helix-turn-helix and the zinc finger. We also consider two other types of such motifs: the homeodomain and the RNA recognition motif, which, as its name implies, also binds RNA; both motifs play prominent roles in some eukaryotic regulatory proteins.

Helix-Turn-Helix The **helix-turn-helix** motif is crucial to the interaction of many regulatory proteins with DNA in bacteria, and similar motifs occur in some eukaryotic regulatory proteins. The helix-turn-helix comprises about 20 amino acid residues in two short α-helical segments, each 7 to 9 residues long, separated by a β turn (**Fig. 28-11**). This structure generally is not stable by itself; it is simply the interactive portion of a somewhat larger DNA-binding domain. One of the two α-helical segments is called the recognition helix, because it usually contains many of the amino acids that interact with DNA in a sequence-specific way. This α helix is stacked on other segments of the protein structure so that it protrudes from the protein surface. When bound to DNA, the recognition helix is positioned in or nearly in the major groove. The Lac repressor has this DNA-binding motif.

Zinc Finger In a **zinc finger**, about 30 amino acid residues form an elongated loop held together at the base by a single Zn^{2+} ion, which is coordinated to 4 of the residues (4 Cys, or 2 Cys and 2 His). The zinc does not itself interact with DNA; rather, the coordination of zinc with the amino acid residues stabilizes this small structural motif. Several hydrophobic side chains in the core of the structure also lend stability. **Figure 28-12** shows the interaction between DNA and three zinc fingers of a single polypeptide from the mouse regulatory protein Zif268.

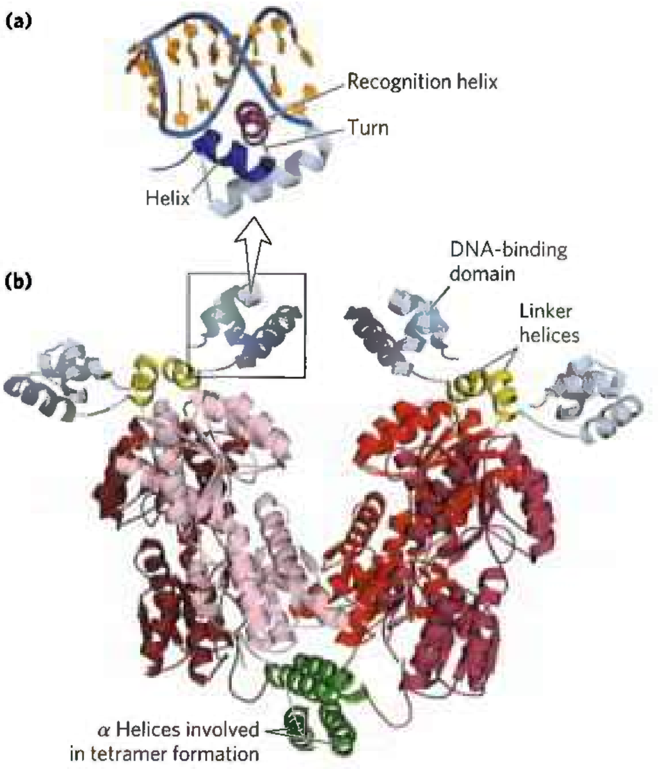


FIGURE 28-11 Helix-turn-helix. (a) DNA-binding domain of the Lac repressor bound to DNA. (b) The entire Lac repressor. The DNA-binding domains and the α helices involved in tetramer formation are labeled. The remainder of the protein has the binding sites for allolactose. The allolactose-binding domains are linked to the DNA-binding domains through linker helices. [Data from PDB ID 2PE5, R. Daber et al., *J. Mol. Biol.* 370:609, 2007.]

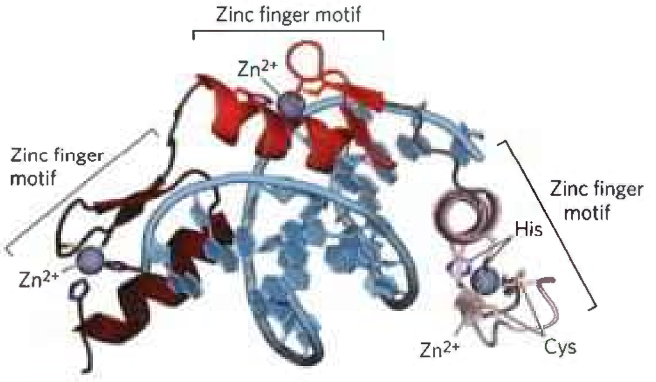


FIGURE 28-12 Zinc fingers. Three zinc fingers of the regulatory protein Zif268, complexed with DNA. Each Zn^{2+} coordinates with two His and two Cys residues. [Data from PDB ID 1ZAA, N. P. Pavletich and C. O. Pabo, *Science* 252:809, 1991.]

Many eukaryotic DNA-binding proteins contain zinc fingers. The interaction of a single zinc finger with DNA is typically weak, and many DNA-binding proteins, like Zif268, have multiple zinc fingers that substantially enhance binding by interacting simultaneously with the DNA. One DNA-binding protein of the frog *Xenopus* has 37 zinc fingers. There are few known examples of the zinc finger motif in bacterial proteins.

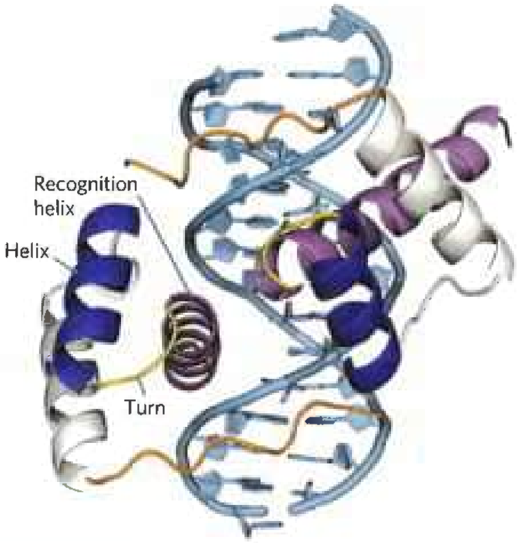


FIGURE 28-13 Homeodomains. Shown here are two homeodomains bound to DNA. In each homeodomain, the recognition helix, layered on two others, can be seen protruding into the major groove. This is only a small part of a larger regulatory protein from a class called Pax, active in the regulation of development in fruit flies (see Section 28.3). [Data from PDB ID 1FJL, D. S. Wilson et al., *Cell* 82:709, 1995.]

The precise manner in which proteins with zinc fingers bind to DNA differs from one protein to the next. Some zinc fingers contain the amino acid residues that are important in sequence discrimination, whereas others seem to bind DNA nonspecifically (the amino acids required for specificity are located elsewhere in the protein). Zinc fingers can also function as RNA-binding motifs, such as in certain proteins that bind eukaryotic mRNAs and act as translational repressors. We discuss this role later (Section 28.3).

Homeodomain Another type of DNA-binding domain has been identified in some proteins that function as transcriptional regulators, especially during eukaryotic development. This domain of 60 amino acid residues—called the **homeodomain**, because it was discovered in homeotic genes (genes that regulate the development of body patterns)—is highly conserved and has now been identified in proteins from a wide variety of organisms, including humans (Fig. 28-13). The DNA-binding segment of the domain is related to the helix-turn-helix motif. The DNA sequence that encodes this domain is known as the **homeobox**.

RNA Recognition Motif New classes of proteins with RNA-binding domains continue to be identified. **RNA recognition motifs (RRMs)** are found in some eukaryotic gene activators, where they may do double duty in binding DNA and RNA. When bound to specific binding sites in DNA, these activators induce transcription. The same activators are sometimes regulated in part by specific lncRNAs that compete with DNA binding and decrease gene transcription. Other proteins with RRM motifs bind to mRNA, rRNA, or any of a range of other smaller, noncoding RNAs. The RRM consists of 90 to 100 amino acid residues, arranged in a four-strand antiparallel β sheet sandwiched against two α helices, with a β_1-α_1-β_2-β_3-α_2-β_4 topology (**Fig. 28-14a**). This motif may be present as part of DNA-binding regulatory proteins that also have other DNA-binding motifs or may occur in proteins that bind uniquely to RNA. The RRM is just one of many diverse protein motifs known to interact with RNA (Fig. 28-14b).

Regulatory Proteins Also Have Protein-Protein Interaction Domains

Regulatory proteins contain domains not only for DNA binding but also for protein-protein interactions—with RNA polymerase, other regulatory proteins, or other subunits of the same regulatory protein. Examples include many eukaryotic transcription factors that function as gene activators, which often bind as dimers to the DNA through DNA-binding domains that contain zinc fingers. Some structural domains are devoted to the interactions required for dimer formation, which is generally a prerequisite for DNA binding. Like DNA-binding motifs, the structural motifs that mediate protein-protein interactions tend to fall within one of a few common categories. Two important examples are the leucine zipper and the basic helix-loop-helix. Structural motifs such as these are the basis for classifying some regulatory proteins into structural families.

Leucine Zipper The **leucine zipper** is an amphipathic α helix with a series of hydrophobic amino acid residues concentrated on one side (**Fig. 28-15**), with the hydrophobic surface forming the area of contact between the two polypeptides of a dimer. A striking

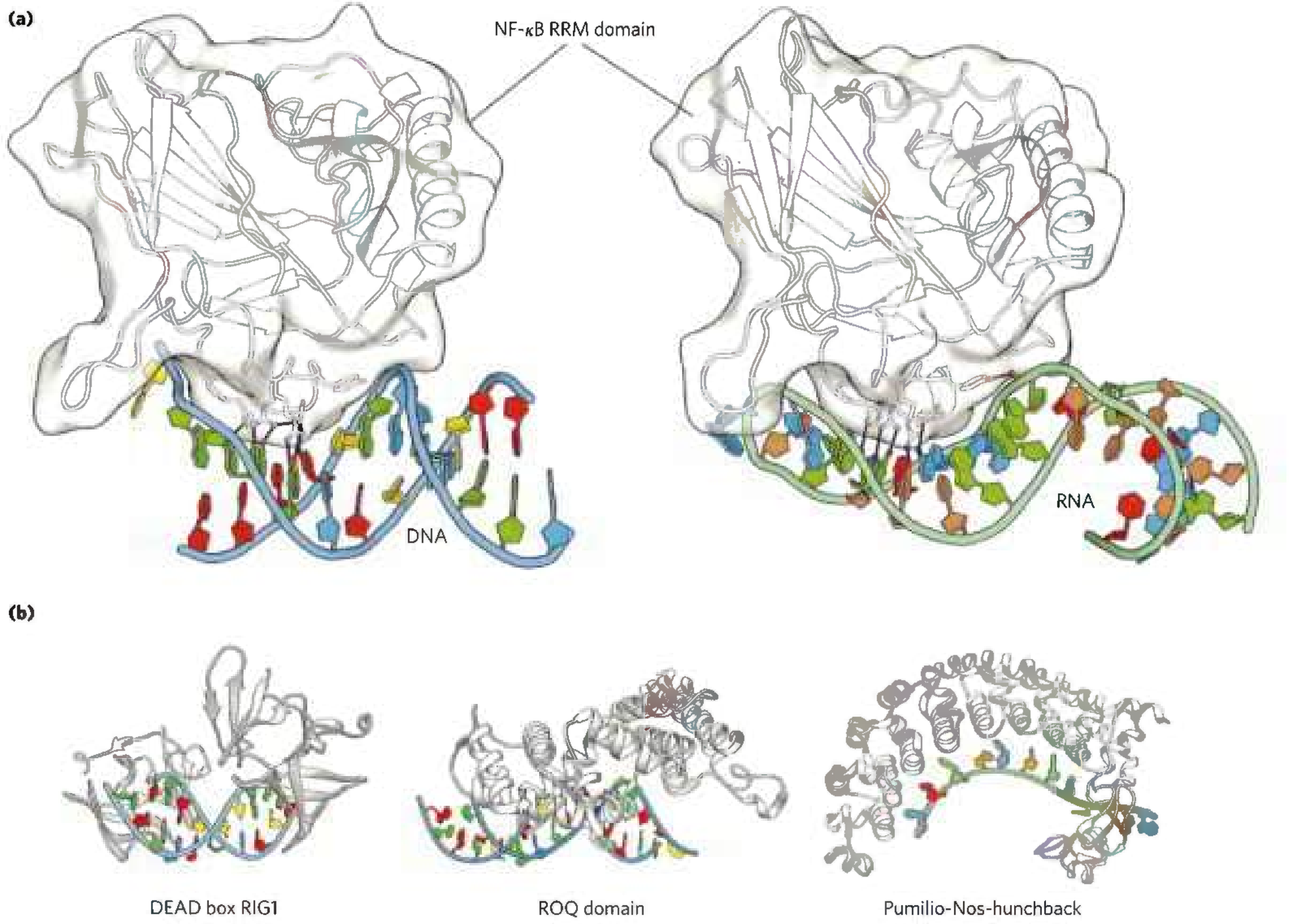


FIGURE 28-14 **RNA recognition motifs (RRMs).** (a) An RRM from the p50 subunit of regulatory protein NF-κB is shown, bound to (left) DNA and (right) RNA. Black lines indicate hydrogen-bonding interactions between particular amino acid residues and bases in the DNA or RNA. NF-κB is the name of a family of structurally related eukaryotic transcription factors that regulate processes ranging from immune and inflammatory responses to cell growth and apoptosis. (b) Three additional RRM motifs from widespread RNA-binding protein families. [(a) Data from (left) PDB ID 1OOA, D. B. Huang et al., *Proc. Natl. Acad. Sci. USA* 100:9268, 2003; (right) PDB ID 1VKX, F. E. Chen et al., *Nature* 391:410, 1998. (b) DEAD box RIG1: PDB ID 3LRR, C. Lu et al., *Structure* 18:1032, 2010; ROQ domain: PDB ID 4QIK, D. Tan et al., *Nat. Struct. Mol. Biol.* 21:679, 2014; Pumilio-Nos-hunchback: PDB ID 5KL1, C. A. Weidmann et al., *eLife* 5, 2016.]

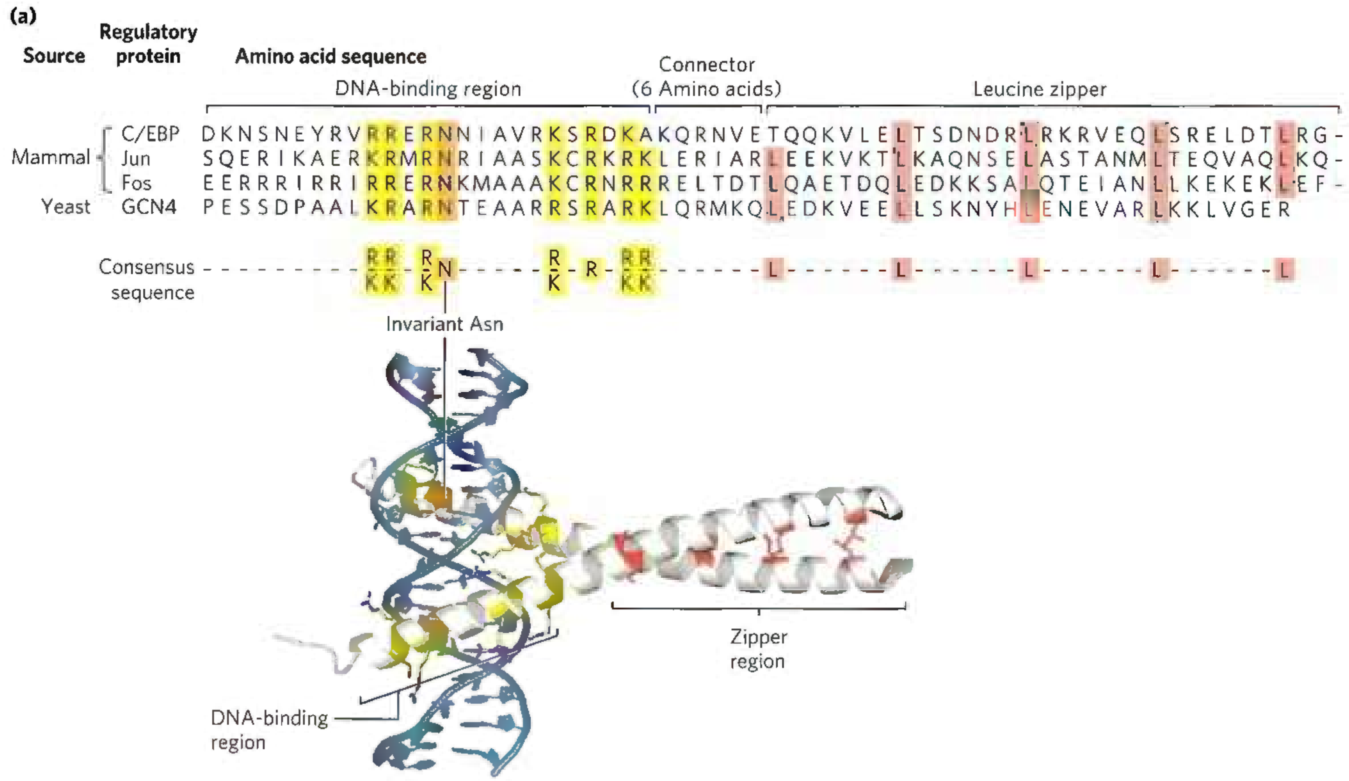

(a)
Source
Regulatory protein
Amino acid sequence
DNA-binding region
Connector (6 Amino acids)
Leucine zipper
Mammal
C/EBP DKNSNEYRVRRERNNIAVRKSRDKAKQRNVETQQKVLELTSDNDRLRKRVEQLSRELDTLRG-
Jun SQERIKAERKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELASTANMLTEQVAQLKQ-
Fos EERRRIRRIRRERNKMAAAKCRNRRRELTDTLQAETDQLEDKKSALQTEIANLLKEKEKLEF-
Yeast
GCN4 PESSDPAALKRARNTEAARRSRARKLQRMKQLEDKVEELLSKNYHLENEVARLKKLVGER
Consensus sequence
RR/KK R/K N R/K R RR/KK L L L L L
Invariant Asn
Zipper region
DNA-binding region

FIGURE 28-15 Leucine zippers. (a) Comparison of amino acid sequences of several leucine zipper proteins. Notice the Leu (L) residues (shaded) at every seventh position in the zipper region, and the number of Lys (K) and Arg (R) residues in the DNA-binding region. (b) Leucine zipper from the yeast activator protein GCN4. Only the "zippered" α helices, derived from different subunits of the dimeric protein, are shown. The two helices wrap around each other in a gently coiled coil. The interacting Leu side chains and the conserved residues in the DNA-binding region are highlighted to correspond to the sequence in (a). [(a) Information from S. L. McKnight, *Sci. Am.* 264 (April):54, 1991. (b) Data from PDB ID 1YSA, T. E. Ellenberger et al., *Cell* 71:1223, 1992.]

feature of these α helices is the occurrence of Leu residues at every seventh position, forming a straight line along the hydrophobic surface. Although researchers initially thought the Leu residues interdigitated (hence the name "zipper"), we now know that they line up, side by side, as the interacting α helices coil around each other (forming a coiled coil; Fig. 28-15b). Regulatory proteins with leucine zippers often have a separate DNA-binding domain with a high concentration of basic (Lys or Arg) residues that can interact with the negatively charged phosphates of the DNA backbone. Leucine zippers have been found in many eukaryotic proteins and a few bacterial proteins.

Basic Helix-Loop-Helix Another common structural motif, the **basic helix-loop-helix**, occurs in some eukaryotic regulatory proteins implicated in the control of gene expression during development of multicellular organisms. These proteins share a conserved region of about 50 amino acid residues important in both DNA binding and protein dimerization. This region can form two short, amphipathic α helices linked by a loop of variable length, the helix-loop-helix (distinct from the helix-turn-helix motif associated with DNA binding). The helix-loop-helix motifs of two polypeptides interact to form dimers (**Fig. 28-16**). In these proteins, DNA binding is mediated by an adjacent short amino acid sequence rich in basic residues, similar to the separate DNA-binding region in proteins containing leucine zippers.

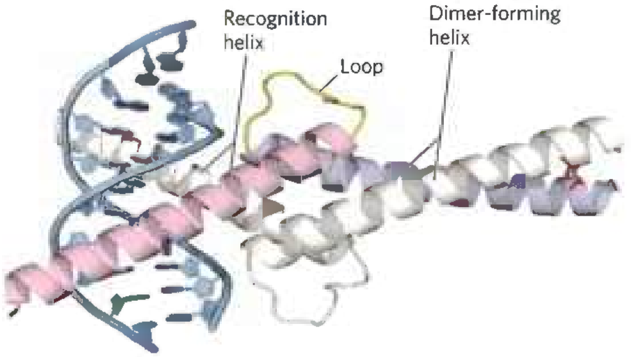

Recognition helix
Loop
Dimer-forming helix

FIGURE 28-16 Helix-loop-helix. The human transcription factor Max, bound to its DNA target site. The protein is dimeric; one subunit is colored. The recognition helix is linked via the loop to the dimer-forming helix, which merges with the carboxyl-terminal end of the subunit. Interaction of the carboxyl-terminal helices of the two subunits describes a coiled coil very similar to that of a leucine zipper (see Fig. 28-15b), but with only one pair of interacting Leu residues (side chains at the right) in this example. The overall structure is sometimes called a helix-loop-helix/leucine zipper motif. [Data from PDB ID 1HLO, P. Brownlie et al., *Structure* 5:509, 1997.]

Protein-Protein Interactions in Eukaryotic Regulatory Proteins In eukaryotes, most genes are regulated by activators, and most genes are monocistronic. If a different activator were required for each gene, the number of activators (and genes encoding them) would need to be equivalent to the number of regulated genes. However, in yeast, about 300 transcription factors (many of them activators) are responsible for the regulation of many thousands of genes. Many of the transcription factors regulate the induction of multiple genes, but most genes are subject to regulation by multiple transcription factors. **P4** Appropriate regulation of different genes is accomplished by different combinations of a limited repertoire of transcription factors at each gene, a mechanism referred to as **combinatorial control**.

Combinatorial control is accomplished in part by mixing and matching the variants within a regulatory protein family to form a series of different active protein dimers. Several families of eukaryotic transcription factors have been defined on the basis of close structural similarities. Within each family, dimers can sometimes form between two identical proteins (a homodimer) or between two different members of the family (a heterodimer). A hypothetical family of four different leucine-zipper proteins could thus form up to 10 different dimeric species. In many cases, the different combinations have distinct regulatory and functional properties and regulate different genes. As we shall see, multiple regulatory proteins of this kind function in the regulation of most eukaryotic genes, further contributing to combinatorial control.

In addition to having structural domains devoted to DNA binding and protein dimerization, which direct a particular protein dimer to a particular gene, many regulatory proteins have domains that interact with RNA polymerase, with regulatory RNAs, with unrelated regulatory proteins, or with some combination of the three. At least three types of additional domains for protein-protein interaction have been characterized (primarily in eukaryotes): glutamine-rich, proline-rich, and acidic domains, the names reflecting the amino acid residues that are especially abundant.

Protein-DNA and protein-RNA binding interactions are the basis of the intricate regulatory circuits fundamental to gene function. We now turn to a closer examination of these gene regulatory schemes, first in bacteria, then in eukaryotes.

SUMMARY 28.1 *The Proteins and RNAs of Gene Regulation*

- Transcription is initiated when an RNA polymerase interacts with a site called a promoter. In bacteria, the frequency of transcription initiation is dictated in part by sequence changes within the promoter. For gene products required all the time at a defined level—the products of housekeeping genes—the promoter sequence may be the sole element of regulation. For genes encoding products that are not always needed, regulation is imposed by additional proteins and RNAs.
- Regulation of gene transcription is imposed primarily by three types of proteins: specificity factors, repressors, and activators. Regulatory RNAs also play an important role in regulating the expression of many genes.
- In bacteria, genes that encode products with interdependent functions are often clustered in an operon, a single transcriptional unit. Transcription of the genes is generally blocked by binding of a specific repressor protein at a DNA site called an operator. Dissociation of the repressor from the operator is mediated by a specific small molecule, an inducer.
- Many principles of gene regulation in bacteria were first elucidated in studies of the lactose (*lac*) operon. The Lac repressor dissociates from the *lac* operator when the repressor binds to its inducer, allolactose.
- Regulatory proteins are DNA-binding proteins that recognize specific DNA sequences; most have distinct DNA-binding domains. Within these domains, common structural motifs that bind DNA (and/or RNA) are the helix-turn-helix, zinc finger, homeodomain, and RNA recognition motif.
- Regulatory proteins often contain domains such as the leucine zipper and helix-loop-helix, required for dimerization or other protein-protein interactions, and other motifs required for activation of transcription. Mixing and matching of protein family variants in dimeric transcription factors provides for more efficient and responsive regulation through combinatorial control.

28.2 Regulation of Gene Expression in Bacteria

As in many other areas of biochemical investigation, the study of the regulation of gene expression advanced earlier and faster in bacteria than in other experimental organisms. The examples of bacterial gene regulation presented here are chosen from among scores of well-studied systems, partly for their historical significance, but primarily because they provide a good overview of the range of regulatory mechanisms in bacteria. Many of the principles of bacterial gene regulation are also relevant to understanding gene expression in eukaryotic cells.

We begin by examining the lactose and tryptophan operons; each system has regulatory proteins, but the overall mechanisms of regulation are very different. This is followed by a short discussion of the SOS response in *E. coli*, illustrating how genes scattered throughout the genome can be coordinately regulated. We then describe two bacterial systems of quite different types, illustrating the diversity of gene regulatory mechanisms: regulation of ribosomal protein synthesis at the level of translation, with many of the regulatory proteins binding to RNA (rather than DNA), and regulation of the process of "phase variation" in *Salmonella*, which results from genetic recombination. Finally, we examine some additional examples of posttranscriptional regulation in which the RNA modulates its own function.

The *lac* Operon Undergoes Positive Regulation

The operator-repressor-inducer interactions described earlier for the *lac* operon (Fig. 28-8) provide an intuitively satisfying model for an on/off switch in the regulation of gene expression, but operon regulation is rarely so simple. A bacterium's environment is too complex for its genes to be controlled by one signal. Other factors besides lactose, such as the availability of glucose, affect the expression of the *lac* genes. Glucose, metabolized directly by glycolysis, is the preferred energy source in *E. coli*. Other sugars can serve as the main or sole nutrient, but extra enzymatic steps are required to prepare them for entry into glycolysis, necessitating the synthesis of additional enzymes. Clearly, expressing the genes for proteins that metabolize sugars such as lactose or arabinose is wasteful when glucose is abundant.

What happens to the expression of the *lac* operon when both glucose and lactose are present? A regulatory mechanism known as **catabolite repression** restricts expression of the genes required for catabolism of lactose, arabinose, and other sugars in the presence of glucose, even when these secondary sugars are also present. The effect of glucose is mediated by cAMP, as a coactivator, and an activator protein known as **cAMP receptor protein**, or **CRP** (the protein is sometimes called CAP, for *c*atabolite gene *a*ctivator *p*rotein). CRP is a homodimer (subunit M_r 22,000) with binding sites for DNA and cAMP. Binding is mediated by a helix-turn-helix motif in the protein's DNA-binding domain (**Fig. 28-17**). When glucose is absent, CRP-cAMP binds to a site near the *lac* promoter (**Fig. 28-18**) and stimulates RNA transcription 50-fold. The wild-type *lac* promoter is a relatively weak promoter, diverging from the consensus shown in Figure 28-2. The open complex of RNA polymerase and the promoter (see Fig. 26-6) does not form readily unless CRP-cAMP is present and also bound (Fig. 28-18a, c). CRP-cAMP is therefore a positive regulatory element responsive to glucose levels, whereas the Lac repressor is a negative regulatory element responsive to lactose.

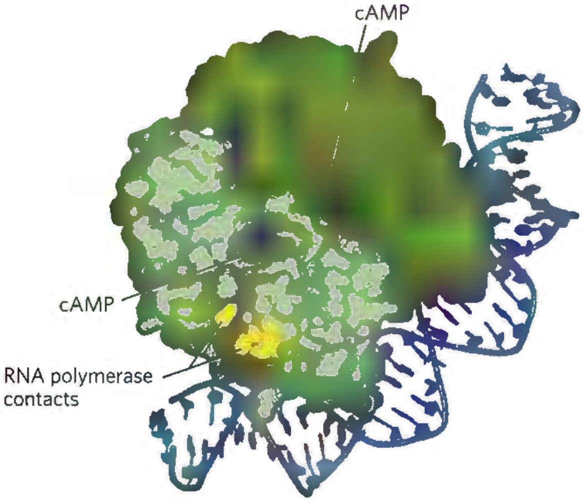
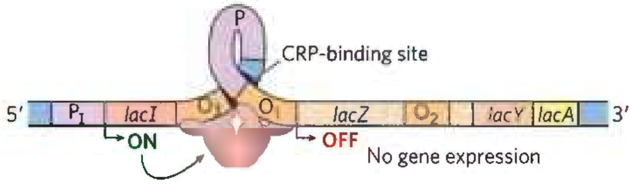
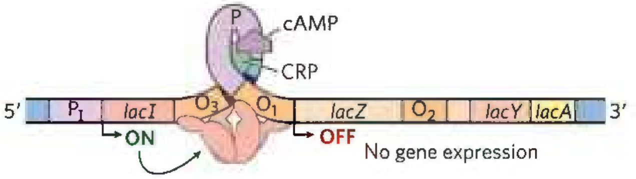


FIGURE 28-17 CRP homodimer with bound cAMP. Note the bending of the DNA around the protein. The region that interacts with RNA polymerase is labeled. [Data from PDB ID 1RUN, G. Parkinson et al., *Nat. Struct. Biol.* 3:837, 1996.]

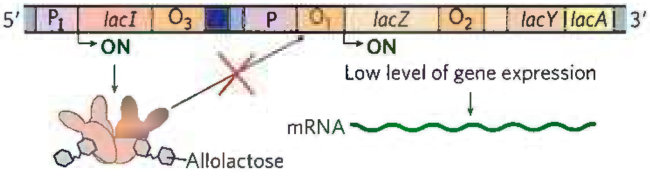
(a) Glucose high, cAMP low, lactose absent



(b) Glucose low, cAMP high, lactose absent



(c) Glucose high, cAMP low, lactose present



(d) Glucose low, cAMP high, lactose present

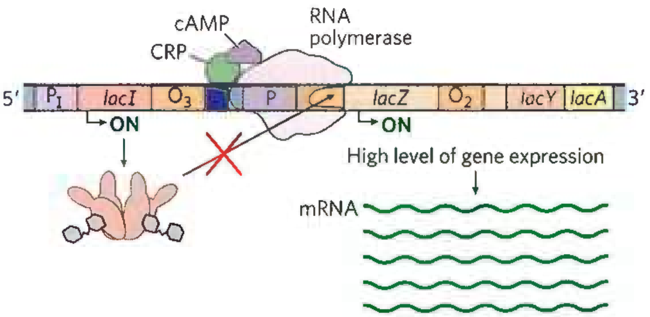


FIGURE 28-18 Positive regulation of the *lac* operon by CRP. The binding site for CRP-cAMP is near the promoter. The combined effects of glucose and lactose availability on *lac* operon expression are shown. When lactose is absent, the repressor binds to the operator and prevents transcription of the *lac* genes. It does not matter whether glucose is (a) present or (b) absent. (c) If lactose is present, the repressor dissociates from the operator. However, if glucose is also available, low cAMP levels prevent CRP-cAMP formation and DNA binding. RNA polymerase may occasionally bind and initiate transcription, resulting in a very low level of *lac* genes transcription. (d) When lactose is present and glucose levels are low, cAMP levels rise. The CRP-cAMP complex forms and facilitates robust binding of RNA polymerase to the *lac* promoter and high levels of transcription.

The two act in concert. CRP-cAMP has little effect on the *lac* operon when the Lac repressor is blocking transcription, and dissociation of the repressor from the *lac* operator has little effect on transcription of the *lac* operon unless CRP-cAMP is present to facilitate transcription. CRP interacts directly with RNA polymerase (at the region shown in Fig. 28-17) through the polymerase's α subunit. Thus, optimal expression of the *lac* operon requires dissociation of the Lac repressor (indicating that lactose is available) and the binding of CRP-cAMP (indicating that glucose is not available).

The effect of glucose on CRP is mediated by the cAMP interaction (Fig. 28-18). CRP binds to DNA most avidly when cAMP concentrations are high. In the presence of glucose, the synthesis of cAMP is inhibited and efflux of cAMP from the cell is stimulated. As [cAMP] declines, CRP binding to DNA declines, thereby decreasing the expression of the *lac* operon.

CRP and cAMP participate in the coordinated regulation of many operons, primarily those that encode enzymes for the metabolism of secondary sugars such as lactose and arabinose. A network of operons with a common regulator is called a **regulon**. This arrangement, which allows coordinated shifts in cellular functions that can require the action of hundreds of genes, is a major theme in the regulated expression of dispersed networks of genes in eukaryotes. Other bacterial regulons include the heat shock gene system that responds to changes in temperature and the genes induced in *E. coli* as part of the SOS response to DNA damage, described later.

Many Genes for Amino Acid Biosynthetic Enzymes Are Regulated by Transcription Attenuation

The 20 common amino acids are required in large amounts for protein synthesis, and *E. coli* can synthesize all of them. The genes for the enzymes needed to synthesize a given amino acid are generally clustered in an operon and are expressed whenever existing supplies of that amino acid are inadequate for cellular requirements. When the amino acid is abundant, the biosynthetic enzymes are not needed and the operon is repressed.

The *E. coli* tryptophan (*trp*) operon (**Fig. 28-19**) includes five genes for the enzymes required to convert chorismate to tryptophan (see Fig. 22-19). Note that two of the enzymes catalyze more than one step in the pathway. The mRNA from the *trp* operon has a half-life of only about 3 min, allowing the cell to respond rapidly to changing needs for this amino acid. The Trp repressor is a homodimer. When tryptophan is abundant, it binds to the Trp repressor, causing a conformational change that permits the repressor to bind to the *trp* operator and inhibit expression of the *trp* operon. The *trp* operator site overlaps the promoter, so binding of the repressor blocks binding of RNA polymerase.

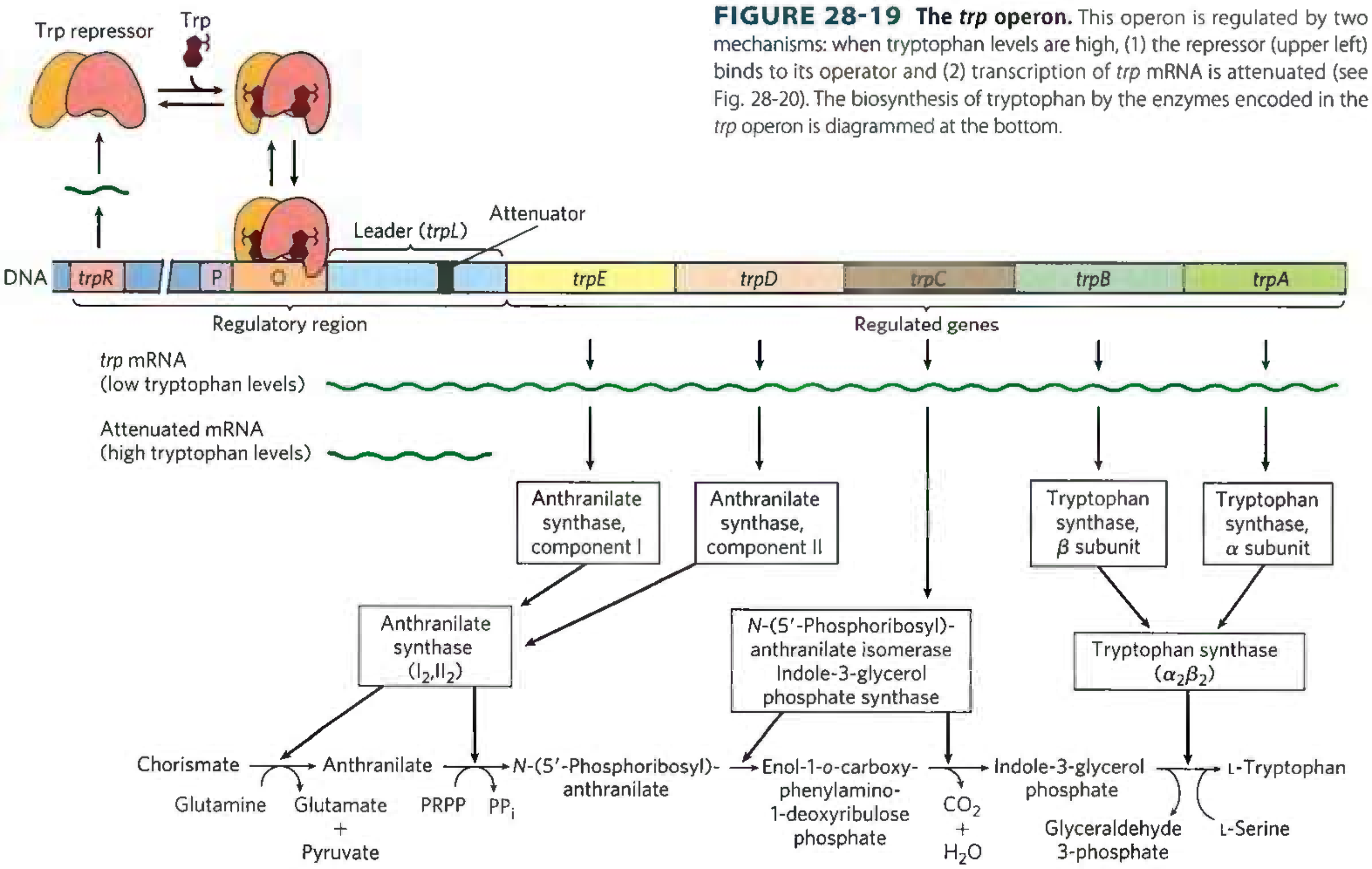

Trp repressor
Trp
DNA
trpR
P
O
Leader (*trpL*)
Attenuator
trpE
trpD
trpC
trpB
trpA
Regulatory region
Regulated genes
trp mRNA (low tryptophan levels)
Attenuated mRNA (high tryptophan levels)
Anthranilate synthase, component I
Anthranilate synthase, component II
Tryptophan synthase, β subunit
Tryptophan synthase, α subunit
Anthranilate synthase (I₂,II₂)
N-(5′-Phosphoribosyl)-anthranilate isomerase Indole-3-glycerol phosphate synthase
Tryptophan synthase (α₂β₂)
Chorismate
Glutamine
Anthranilate
Glutamate + Pyruvate
PRPP
PPᵢ
N-(5′-Phosphoribosyl)-anthranilate
Enol-1-o-carboxy-phenylamino-1-deoxyribulose phosphate
CO₂ + H₂O
Indole-3-glycerol phosphate
Glyceraldehyde 3-phosphate
L-Tryptophan
L-Serine

FIGURE 28-19 The *trp* operon. This operon is regulated by two mechanisms: when tryptophan levels are high, (1) the repressor (upper left) binds to its operator and (2) transcription of *trp* mRNA is attenuated (see Fig. 28-20). The biosynthesis of tryptophan by the enzymes encoded in the *trp* operon is diagrammed at the bottom.

Once again, this simple on/off circuit mediated by a repressor is not the entire regulatory story. Different cellular concentrations of tryptophan can vary the rate of synthesis of the biosynthetic enzymes over a 700-fold range. P1 Once repression is lifted and transcription begins, the rate of transcription is fine-tuned to cellular tryptophan requirements by a second regulatory process, called **transcription attenuation**, in which transcription is initiated normally but is abruptly halted *before* the operon genes are transcribed. The frequency with which transcription is attenuated is regulated by the availability of tryptophan and relies on the very close coupling of transcription and translation in bacteria.

The *trp* operon attenuation mechanism uses signals encoded in four sequences within a 162 nucleotide **leader** region at the 5′ end of the mRNA, preceding the initiation codon of the first gene (**Fig. 28-20a**). The leader contains a region known as the **attenuator**, made up of sequences 3 and 4. These sequences base-pair to form a G≡C-rich stem-and-loop structure closely followed by a series of U residues. The attenuator structure acts as a transcription terminator (Fig. 28-20b; see also Fig. 26-7a). Sequence 2 is an alternative complement for sequence 3 (Fig. 28-20c). If sequences 2 and 3 base-pair, the attenuator structure cannot form and transcription continues into the *trp* biosynthetic genes; the loop formed by the pairing of sequences 2 and 3 does not obstruct transcription.

Regulatory sequence 1 is crucial for a tryptophan-sensitive mechanism that determines whether sequence 3 pairs with sequence 2 (allowing transcription to continue) or with sequence 4 (attenuating transcription). Formation of the attenuator stem-and-loop structure depends on events that occur during *translation* of regulatory sequence 1, which encodes a leader peptide (so called because it is encoded by the leader region of the mRNA) of 14 amino acids, two of which are Trp residues. The leader peptide has no other known cellular function; its synthesis is simply an operon regulatory device. This peptide is translated immediately after it is transcribed, by a ribosome that follows closely behind RNA polymerase as transcription proceeds.

When tryptophan concentrations are high, concentrations of charged tryptophan tRNA (Trp-tRNA^{Trp}) are also high. This allows translation to proceed rapidly past the two Trp codons of sequence 1 and into sequence 2, before sequence 3 is synthesized by RNA polymerase. In this situation, sequence 2 is covered by the ribosome and unavailable for pairing to sequence 3 when sequence 3 is synthesized; the attenuator structure (sequences 3 and 4) forms and transcription halts (Fig. 28-20b, top). When tryptophan concentrations are low, however, the ribosome stalls at the two Trp codons in sequence 1, because charged tRNA^{Trp} is less available. Sequence 2 remains free while sequence 3 is synthesized, allowing these two sequences to base-pair and permitting transcription to proceed (Fig. 28-20b, bottom). In this way, the proportion of transcripts that are attenuated declines as tryptophan concentration declines.

Many other amino acid biosynthetic operons use a similar attenuation strategy to fine-tune biosynthetic enzymes to meet the prevailing cellular requirements. The 15 amino acid leader peptide produced by the *phe* operon contains seven Phe residues. The *leu* operon leader peptide has four contiguous Leu residues. The leader peptide for the *his* operon contains seven contiguous His residues. In fact, in the *his* operon and several others, attenuation is sufficiently sensitive to be the *only* regulatory mechanism.

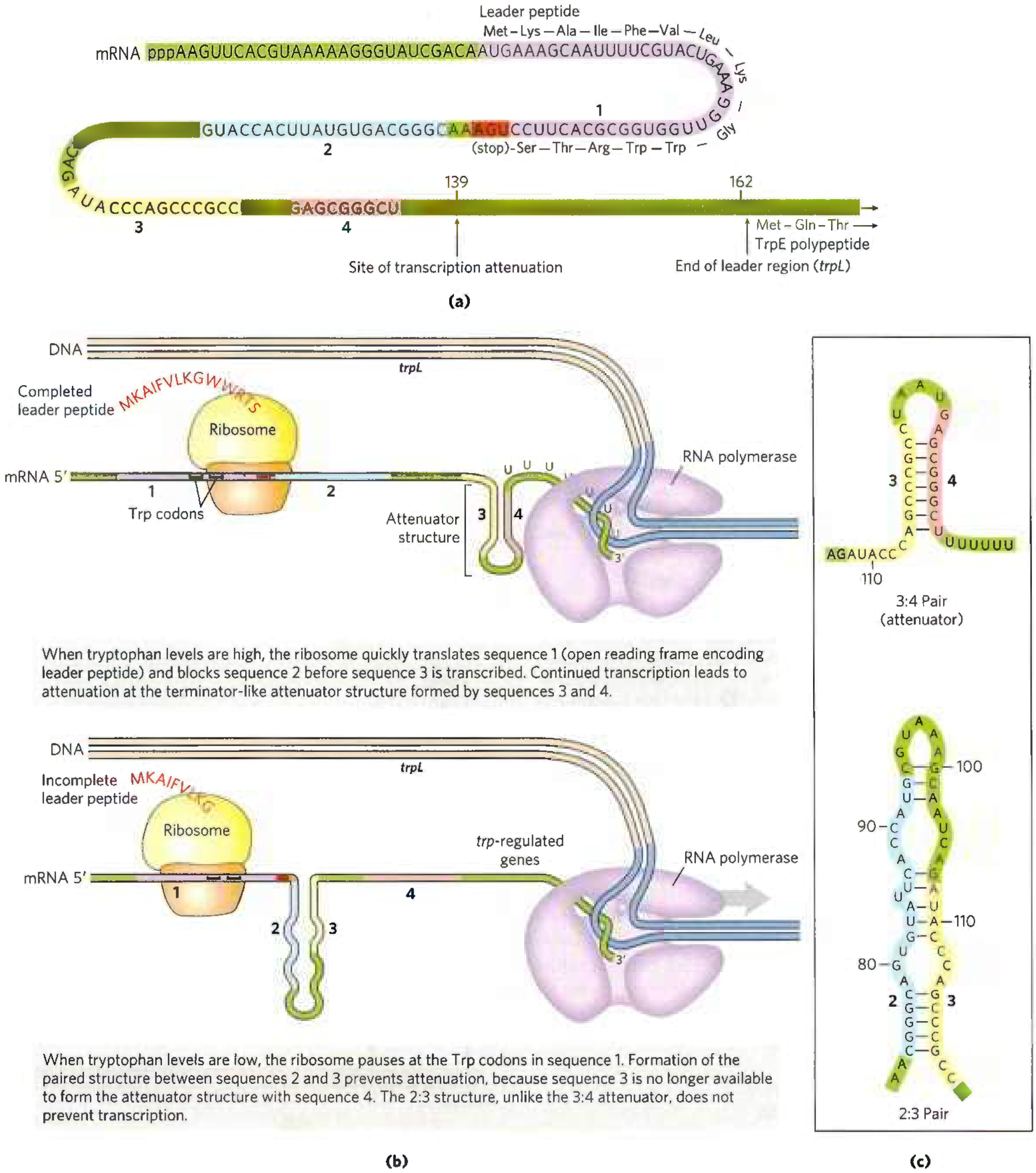
Induction of the SOS Response Requires Destruction of Repressor Proteins

Extensive DNA damage in the bacterial chromosome triggers the induction of nearly 60 genes scattered about the chromosome. P3 The genes involved in the coordinated inducible response, called the SOS response (p. 939), constitute the SOS regulon. Many of the induced genes are involved in DNA repair. The key regulatory proteins are the RecA protein and the LexA repressor.

The LexA repressor (M_r 22,700) inhibits transcription of all the SOS genes (**Fig. 28-21**), and induction of the SOS response requires removal of LexA. This is not a simple dissociation from DNA in response to binding of a small molecule, as in the regulation of the *lac* operon described above. Instead, the LexA repressor is inactivated when it catalyzes its own cleavage at a specific Ala–Gly peptide bond, producing two roughly equal protein fragments. At physiological pH, this autocleavage reaction requires the RecA protein. RecA is not a protease in the classical sense, but its interaction with LexA enables the repressor's self-cleavage reaction. This function of RecA is sometimes called a co-protease activity.

The RecA protein provides the functional link between the biological signal (DNA damage) and induction of the SOS genes. Heavy DNA damage leads to numerous single-strand gaps in the DNA, and only RecA that is bound to single-stranded DNA can facilitate cleavage of the LexA repressor (Fig. 28-21, bottom). Binding of RecA at the gaps eventually activates its co-protease activity, leading to cleavage of the LexA repressor and SOS induction.

During induction of the SOS response in a severely damaged cell, RecA also promotes the autocatalytic cleavage of, and thus inactivates, the repressors that otherwise allow propagation of certain viruses in a dormant lysogenic state within the bacterial host. This provides a remarkable illustration of evolutionary adaptation. These repressors, like LexA, undergo self-cleavage at a specific Ala–Gly peptide bond, so induction of the SOS response permits replication of the virus and lysis of the cell, releasing new viral particles. Thus, the bacteriophage can make a hasty exit from a compromised bacterial host cell.


Leader peptide
Met – Lys – Ala – Ile – Phe – Val – Leu – Lys – Gly – Trp – Trp – Arg – Thr – Ser – (stop)
mRNA pppAAGUUCACGUAAAAAGGGUAUCGACAAUGAAAGCAAUUUUCGUACUGAAAGG
1
GUACCACUUAUGUGACGGGCAAAGUCCUUCACGCGGUGGUUGG
2
GACGAUACCCAGCCCGCC GAGCGGGCU
3
4
139
162
Met – Gln – Thr
TrpE polypeptide
Site of transcription attenuation
End of leader region (trpL)
(a)
DNA
trpL
Completed leader peptide
MKAIFVLKGWWRTS
Ribosome
mRNA 5′
1
2
Trp codons
U U U
RNA polymerase
Attenuator structure
3
4
3′
DNA
trpL
Incomplete leader peptide
MKAIFVLKG
Ribosome
mRNA 5′
1
2
3
4
trp-regulated genes
RNA polymerase
3′
(b)
3
4
AGAUACC
UUUUUU
110
3:4 Pair (attenuator)
100
90
110
80
2
3
2:3 Pair
(c)

When tryptophan levels are high, the ribosome quickly translates sequence 1 (open reading frame encoding leader peptide) and blocks sequence 2 before sequence 3 is transcribed. Continued transcription leads to attenuation at the terminator-like attenuator structure formed by sequences 3 and 4.

When tryptophan levels are low, the ribosome pauses at the Trp codons in sequence 1. Formation of the paired structure between sequences 2 and 3 prevents attenuation, because sequence 3 is no longer available to form the attenuator structure with sequence 4. The 2:3 structure, unlike the 3:4 attenuator, does not prevent transcription.

FIGURE 28-20 Transcriptional attenuation in the *trp* operon. Transcription is initiated at the beginning of the 162 nucleotide mRNA leader encoded by a DNA region called *trpL* (see Fig. 28-19). A regulatory mechanism determines whether transcription is attenuated at the end of the leader or continues into the structural genes. (a) The *trp* mRNA leader (*trpL*). The attenuation mechanism in the *trp* operon involves sequences 1 to 4 (highlighted). (b) Sequence 1 encodes a small peptide, the leader peptide, containing two Trp residues (W); it is translated immediately after transcription begins. Sequences 2 and 3 are complementary, as are sequences 3 and 4. The attenuator structure forms by the pairing of sequences 3 and 4 (top). Its structure and function are similar to those of a transcription terminator. Pairing of sequences 2 and 3 (bottom) prevents the attenuator structure from forming. Note that the leader peptide has no other cellular function. Translation of its open reading frame has a purely regulatory role that determines which complementary sequences (2 and 3, or 3 and 4) are paired. (c) Base-pairing schemes for the complementary regions of the *trp* mRNA leader.

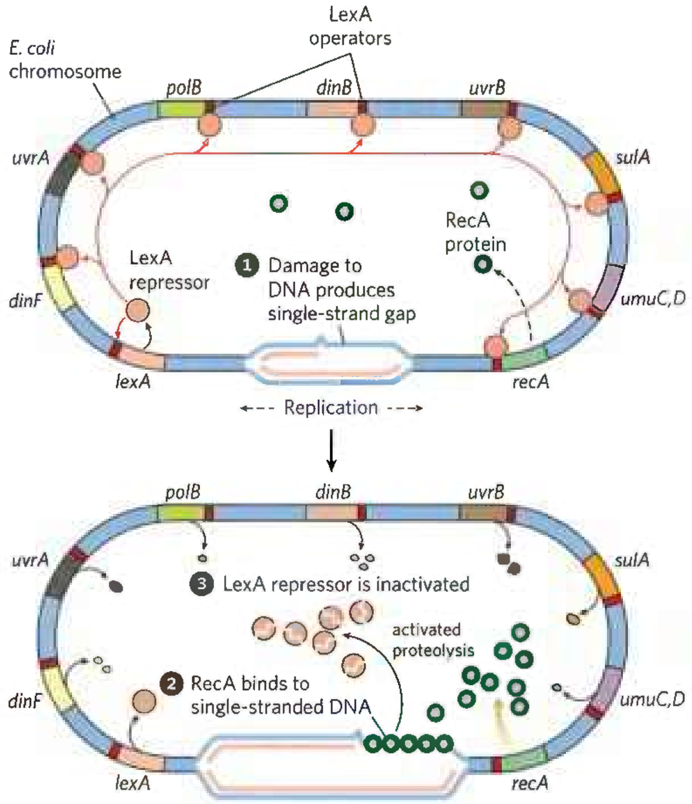

LexA operators
E. coli chromosome
polB
dinB
uvrB
uvrA
sulA
RecA protein
LexA repressor
1 Damage to DNA produces single-strand gap
dinF
umuC,D
lexA
recA
Replication
polB
dinB
uvrB
uvrA
sulA
3 LexA repressor is inactivated
activated proteolysis
2 RecA binds to single-stranded DNA
dinF
umuC,D
lexA
recA

FIGURE 28-21 SOS response in *E. coli*. The LexA protein is the repressor in this system, which has an operator site near each gene. Because the *recA* gene is not entirely repressed by the LexA repressor, the normal cell contains about 1,000 RecA monomers. ❶ When DNA is extensively damaged (such as by UV light), DNA replication is halted and the number of single-strand gaps in the DNA increases. ❷ RecA protein binds to this damaged, single-stranded DNA, activating the protein's co-protease activity. ❸ While bound to DNA, the RecA protein facilitates cleavage and inactivation of the LexA repressor. When the repressor is inactivated, the SOS genes, including *recA*, are induced; RecA levels increase 50- to 100-fold.

The destruction of the LexA repressor proteins as part of the response means that LexA must be resynthesized in order to reestablish gene control when the DNA damage is no longer present. P5 The considerable amount of ATP and GTP needed for protein synthesis to maintain SOS regulon repression provides one example of the energetic cost of regulation.

Synthesis of Ribosomal Proteins Is Coordinated with rRNA Synthesis

In bacteria, an increased cellular demand for protein synthesis is met by increasing the number of ribosomes rather than altering the activity of individual ribosomes. In general, the number of ribosomes increases as the cellular growth rate increases. At high growth rates, ribosomes make up approximately 45% of the cell's dry weight. The proportion of cellular resources devoted to making ribosomes is so large, and the function of ribosomes so important, that cells must coordinate the synthesis of the ribosomal components: the ribosomal proteins (r-proteins) and RNAs (rRNAs). P1 This regulation is distinct from the mechanisms described so far: it occurs largely at the level of *translation*.

The 52 genes that encode the r-proteins are distributed across at least 20 operons, each with 1 to 11 genes. Some of these operons also contain the genes for the subunits of DNA primase, RNA polymerase, and protein synthesis elongation factors—reflecting the close coupling of replication, transcription, and protein synthesis during bacterial cell growth.

The r-protein operons are regulated primarily through a translational feedback mechanism. One r-protein encoded by each operon also functions as a **translational repressor**, which binds to the mRNA transcribed from that operon and blocks translation of all the genes the messenger encodes (**Fig. 28-22**). In general, the r-protein that plays the role of repressor also binds directly to an rRNA. Each translational repressor r-protein binds with higher affinity to the appropriate rRNA than to its mRNA, so the mRNA is bound and translation repressed only when the level of the r-protein exceeds that of the rRNA. This ensures that translation of the mRNAs encoding r-proteins is repressed only when synthesis of these r-proteins exceeds that needed to make functional ribosomes. In this way, the rate of r-protein synthesis is kept in balance with rRNA availability.

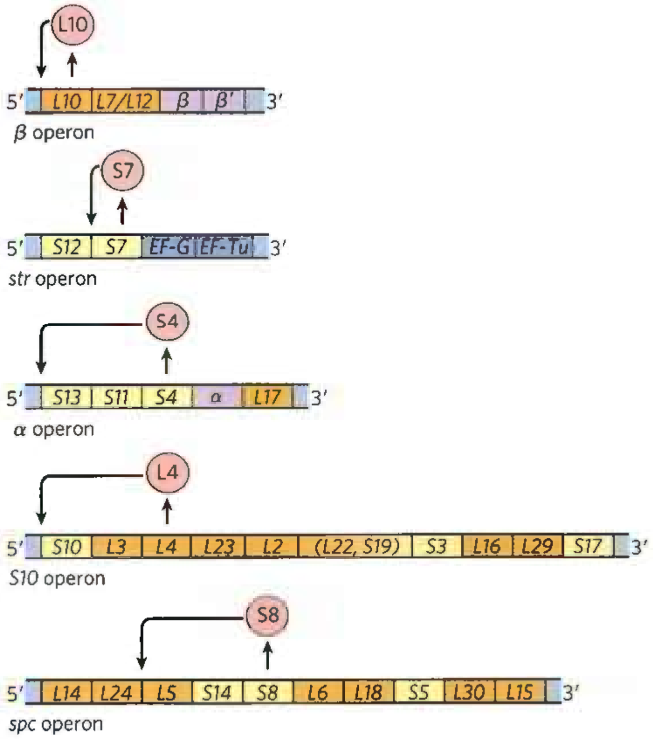

L10
5'
L10
L7/L12
β
β'
3'
β operon
S7
5'
S12
S7
EF-G
EF-Tu
3'
str operon
S4
5'
S13
S11
S4
α
L17
3'
α operon
L4
5'
S10
L3
L4
L23
L2
(L22, S19)
S3
L16
L29
S17
3'
S10 operon
S8
5'
L14
L24
L5
S14
S8
L6
L18
S5
L30
L15
3'
spc operon

FIGURE 28-22 Translational feedback in some ribosomal protein operons. The r-proteins that act as translational repressors are shown (red circles). Each translational repressor blocks the translation of all genes in that operon by binding to the indicated site on the mRNA. The operons include the genes that encode the α, β, and β' subunits of RNA polymerase and the elongation factors EF-G and EF-Tu (labeled). The r-proteins of the large (50S) ribosomal subunit are designated L1 to L34; those of the small (30S) subunit are designated S1 to S21.

The mRNA-binding site for the translational repressor is near the translational start site of one of the genes in the operon, often but not always the first gene (Fig. 28-22). In other operons this would affect only that one gene, because in bacterial polycistronic mRNAs, most genes have independent translation signals. In the r-protein operons, however, the translation of one gene depends on the translation of all the others. The translation of multiple genes seems to be blocked by folding of the mRNA into an elaborate three-dimensional structure that is stabilized both by internal base pairing and by binding of the translational repressor protein. When the translational repressor is absent, ribosome binding and translation of one or more of the genes disrupts the folded structure of the mRNA and allows all the genes to be translated.

Because the synthesis of r-proteins is coordinated with the availability of rRNA, the regulation of ribosome production reflects the regulation of rRNA synthesis. In *E. coli*, rRNA synthesis from the seven rRNA operons responds to cellular growth rate and to changes in the availability of crucial nutrients, particularly amino acids. The regulation coordinated with amino acid concentrations is known as the **stringent response** (**Fig. 28-23**). When amino acid concentrations are low, rRNA synthesis is halted. Amino acid starvation leads to the binding of uncharged tRNAs to the ribosomal A site; this triggers a sequence of events that begins with the binding of an enzyme called **stringent factor** (RelA protein) to the ribosome. When bound to the ribosome, stringent factor catalyzes formation of the unusual nucleotide guanosine tetraphosphate (ppGpp); it adds pyrophosphate to the 3′ position of GTP, in the reaction

$$\mathrm{GTP} + \mathrm{ATP} \rightarrow \mathrm{pppGpp} + \mathrm{AMP}$$

Then a phosphohydrolase cleaves off one phosphate to convert some pppGpp to ppGpp. The abrupt rise in pppGpp and ppGpp levels in response to amino acid starvation results in a great reduction in rRNA synthesis, mediated at least in part by the binding of ppGpp to RNA polymerase.

The nucleotides pppGpp and ppGpp, along with cAMP, belong to a class of modified nucleotides that act as cellular second messengers. In *E. coli*, these two nucleotides serve as starvation signals; they cause large changes in cellular metabolism by increasing or decreasing the transcription of hundreds of genes. In eukaryotic cells, similar nucleotide second messengers also have multiple regulatory functions. The coordination of cellular metabolism with cell growth is highly complex, and further regulatory mechanisms undoubtedly remain to be discovered.

The Function of Some mRNAs Is Regulated by Small RNAs in Cis or in Trans

As described throughout this chapter, proteins play an important and well-documented role in regulating gene expression. But RNA also has a crucial role—one that is becoming increasingly recognized as more examples of regulatory RNAs are discovered. Once an mRNA is synthesized, its functions can be controlled by RNA-binding proteins, as seen for the r-protein operons just described, or by an RNA. A separate RNA molecule may bind to the mRNA "in trans" and affect its activity. Alternatively, a portion of the mRNA itself may regulate its own function. When part of a molecule affects the function of another part of the same molecule, it is said to act "in cis."

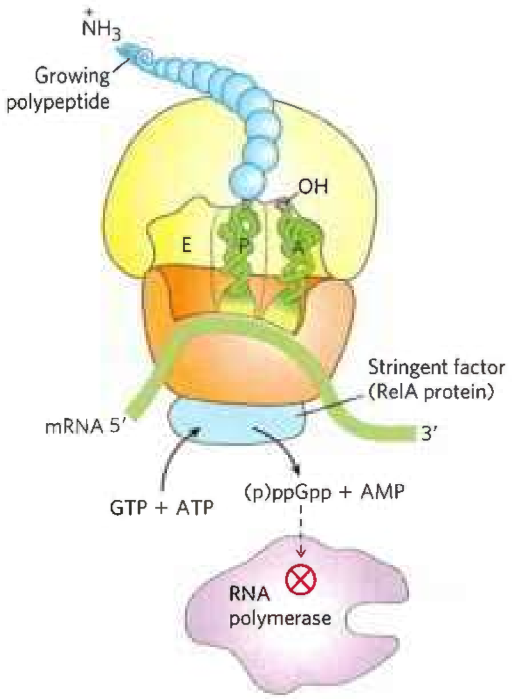


FIGURE 28-23 Stringent response in *E. coli*. This response to amino acid starvation is triggered by binding of an uncharged tRNA in the ribosomal A site. A protein called stringent factor binds to the ribosome and catalyzes the synthesis of pppGpp, which is converted by a phosphohydrolase to ppGpp. The signal ppGpp reduces transcription of some genes and increases transcription of others, in part by binding to the β subunit of RNA polymerase and altering the enzyme's promoter specificity. Synthesis of rRNA is reduced when ppGpp levels increase.

A well-characterized example of RNA regulation in trans is regulation of the mRNA of the gene *rpoS* (*R*NA *po*lymerase *s*igma factor), which encodes σ^S (formerly known as σ^{38}), one of seven *E. coli* sigma factors. The cell uses this specificity factor in certain stress situations, such as when it enters the stationary phase (a state of no growth, necessitated by lack of nutrients) and σ^S is needed to transcribe large numbers of stress response genes. The σ^S mRNA is present at low levels under most conditions but is not translated, because a large hairpin structure upstream of the coding region inhibits ribosome binding (**Fig. 28-24**). **P2** Under certain stress conditions, one or both of two small ncRNAs, DsrA (*d*own*s*tream *r*egion *A*) and RprA (*rpoS* *r*egulator RNA *A*), are induced. Both can pair with one strand of the hairpin in the σ^S mRNA, disrupting the hairpin and thus allowing translation of *rpoS*.

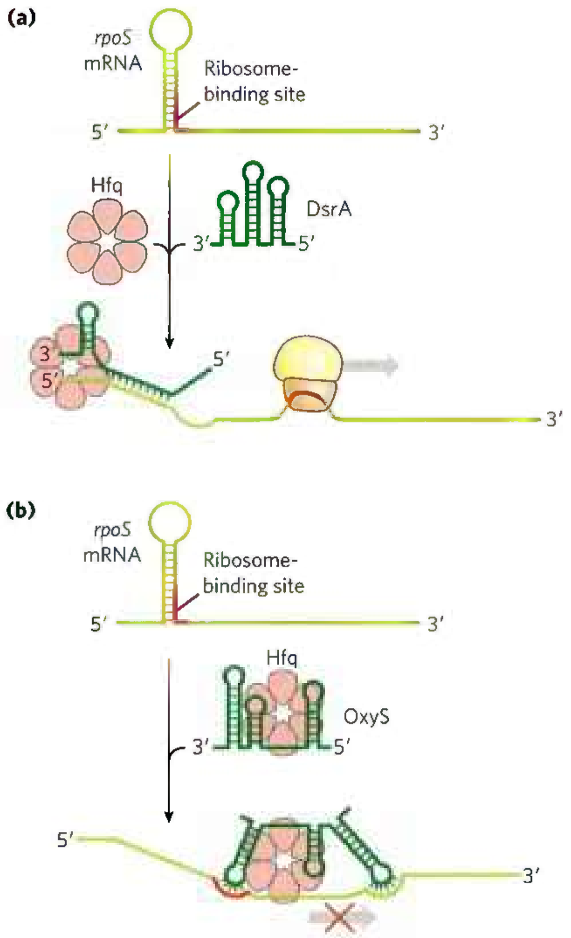

(a)
rpoS mRNA
Ribosome-binding site
5′
3′
Hfq
DsrA
3′
5′
3
5′
5′
3′
(b)
rpoS mRNA
Ribosome-binding site
5′
3′
Hfq
OxyS
3′
5′
5′
3′

FIGURE 28-24 Regulation of bacterial mRNA function in trans by sRNAs. Several sRNAs (small RNAs)—DsrA, RprA, and OxyS—participate in regulation of the *rpoS* gene. All require the protein Hfq, an RNA chaperone that facilitates RNA-RNA pairing. Hfq has a toroid structure, with a pore in the center. (a) DsrA promotes translation by pairing with one strand of a stem-loop structure that otherwise blocks the ribosome-binding site. RprA (not shown) acts in a similar way. (b) OxyS blocks translation by pairing with the ribosome-binding site. [Information from M. Szymański and J. Barciszewski, *Genome Biol.* 3:reviews0005.1, 2002.]

Another small RNA, OxyS (*ox*idative stress gene *S*), is induced under conditions of oxidative stress and inhibits the translation of *rpoS*, probably by pairing with and blocking the ribosome-binding site on the mRNA. OxyS is expressed as part of a system that responds to a different type of stress (oxidative damage) than does the *rpoS* RNA, and its task is to prevent expression of unneeded repair pathways. DsrA, RprA, and OxyS are all relatively small bacterial RNA molecules (less than 300 nucleotides), designated sRNAs (*s* for small; there are, of course, other "small" RNAs with other designations in eukaryotes). All sRNAs require for their function a protein called Hfq, an RNA chaperone that facilitates RNA-RNA pairing. The known bacterial genes regulated in this way are few in number, just a few dozen in a typical bacterial species. However, these examples provide good model systems for understanding patterns present in the more complex and numerous examples of RNA-mediated regulation in eukaryotes.

Regulation in cis involves a class of RNA structures known as **riboswitches**. As described in Box 26-4, aptamers are RNA molecules, generated in vitro, that are capable of specific binding to a particular ligand. As one might expect, such ligand-binding RNA domains are also present in nature—in riboswitches—in a significant number of bacterial mRNAs (and even in some eukaryotic mRNAs). These natural aptamers are structured domains found in untranslated regions at the 5′ ends of certain bacterial mRNAs. Some riboswitches also regulate the transcription of certain noncoding RNAs. Binding of an mRNA's riboswitch to its appropriate ligand results in a conformational change in the mRNA, and transcription is inhibited by stabilization of a premature transcription termination structure, or translation is inhibited (in cis) by occlusion of the ribosome-binding site (**Fig. 28-25**). In most cases, the riboswitch acts in a kind of feedback loop. Most genes

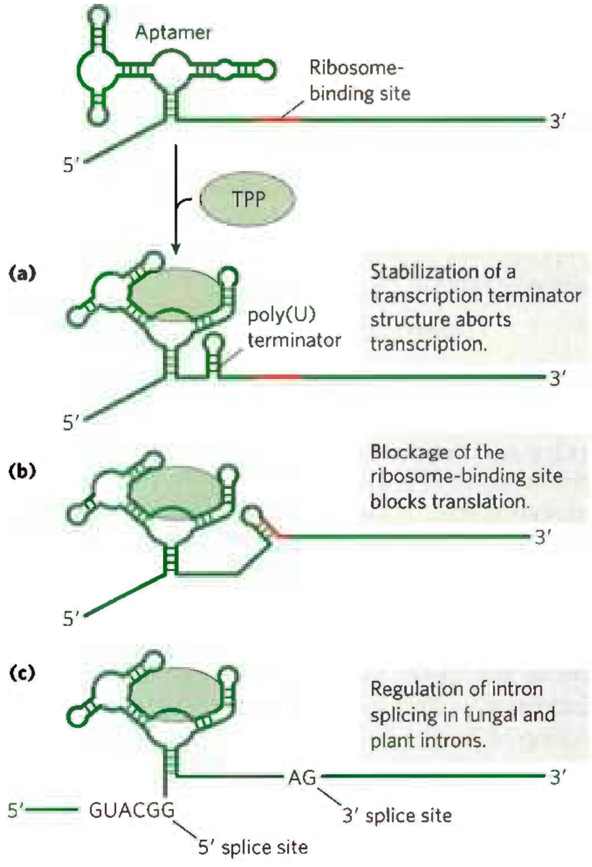

Aptamer
Ribosome-binding site
5′
3′
TPP
(a)
poly(U) terminator
Stabilization of a transcription terminator structure aborts transcription.
5′
3′
(b)
Blockage of the ribosome-binding site blocks translation.
5′
3′
(c)
Regulation of intron splicing in fungal and plant introns.
5′
GUACGG
AG
3′
3′ splice site
5′ splice site

FIGURE 28-25 Regulation of bacterial mRNA function in cis by riboswitches. The known modes of action are illustrated by several different riboswitches, based on a widespread natural aptamer that binds thiamine pyrophosphate. TPP binding to the aptamer leads to a conformational change that produces the varied results illustrated in (a), (b), and (c) in several different systems in which the aptamer is utilized. [Information from W. C. Winkler and R. R. Breaker, *Annu. Rev. Microbiol.* 59:487, 2005.]

regulated in this way are involved in the synthesis or transport of the ligand that is bound by the riboswitch; thus, when the ligand is present in high concentrations, the riboswitch inhibits expression of the genes needed to replenish this ligand.

Each riboswitch binds only one ligand. Distinct riboswitches have been detected that respond to more than a dozen different ligands, including thiamine pyrophosphate (TPP, vitamin B_1), cobalamin (vitamin B_{12}), flavin mononucleotide, lysine, *S*-adenosylmethionine (adoMet), purines, *N*-acetylglucosamine 6-phosphate, glycine, and some metal cations such as Mn^{2+}. It is likely that many more remain to be discovered. The riboswitch that responds to TPP seems to be the most widespread; it is found in many bacteria, fungi, and some plants. The bacterial TPP riboswitch inhibits translation in some species and induces premature transcription termination in others (Fig. 28-25). The eukaryotic TPP riboswitch is found in the introns of certain genes and modulates the alternative splicing of those genes. It is not yet clear how common riboswitches are. However, estimates suggest that more than 4% of the genes of *Bacillus subtilis* are regulated by riboswitches.

Most of the riboswitches described to date, including the one that responds to adoMet, have been found only in bacteria. A drug that bound to and activated the adoMet riboswitch would shut down the genes encoding the enzymes that synthesize and transport adoMet, effectively starving the bacterial cells of this essential cofactor. Drugs of this type are being sought for use as a new class of antibiotics. ■

The pace of discovery of functional RNAs shows no signs of abating and continues to bolster the hypothesis that RNA played a special role in the evolution of life (Chapter 26). The sRNAs and riboswitches, like ribozymes and ribosomes, may be vestiges of an RNA world obscured by time but persisting as a rich array of biological devices still functioning in the biosphere. The laboratory selection of aptamers and ribozymes with novel ligand-binding and enzymatic functions tells us that the RNA-based activities necessary for a viable RNA world are possible. Discovery of many of the same RNA functions in living organisms tells us that key components for RNA-based metabolism do exist. For example, the natural aptamers of riboswitches may be derived from RNAs that, billions of years ago, bound to cofactors needed to promote the enzymatic processes required for metabolism in the RNA world.

Some Genes Are Regulated by Genetic Recombination

We turn now to another mode of bacterial gene regulation, at the level of DNA rearrangement—recombination. *Salmonella typhimurium*, which inhabits the mammalian intestine, moves by rotating the flagella on its cell surface (**Fig. 28-26**). The many copies of the protein flagellin (M_r 53,000) that make up the flagella are prominent targets of mammalian immune systems. But *Salmonella* cells have a mechanism that evades the immune response: they switch between two distinct flagellin proteins (FljB and FliC) roughly once every 1,000 generations, using a process called **phase variation**.

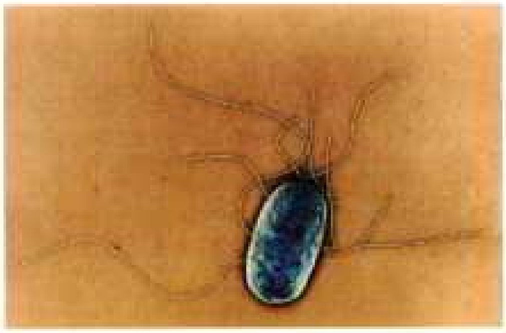


FIGURE 28-26 ***Salmonella typhimurium.*** The appendages emanating from the cell are flagella. [Eye of Science/Science Source.]

The switch is accomplished by periodic inversion of a segment of DNA containing the promoter for a flagellin gene. The inversion is a site-specific recombination reaction (see Fig. 25-37) mediated by the Hin recombinase at specific 14 bp sequences (*hix* sequences) at each end of the DNA segment. When the DNA segment is in one orientation, the gene for FljB flagellin and the gene encoding a repressor, FljA, are expressed (**Fig. 28-27a**); the repressor shuts down expression of the gene for FliC flagellin. When the DNA segment is inverted (Fig. 28-27b), the *fljA* and *fljB* genes are no longer transcribed, and the *fliC* gene is induced as the repressor becomes depleted. The Hin recombinase, encoded by the *hin* gene in the DNA segment that undergoes inversion, is expressed when the DNA segment is in either orientation, so the cell can always switch from one state to the other.

This type of regulatory mechanism has the advantage of being absolute: gene expression is impossible when the gene is physically separated from its promoter (note the position of the *fljB* promoter in Fig. 28-27b). An absolute on/off switch may be important in this system (even though it affects only one of the two flagellin genes) because a flagellum with just one copy of the wrong flagellin might be vulnerable to host antibodies against that protein. The *Salmonella* system is by no means unique. Similar regulatory systems occur in some other bacteria and in some bacteriophages, and recombination systems with similar functions have been found in eukaryotes (**Table 28-1**). Gene regulation by DNA rearrangements that move genes and/or promoters is particularly common in pathogens that benefit by changing their host range or by changing their surface proteins, thereby staying ahead of host immune systems.

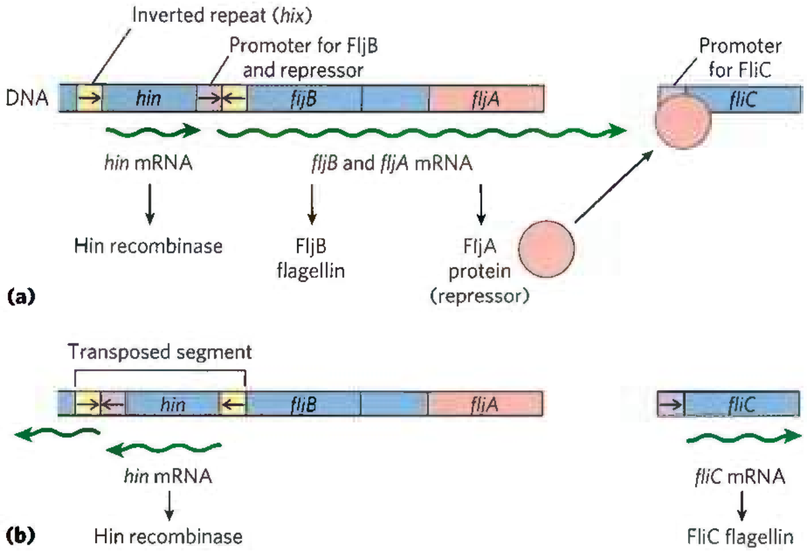


FIGURE 28-27 Regulation of flagellin genes in *Salmonella*: phase variation. The products of genes *fliC* and *fljB* are different flagellins. The *hin* gene encodes the recombinase that catalyzes inversion of the DNA segment containing the *fljB* promoter and the *hin* gene. The recombination sites (inverted repeats) are called *hix*. (a) In one orientation, *fljB* is expressed along with a repressor protein (product of the *fljA* gene) that represses transcription of the *fliC* gene. (b) In the opposite orientation, only the *fliC* gene is expressed; the *fljA* and *fljB* genes cannot be transcribed. The interconversion between these two states, known as phase variation, also requires two other nonspecific DNA-binding proteins (not shown), HU and FIS.

TABLE 28-1 Examples of Gene Regulation by Recombination

System	Recombinase/recombination site	Type of recombination	Function
Phase variation (*Salmonella*)	Hin/*hix*	Site-specific	Alternative expression of two flagellin genes allows evasion of host immune response.
Host range (bacteriophage μ)	Gin/*gix*	Site-specific	Alternative expression of two sets of tail fiber genes affects host range.
Mating-type switch (yeast)	HO endonuclease, RAD52 protein, other proteins/*MAT*	Nonreciprocal gene conversion[a]	Alternative expression of two mating types of yeast, a and α, creates cells of different mating types that can mate and undergo meiosis.
Antigenic variation (trypanosomes)[b]	Varies	Nonreciprocal gene conversion[a]	Successive expression of different genes encoding the variable surface glycoproteins (VSGs) allows evasion of host immune response.

[a] In nonreciprocal gene conversion (a class of recombination events not discussed in Chapter 25), genetic information is moved from one part of the genome (where it is silent) to another (where it is expressed). The reaction is similar to replicative transposition (see Fig. 25-41).

[b] Trypanosomes cause African sleeping sickness and other diseases (see Box 6-1). The outer surface of a trypanosome is made up of multiple copies of a single VSG, the major surface antigen. A cell can change surface antigens to more than 100 different forms, precluding an effective defense by the host immune system.

SUMMARY 28.2 *Regulation of Gene Expression in Bacteria*

- In addition to repression by the Lac repressor, the *E. coli lac* operon undergoes positive regulation by the cAMP receptor protein (CRP). When [glucose] is low, [cAMP] is high and CRP-cAMP binds to a specific site on the DNA, stimulating transcription of the *lac* operon and production of lactose-metabolizing enzymes. The presence of glucose depresses [cAMP], decreasing expression of *lac* and other genes involved in metabolism of secondary sugars. A group of coordinately regulated operons is referred to as a regulon.
- Operons that produce the enzymes of amino acid synthesis have a regulatory circuit called attenuation, which uses a transcription termination site, called the attenuator, in the mRNA. Formation of the attenuator is modulated by a mechanism that couples transcription and translation while responding to small changes in amino acid concentration.
- In the SOS system, multiple unlinked genes repressed by a single repressor are induced simultaneously when DNA damage triggers RecA protein–facilitated autocatalytic proteolysis of the repressor.
- In the synthesis of ribosomal proteins, one protein in each r-protein operon acts as a translational repressor. The mRNA is bound by the repressor, and translation is blocked only when the r-protein is present in excess of available rRNA.
- Posttranscriptional regulation of some mRNAs is mediated by sRNAs that act in trans or by riboswitches, part of the mRNA structure itself, that act in cis.

- Some genes are regulated by genetic recombination processes that move promoters relative to the genes being regulated. Regulation can also take place at the level of translation.

28.3 Regulation of Gene Expression in Eukaryotes

Initiation of transcription is a crucial regulation point for gene expression in all organisms. Although eukaryotes and bacteria use some of the same regulatory mechanisms, the regulation of transcription in the two systems is fundamentally different.

P4 We can define a transcriptional ground state as the inherent activity of promoters and transcriptional machinery in vivo in the absence of regulatory sequences. In bacteria, RNA polymerase generally has access to every promoter and can bind and initiate transcription at some level of efficiency in the absence of activators or repressors. In eukaryotes, however, strong promoters are generally inactive in vivo in the absence of regulatory proteins. This fundamental difference gives rise to at least five important features that distinguish the regulation of gene expression at eukaryotic promoters from that observed in bacteria.

First, access to eukaryotic promoters is restricted by the structure of chromatin, and activation of transcription is associated with many changes in chromatin structure in the transcribed region. Second, although eukaryotic cells have both positive and negative regulatory mechanisms, positive mechanisms are more prominent. Almost every eukaryotic gene requires activation to be transcribed. Third, regulatory mechanisms involving lncRNAs are more common in eukaryotic transcriptional regulation. Fourth, eukaryotic cells have larger, more complex multimeric regulatory proteins than do bacteria. Finally, transcription in the eukaryotic nucleus is separated from translation in the cytoplasm in both space and time.

The complexity of regulatory circuits in eukaryotic cells is extraordinary, as is evident from the following discussion. The section ends with an illustrated description of one of the most elaborate circuits: the regulatory cascade that controls development in fruit flies.

Transcriptionally Active Chromatin Is Structurally Distinct from Inactive Chromatin

The effects of chromosome structure on gene regulation in eukaryotes have no clear parallel in bacteria. In the eukaryotic cell cycle, interphase chromosomes appear, at first viewing, to be dispersed and amorphous (see Fig. 24-22). Nevertheless, several forms of chromatin can be found along these chromosomes. About 10% of the chromatin in a typical eukaryotic cell is in a more condensed form than the rest of the chromatin. This form, **heterochromatin**, is transcriptionally inactive. Heterochromatin is generally associated with particular chromosome structures—the centromeres, for example. The remaining, less condensed chromatin is called **euchromatin**.

Transcription of a eukaryotic gene is strongly repressed when its DNA is condensed within heterochromatin. Some, but not all, of the euchromatin is transcriptionally active. Transcriptionally active chromosomal regions are distinguished from heterochromatin in at least three ways: the positioning of nucleosomes, the presence of histone variants, and the covalent modification of nucleosomes. These transcription-associated structural changes in chromatin are collectively called **chromatin remodeling**. The remodeling employs a set of enzymes that promote these changes (**Table 28-2**).

Four known families of chromatin remodeling complexes, distinguished by their structural features, act directly to alter nucleosome composition in transcribed regions. They may unwrap, translocate, remove, or exchange nucleosomes on the DNA, hydrolyzing ATP in the process (Table 28-2; see the table footnote for an explanation of the abbreviated names of enzyme complexes described here). In some cases, the enzymes catalyze the exchange of pairs of histones within nucleosomes to alter nucleosome composition. The multitude of different complexes are specialized to function at particular genes or chromosomal regions. There are two related complexes in the **SWI/SNF** family in all eukaryotic cells, both of which remodel chromatin so that nucleosomes are ejected from the DNA near transcription start sites. They appear to be involved in a dynamic cycle to allow replacement of nucleosomes with transcription factors (**Fig. 28-28**). The two distinct complexes generally function at different sets of genes. Most of the **ISWI** family complexes optimize nucleosome spacing to allow chromatin assembly and transcriptional silencing. There are generally 9 or 10 different **CHD** family complexes in eukaryotic cells, separated into three subfamilies. The different family members have specialized roles, either ejecting nucleosomes to activate transcription or assembling chromatin to repress transcription. The **INO80** family complexes have a variety of roles in remodeling chromatin for transcriptional activation and DNA repair. One family member, SWR1, promotes subunit exchange in nucleosomes to introduce histone variants such as H2AZ (see Box 24-1), found in transcriptionally active regions.

The covalent modification of histones is altered dramatically within transcriptionally active chromatin. The core histones of nucleosome particles (H2A, H2B, H3, H4; see Fig. 24-24) are modified by methylation of Lys or Arg residues, phosphorylation of Ser or Thr residues, acetylation (see below), ubiquitination (see Fig. 27-47), or SUMOylation (SUMOs are *s*mall *u*biquitin-like *mo*difiers). Each of the core histones has two distinct structural domains. A central domain is involved in histone-histone interaction and the wrapping of DNA

TABLE 28-2 Some Enzyme Complexes That Catalyze Chromatin Structural Changes Associated with Transcription

Enzyme complex[a]	Oligomeric structure (number of polypeptides)	Source	Activities
Histone movement, replacement, or editing, requiring ATP			
SWI/SNF family	8–17, $M_r > 10^6$	Eukaryotes	Nucleosome remodeling; transcriptional activation
ISWI family	2–4	Eukaryotes	Nucleosome remodeling; transcriptional repression; transcriptional activation in some cases
CHD family	1–10	Eukaryotes	Nucleosome remodeling; nucleosome ejection for transcriptional activation; some have repressive roles
INO80 family	>10	Eukaryotes	Nucleosome remodeling; transcriptional activation; family member SWR1 engages in replacement of H2A-H2B with H2AZ-H2B
Histone modification			
GCN5-ADA2-ADA3	3	Yeast	GCN5 has type A HAT activity
SAGA/PCAF	>20	Eukaryotes	Includes GCN5-ADA2-ADA3; acetylates residues in H3, H2B, H2AZ
NuA4	≥12	Eukaryotes	EsaI component has HAT activity; acetylates H4, H2A, and H2AZ
Histone chaperones not requiring ATP			
HIRA	1	Eukaryotes	Deposition of H3.3 during transcription

[a] The abbreviations for eukaryotic genes and proteins are often more confusing or obscure than those used for bacteria. SWI (*sw*itching) was discovered as a protein required for expression of certain genes involved in mating-type switching in yeast, and SNF (*s*ucrose *n*on*f*ermenting) as a factor for expression of the yeast gene for sucrase. Subsequent studies revealed multiple SWI and SNF proteins that act in a complex. The SWI/SNF complex has a role in expression of a wide range of genes and has been found in many eukaryotes, including humans. ISWI is *i*mitation *SWI*. CHD is *c*hromodomain, *h*elicase, *D*NA binding; INO80 is *ino*sitol-requiring *80*; and SWR1 is *SW*i2/Snf2-*r*elated ATPase 1. The complex of GCN5 (*g*eneral *c*ontrol *n*onderepressible) and ADA (*a*lteration/*d*eficiency in *a*ctivation) proteins was discovered during investigation of the regulation of nitrogen metabolism genes in yeast. These proteins can be part of the larger SAGA (SPF, ADA2,3, GCN5, *a*cetyltransferase) complex in yeasts. The equivalent of SAGA in humans is PCAF (*p*300/*C*BP-*a*ssociated *f*actor). NuA4 is *nu*cleosome *a*cetyltransferase of H*4*; ESA1 is *e*ssential *S*AS2-related *a*cetyltransferase; HIRA is *hi*stone *r*egulator *A*

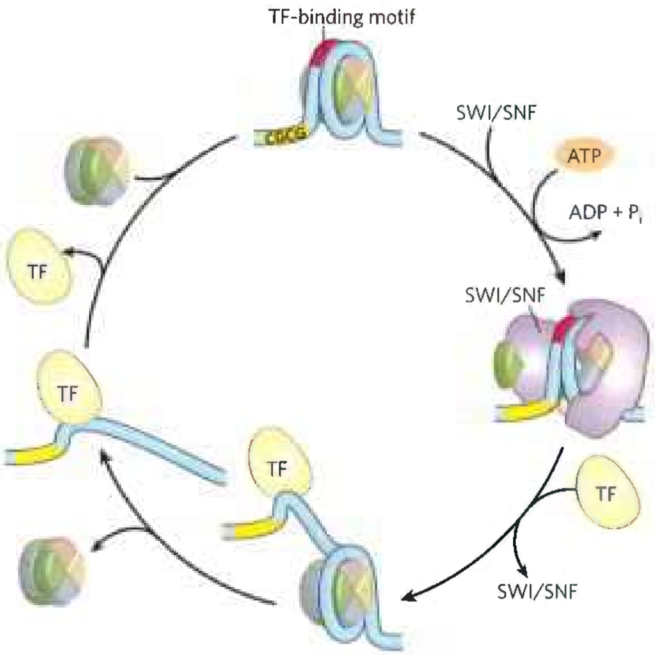


FIGURE 28-28 Nucleosome ejection by a SWI/SNF remodeler. The SWI/SNF enzyme engulfs the nucleosome, interacting with short CGCG sequences nearby. With the aid of ATP hydrolysis, the DNA is partially separated from the nucleosome, exposing a site for transcription factor (TF) binding. After the transcription factor is bound, the nucleosome is ejected. When transcription is no longer needed, the nucleosome can again replace the transcription factor or factors, completing the cycle. [Information from S. Brahma and S. Henikoff, *Trends Biochem. Sci.* 45:13, 2020.]

around the nucleosome. A lysine-rich amino-terminal domain is generally positioned near the exterior of the assembled nucleosome particle; the covalent modifications occur at specific residues concentrated in this amino-terminal domain. The patterns of modification have led some researchers to propose the existence of a histone code, in which modification patterns are recognized by enzymes that alter the structure of chromatin. Indeed, some of the modifications are essential for interactions with proteins that play key roles in transcription.

The acetylation and methylation of histones figure prominently in the processes that activate chromatin for transcription. During transcription, histone H3 in nucleosomes is methylated (by specific histone methylases) at Lys^4 near the 5′ end of the coding region and at Lys^{36} within the coding region. These methylations enable the binding of **histone acetyltransferases (HATs)**, enzymes that acetylate particular Lys residues. Cytosolic (type B) HATs acetylate newly synthesized histones before the histones are imported into the nucleus. The subsequent assembly of the histones into chromatin after replication is facilitated by histone chaperones: CAF1 for H3 and H4 (see Box 24-1), and NAP1 for H2A and H2B.

Where chromatin is being activated for transcription, the nucleosomal histones are further acetylated by nuclear (type A) HATs. The acetylation of multiple Lys residues in the amino-terminal domains of histones

H3 and H4 can reduce the affinity of the entire nucleosome for DNA. Acetylation of particular Lys residues is critical for the interaction of nucleosomes with other proteins.

When transcription of a gene is no longer required, the extent of methylation and acetylation of nucleosomes in that vicinity is reduced as part of a general gene-silencing process that restores the chromatin to a transcriptionally inactive state. There are two known classes of demethylases. One class, called LSD (*l*ysine-*s*pecific histone *d*emethylases), first converts the CH_3—N linkage to an imine (CH_2=N) linkage, followed by hydrolysis to generate formaldehyde and the demethylated lysine. The other class of demethylases contains JmjC (Jumonji-C) domains, first hydroxylating the methyl group, which is again subsequently removed as formaldehyde. More than 20 JmjC domain–containing histone demethylases are encoded by mammalian genomes. They are part of the same α-ketoglutarate-dependent hydroxylase enzyme family that includes the enzyme that hydroxylates proline residues in collagen (see Box 4-2). These enzymes are strongly inhibited by 2-hydroxyglutarate, an unusual metabolite produced in abundance by a mutated form of isocitrate dehydrogenase that is common in human cancers (see Fig. 16-20). Within the tumors, the high levels of 2-hydroxyglutarate produce global changes in gene expression. ■

Histone acetylation is reduced by the action of **histone deacetylases (HDACs)**. The deacetylases include SIRT1, SIRT2, SIRT6, and SIRT7, which are NAD^+-dependent enzymes in the sirtuin family (SIRT1–7 in mammals). These enzymes deacetylate specific Lys residues in histones and other, cytoplasmic targets. In addition to the removal of certain acetyl groups, new covalent modification of histones marks chromatin as transcriptionally inactive. For example, Lys^9 of histone H3 is often methylated in heterochromatin.

The net effect of chromatin remodeling in the context of transcription is to make a segment of the chromosome more accessible and to "label" (chemically modify) it so as to facilitate the binding and activity of transcription factors that regulate expression of the gene or genes in that region.

Most Eukaryotic Promoters Are Positively Regulated

As already noted, eukaryotic RNA polymerases have little or no intrinsic affinity for their promoters. P4 The default state of eukaryotic genes is "off," and initiation of transcription is almost always dependent on the action of multiple activator proteins. One important reason for the apparent predominance of positive regulation seems obvious: the storage of DNA within chromatin effectively renders most promoters inaccessible, so genes are silent in the absence of other regulation. The structure of chromatin affects access to some promoters more than others, but repressors that bind to DNA so as to preclude access of RNA polymerase (negative regulation) would often be simply redundant. Other factors must be at play in the use of positive regulation, and speculation generally centers around two: the large size of eukaryotic genomes and the greater efficiency of positive regulation.

First, nonspecific DNA binding of regulatory proteins becomes a more important problem in the much larger genomes of higher eukaryotes. And the chance that a single specific binding sequence will occur randomly at an inappropriate site also increases with genome size. Combinatorial control thus becomes important in a large genome (**Fig. 28-29**). Specificity for transcriptional activation can be improved if each of several positive regulatory proteins must bind specific DNA sequences to activate a gene. The average number of regulatory sites for a gene in a multicellular organism is six, and genes that are regulated by a dozen such sites are common. The requirement for binding of several positive regulatory proteins to specific DNA sequences vastly reduces the probability of the random occurrence of a functional juxtaposition of all the necessary binding sites. This requirement also reduces the number of regulatory proteins that must be encoded by a genome to regulate all of its genes (Fig. 28-28). Thus, a new regulator is not needed for every gene, although regulation is complex enough in higher eukaryotes that regulatory proteins may represent 5% to 10% of all protein-coding genes.

In principle, a similar combinatorial strategy could be used by multiple negative regulatory elements, but this brings us to the second reason for the use of positive regulation: it is simply more efficient. If the ~20,000 genes in the human genome were negatively regulated, each cell would have to synthesize, at all times, all of the different repressors in concentrations sufficient to permit specific binding to each "unwanted" gene. In positive regulation, most of the genes are usually inactive (that is, RNA polymerases do not bind to the promoters) and the cell synthesizes only the activator proteins needed to promote transcription of the subset of genes required in the cell at that time.

These arguments notwithstanding, there are examples of negative regulation in eukaryotes, from yeasts to humans, as we shall see. Some of that negative regulation involves lncRNAs, which are more economical to synthesize than repressor proteins.

DNA-Binding Activators and Coactivators Facilitate Assembly of the Basal Transcription Factors

To continue our exploration of the regulation of gene expression in eukaryotes, we return to the interactions between promoters and RNA polymerase II (Pol II), the enzyme responsible for the synthesis of eukaryotic

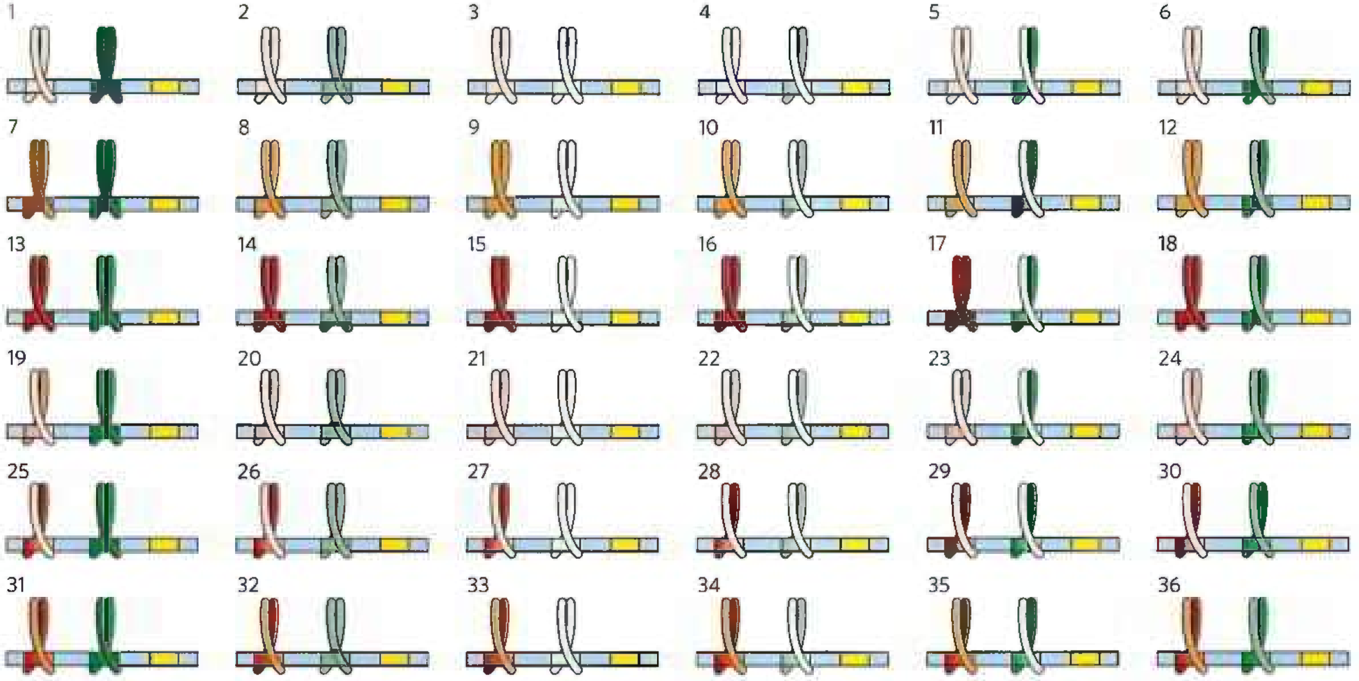


FIGURE 28-29 The advantages of combinatorial control. Combinatorial control allows specific regulation of many genes using a limited repertoire of regulatory proteins. Consider the possibilities inherent in regulation by two different families of leucine zipper proteins (red and green). If each regulatory gene family had three members (as shown here, in dark, medium, and light shades, each binding to a different DNA sequence) that could freely form either homo- or heterodimers, there would be six possible dimeric species in each family and each dimer would recognize a different bipartite regulatory DNA sequence. If a gene had a regulatory site for each protein family, 36 different regulatory combinations would be possible, using just the six proteins from these two families. With six or more sites used in the regulation of a typical eukaryotic gene, the number of possible variants is much greater than this example suggests.

mRNAs. Although many (but not all) Pol II promoters include the TATA box and Inr (initiator) sequences, with their standard spacing (see Fig. 26-8), they vary greatly in both the number and the location of additional sequences required for the regulation of transcription.

The additional regulatory sequences, generally bound by transcription activators, are usually called **enhancers** in higher eukaryotes and **upstream activator sequences (UASs)** in yeast. A typical enhancer may be found hundreds or even thousands of base pairs upstream from the transcription start site, or may even be downstream, within the gene itself. When bound by the appropriate regulatory proteins, an enhancer increases transcription at nearby promoters regardless of its orientation in the DNA. The UASs of yeast function in a similar way, although generally they must be positioned upstream and within a few hundred base pairs of the transcription start site.

Successful binding of the active Pol II holoenzyme at one of its promoters usually requires the combined action of proteins of five types: (1) **transcription activators**, which bind to enhancers or UASs and facilitate transcription; (2) **architectural regulators**, which facilitate DNA looping; (3) **chromatin modification and remodeling proteins**, described above; (4) **coactivators**; and (5) **basal transcription factors**, also called general transcription factors (see Fig. 26-9, Table 26-2), required at most Pol II promoters (**Fig. 28-30**). The coactivators are required for essential communication between activators and the complex composed of Pol II and the basal transcription factors. Coactivators also play a direct role in assembly of the **preinitiation complex (PIC)**. Furthermore, a variety of repressor proteins can interfere with communication between Pol II and the activators, resulting in repression of transcription (Fig. 28-30b). Here we focus on the protein complexes shown in Figure 28-30 and how they interact to activate transcription.

Transcription Activators The requirements for activators vary greatly from one promoter to another. A few are known to activate transcription at hundreds of promoters, whereas others are specific for a few promoters. Many activators are sensitive to the binding of signal molecules, providing the capacity to activate or deactivate transcription in response to a changing cellular environment. Some enhancers bound by activators are quite distant from the promoter's TATA box. Multiple enhancers (often six or more) are bound by a similar number of activators for a typical gene, providing combinatorial control and response to multiple signals.

Some transcription activators can bind to both DNA and RNA, and their function is affected by one or more

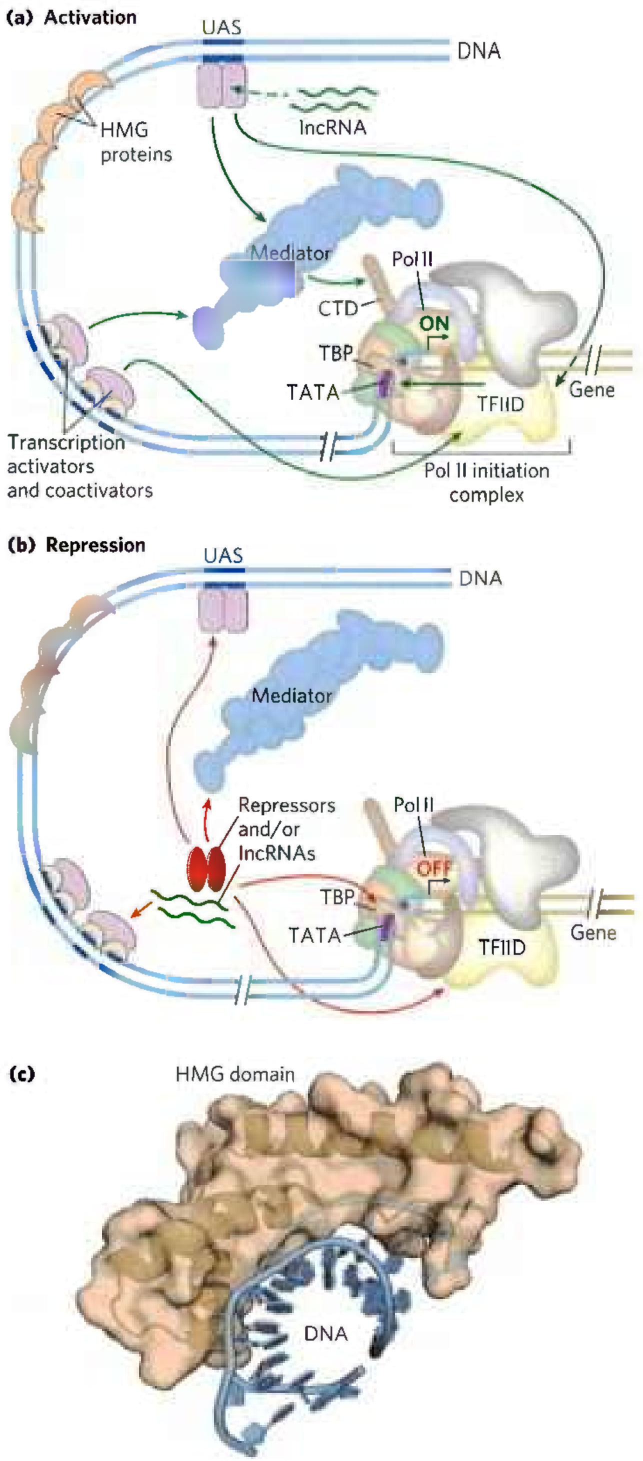


FIGURE 28-30 Eukaryotic promoters and regulatory proteins. RNA polymerase II and its associated basal (general) transcription factors form a preinitiation complex at the TATA box and Inr site of the cognate promoters, a process facilitated by transcription activators, acting through coactivators (Mediator, TFIID, or both). (a) A composite promoter with typical sequence elements and protein complexes found in both yeast and higher eukaryotes. The carboxyl-terminal domain (CTD) of Pol II (see Fig. 26-9) is an important point of interaction with Mediator and other protein complexes. Histone modification enzymes (not shown) catalyze methylation and acetylation; remodeling enzymes alter the content and placement of nucleosomes. The transcription activators have distinct DNA-binding domains and activation domains. In some cases, their function is affected by interaction with lncRNAs. Arrows indicate common modes of interaction often required for the activation of transcription. The HMG proteins are a common type of architectural regulator (see Fig. 28-5), allowing the looping of the DNA required to bring together system components bound at distant binding sites. (b) Eukaryotic transcriptional repressors function through a range of mechanisms. Some bind directly to DNA, displacing a protein complex required for activation (not shown); many others interact with various parts of the transcription or activation protein complexes to prevent activation. Possible points of interaction are indicated with arrows. (c) The structure of an HMG protein complex with DNA shows how HMG proteins facilitate DNA looping. The binding is relatively nonspecific, although DNA sequence preferences have been identified for many HMG proteins. Shown here is the HMG domain of the protein HMG-D of *Drosophila*, bound to DNA. [(c) Data from PDB ID 1QRV, F. V. Murphy IV et al., *EMBO J.* 18:6610, 1999.]

lncRNAs. The protein NF-κB, for example (Fig. 28-14), activates transcription of many genes involved in the immune response and cytokine production. It can bind to a DNA enhancer site or, alternatively, to an lncRNA called *lethe*, named after the river of forgetfulness in Greek mythology. The lncRNA reduces transcription of genes controlled by NF-κB.

Architectural Regulators How do activators function at a distance? The answer in most cases seems to be that, as indicated earlier, the intervening DNA is looped so that the various protein complexes can interact directly. The looping is promoted by architectural regulators that are abundant in chromatin and bind to DNA with limited specificity. Most prominently, the **high mobility group (HMG)** proteins (Fig. 28-29c; "high mobility" refers to their electrophoretic mobility in polyacrylamide gels) play an important structural role in chromatin remodeling and transcriptional activation.

Coactivator Protein Complexes Most transcription requires the presence of additional protein complexes. Some major regulatory protein complexes that interact with Pol II have been defined both genetically and biochemically. These coactivator complexes act as intermediaries between the transcription activators and the Pol II complex.

Mediator, a complex consisting of 25 (yeast) to 30 (human) polypeptides, is a major eukaryotic coactivator (Fig. 28-30). Many of the 25 core polypeptides are highly conserved from fungi to humans. A subcomplex of four subunits has a kinase role, interacting transiently with the remainder of the Mediator complex, and may dissociate prior to transcription initiation. Mediator binds tightly to the carboxyl-terminal domain (CTD) of the largest subunit of Pol II. The Mediator complex is required for both basal and regulated transcription at many promoters used by Pol II, and it also stimulates phosphorylation of the CTD by TFIIH (a basal transcription factor). Transcription activators interact with one or more components of the Mediator complex, with the precise interaction sites differing from one activator to another. Coactivator complexes function at or near the promoter's TATA box.

Additional coactivators, functioning with one or a few genes, have also been described. Some of these operate in conjunction with Mediator, and some may act in systems that do not employ Mediator.

TATA-Binding Protein and Basal Transcription Factors The first component to bind in the assembly of a preinitiation complex (PIC) at the TATA box of a typical Pol II promoter is the **TATA-binding protein (TBP)**. At promoters lacking a TATA box, TBP is usually delivered as part of a larger complex (13 to 14 subunits) called TFIID. The complete complex also includes the basal transcription factors TFIIB, TFIIE, TFIIF, TFIIH; Pol II; and perhaps TFIIA. This minimal PIC, however, is often insufficient for initiation of transcription and generally does not form at all if the promoter is obscured within chromatin. Positive regulation, leading to transcription, is imposed by the activators and coactivators. Mediator interacts directly with TFIIH and TFIIE, allowing their recruitment to the PIC.

Choreography of Transcriptional Activation We can now begin to piece together the sequence of transcriptional activation events at a typical Pol II promoter (**Fig. 28-31**). The exact order of binding of some components may vary, but the model in Figure 28-31 illustrates the principles of activation as well as one common path. Many transcription activators have significant affinity for their binding sites even when the sites are within condensed chromatin. The binding of activators is often the event that triggers subsequent activation of the promoter. Binding of one activator may enable the binding of others, gradually displacing some nucleosomes.

Crucial remodeling of the chromatin then takes place in stages, facilitated by interactions between activators and HATs or enzyme complexes such as SWI/SNF, or both. In this way, a bound activator can draw in other components necessary for further chromatin remodeling to permit transcription of specific genes. The bound activators interact with the large Mediator complex. Mediator, in turn, provides an assembly surface for the binding of, first, TBP (or TFIID), then TFIIB, and then other components of the PIC, including Pol II. Mediator stabilizes the binding of Pol II and its associated transcription factors and greatly facilitates formation of the PIC. Complexity in these regulatory circuits is the rule rather than the exception, with multiple DNA-bound activators promoting transcription.

The script can change from one promoter to another. For example, many promoters have a different set of recognition sequences and may not have a TATA box, and in multicellular eukaryotes the subunit composition of factors such as TFIID can vary from one tissue to another. However, most promoters seem to require a precisely ordered assembly of components to initiate transcription. The assembly process is not always fast. For some genes it may take minutes; for certain genes of higher eukaryotes, the process can take days.

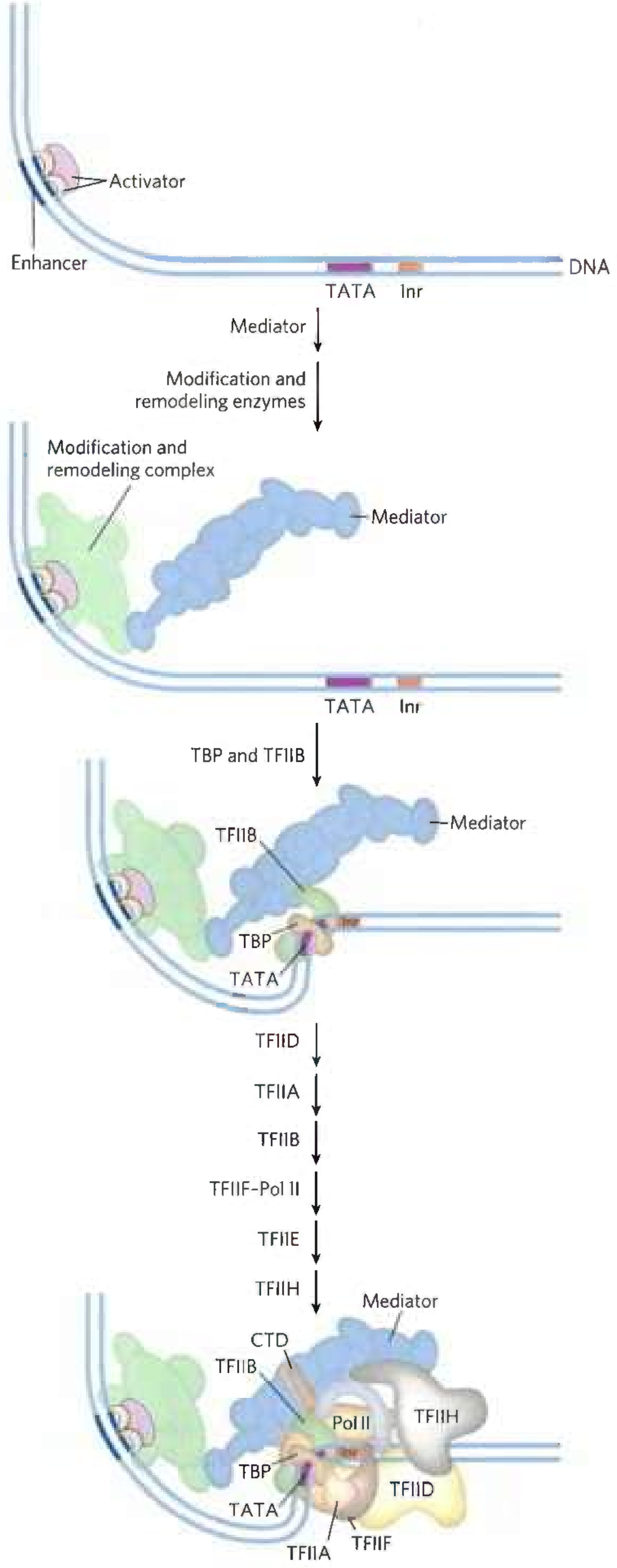


FIGURE 28-31 The components of transcriptional activation. Activators bind the DNA first. The activators recruit the histone modification/nucleosome remodeling complexes and a coactivator such as Mediator. Mediator facilitates the binding of TBP (or TFIID) and TFIIB, and the other basal transcription factors and Pol II then bind. Phosphorylation of the CTD of Pol II leads to transcription initiation (not shown). [Information from J. A. D'Alessio et al., *Mol. Cell* 36:924, 2009.]

The Genes of Galactose Metabolism in Yeast Are Subject to Both Positive and Negative Regulation

Some of the general principles described above can be illustrated by one well-studied eukaryotic regulatory circuit (**Fig. 28-32**). The enzymes required for the importation and metabolism of galactose in yeast are encoded by genes scattered over several chromosomes (**Table 28-3**). Each of the *GAL* genes is transcribed separately, and yeast cells have no operons like those in bacteria. However, all the *GAL* genes have similar promoters and are regulated coordinately by a common set of proteins. The promoters for the *GAL* genes consist of the TATA box and Inr sequences, as well as an upstream activator sequence (UAS_G) recognized by the transcription activator Gal4 protein (Gal4p). Regulation of gene expression by galactose entails an interplay between Gal4p and two other proteins, Gal80p and Gal3p. Gal80p forms a complex with Gal4p, preventing Gal4p from functioning as an activator of the *GAL* promoters. When galactose is present, it binds Gal3p, which then interacts with the Gal80p-Gal4p complex and allows Gal4p to function as an activator at the *GAL* promoters. As the various galactose genes are induced and their products build up, Gal3p may be replaced with Gal1p (a galactokinase needed for galactose metabolism that also acts as a regulator) for sustained activation of the regulatory circuit.

Other protein complexes also have a role in activating transcription of the *GAL* genes. These include the SAGA complex for histone acetylation and chromatin remodeling, the SWI/SNF complex for chromatin remodeling, and Mediator. The Gal4 protein is responsible for recruitment of these additional factors needed for transcriptional activation. SAGA may be the first and primary recruitment target for Gal4p.

Glucose is the preferred carbon source for yeast, as it is for bacteria. When glucose is present, most of the *GAL* genes are repressed—whether galactose is present or not. The *GAL* regulatory system described above is effectively overridden by a complex catabolite repression system that includes several proteins (not depicted in Fig. 28-32).

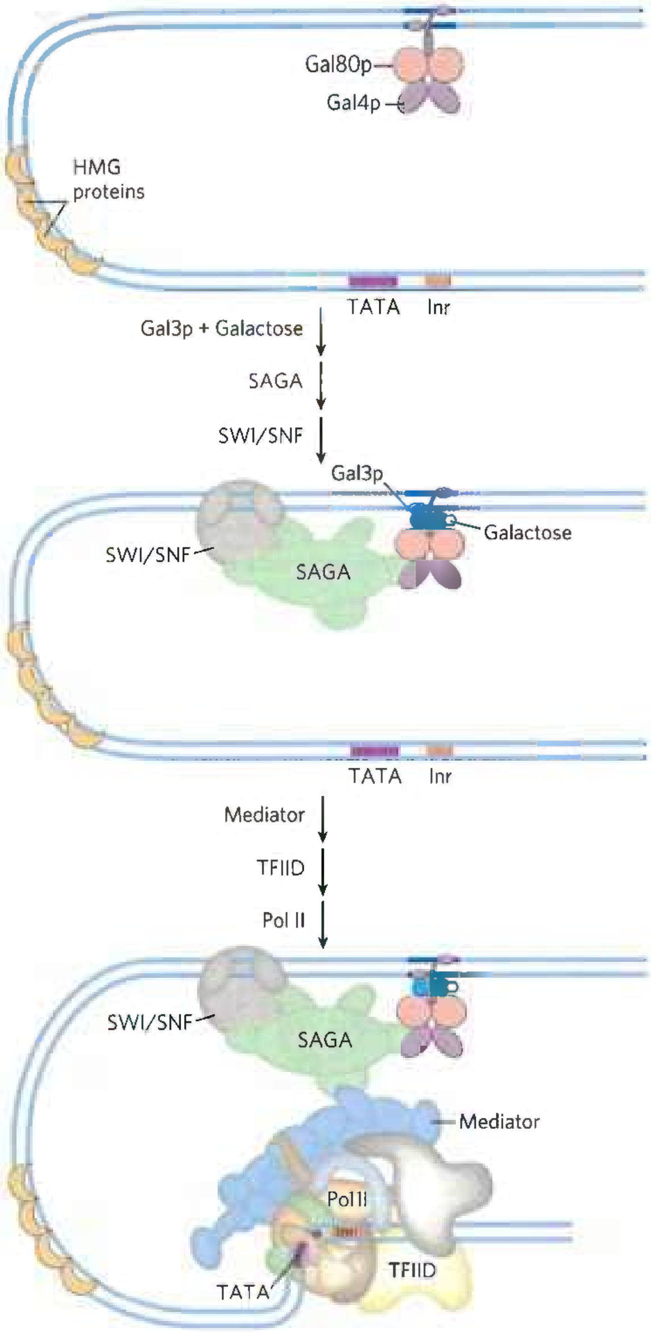


FIGURE 28-32 Regulation of transcription of *GAL* genes in yeast. Galactose imported into the yeast cell is converted to glucose 6-phosphate by a pathway involving five enzymes, whose genes are scattered over three chromosomes (see Table 28-3). Transcription of these genes is regulated by the combined actions of the proteins Gal4p, Gal80p, and Gal3p, with Gal4p playing the central role of transcription activator. The Gal4p-Gal80p complex is inactive. Binding of galactose to Gal3p leads to interaction of Gal3p with the Gal80p-Gal4p complex and activates Gal4p. The Gal4p subsequently recruits SAGA, Mediator, and TFIID to the galactose promoters, leading to recruitment of RNA polymerase II and initiation of transcription. Chromatin remodeling to allow transcription also requires a SWI/SNF complex.

Transcription Activators Have a Modular Structure

Transcription activators typically have a distinct structural domain for specific DNA binding and one or more additional domains for transcriptional activation or for interaction with other regulatory proteins. Interaction of two regulatory proteins is often mediated by domains containing leucine zippers (Fig. 28-15) or helix-loop-helix motifs (Fig. 28-16). We consider here three distinct types of structural domains used in activation by the transcription activators Gal4p, Sp1, and CTF1 (**Fig. 28-33a**).

Gal4p contains a zinc finger–like structure in its DNA-binding domain, near the amino terminus; this domain has six Cys residues that coordinate two Zn^{2+}. The protein functions as a homodimer (with dimerization mediated by interactions between two coiled coils) and binds to UAS_G, a palindromic DNA sequence about 17 bp long. Gal4p has a separate activation domain with many acidic amino acid residues. Experiments that substitute

TABLE 28-3 Genes of Galactose Metabolism in Yeast

Gene	Protein function	Chromosomal location	Protein size (number of residues)	Relative protein expression in different carbon sources		
				Glucose	Glycerol	Galactose
Regulated genes						
GAL1	Galactokinase	II	528	−	−	+++
GAL2	Galactose permease	XII	574	−	−	+++
PGM2	Phosphoglucomutase	XIII	569	+	+	++
GAL7	Galactose 1-phosphate uridylyltransferase	II	365	−	−	+++
GAL10	UDP-glucose 4-epimerase	II	699	−	−	+++
MEL1	α-Galactosidase	II	453	−	+	++
Regulatory genes						
GAL3	Inducer	IV	520	−	+	++
GAL4	Transcriptional activator	XVI	881	+/−	+	+
GAL80	Transcriptional inhibitor	XIII	435	+	+	++

Information from R. Reece and A. Platt, *Bioessays* 19:1001, 1997.

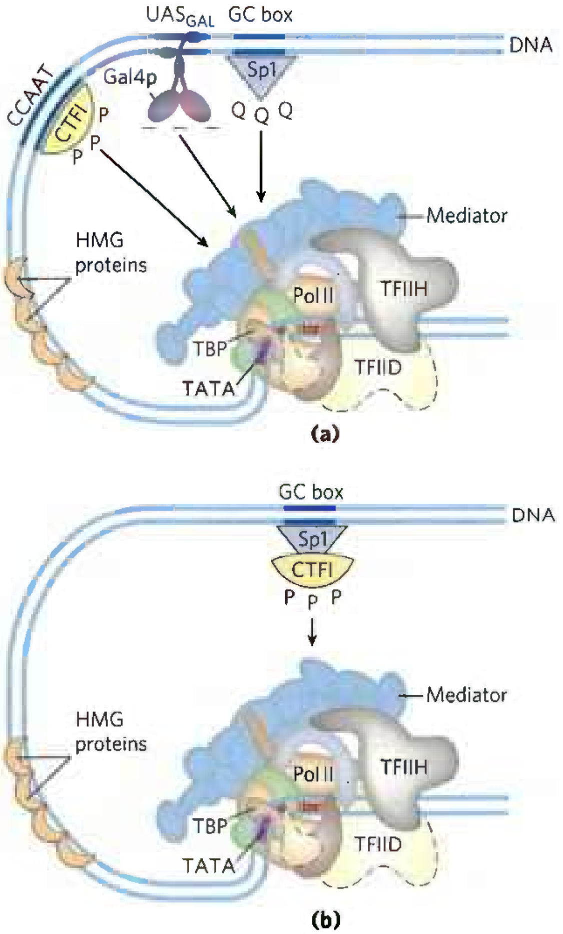


FIGURE 28-33 Transcription activators. (a) Typical activators such as CTF1, Gal4p, and Sp1 have a DNA-binding domain and an activation domain. The nature of the activation domain is indicated by symbols: − − −, acidic; Q Q Q, glutamine-rich; P P P, proline-rich. These proteins generally activate transcription by interacting with coactivator complexes such as Mediator. Note that the binding sites illustrated here are not generally found together near a single gene. (b) A chimeric protein containing the DNA-binding domain of Sp1 and the activation domain of CTF1 activates transcription if a GC box is present.

a variety of different peptide sequences for the **acidic activation domain** of Gal4p suggest that the acidic nature of this domain is critical to its function, although its precise amino acid sequence can vary considerably.

Sp1 (M_r 80,000) is a transcription activator for many genes in higher eukaryotes. Its DNA-binding site, the GC box (consensus sequence GGGCGG), is usually quite near the TATA box. The DNA-binding domain of the Sp1 protein is near its carboxyl terminus and contains three zinc fingers. Two other domains in Sp1 function in activation and are notable in that 25% of their amino acid residues are Gln. A wide variety of other activator proteins also have these **glutamine-rich domains**.

CTF1 (*C*CAAT-binding *t*ranscription *f*actor 1) belongs to a family of transcription activators that bind a sequence called the CCAAT site (its consensus sequence is TGGN$_6$GCCAA, where N is any nucleotide). The DNA-binding domain of CTF1 contains many basic amino acid residues, and the binding region is probably arranged as an α helix. This protein has neither a helix-turn-helix motif nor a zinc finger motif; its DNA-binding mechanism is not yet clear. CTF1 has a **proline-rich activation domain**, with Pro accounting for more than 20% of the amino acid residues.

The discrete activation and DNA-binding domains of regulatory proteins often act completely independently, as has been demonstrated in "domain-swapping" experiments. Genetic engineering techniques (Chapter 9) can join the proline-rich activation domain of CTF1 to the DNA-binding domain of Sp1 to create a protein that, like intact Sp1, binds to GC boxes on the DNA and activates transcription at a nearby promoter (as in Fig. 28-33b). The DNA-binding domain of Gal4p has similarly been replaced experimentally with the DNA-binding domain of the *E. coli* LexA repressor (of the SOS response; Fig. 28-21). This chimeric protein neither binds at UAS$_G$

nor activates the yeast *GAL* genes (as would intact Gal4p) unless the UAS_G sequence in the DNA is replaced by the LexA recognition site.

Eukaryotic Gene Expression Can Be Regulated by Intercellular and Intracellular Signals

The effects of steroid hormones (and of thyroid and retinoid hormones, which have a similar mode of action) provide additional well-studied examples of the modulation of eukaryotic regulatory proteins by direct interaction with molecular signals (see Fig. 12-34). Unlike other types of hormones, steroid hormones do not have to bind to plasma membrane receptors. Instead, they can interact with intracellular receptors that are transcription activators. Steroid hormones too hydrophobic to dissolve readily in the blood (estrogen, progesterone, and cortisol, for example) travel on specific carrier proteins from their point of release to their target tissues. In the target tissue, the hormone passes through the plasma membrane by simple diffusion. Once inside the cell, the hormone interacts with one of two types of steroid-binding nuclear receptor (**Fig. 28-34**). In both cases, the hormone-receptor complex acts by binding to highly specific DNA sequences called **hormone response elements (HREs)**, thereby altering gene expression. Acting at these sites, the receptors act as transcription activators, recruiting coactivators and Pol II (plus its associated transcription factors) to trigger transcription of the gene.

The DNA sequences (HREs) to which hormone-receptor complexes bind are similar in length and arrangement for the various steroid hormones, but they differ in sequence. Each receptor has a consensus HRE sequence (**Table 28-4**) to which the hormone-receptor complex binds well, with each consensus consisting of two six-nucleotide sequences, either contiguous or separated by three nucleotides, in tandem or in a palindromic arrangement. The hormone receptors have a highly conserved DNA-binding domain with two zinc fingers (**Fig. 28-35**). The hormone-receptor complex binds to the DNA as a dimer, with the zinc finger domains of each monomer recognizing one of the six-nucleotide sequences. The ability of a given hormone to act through the hormone-receptor complex to alter the expression of a specific

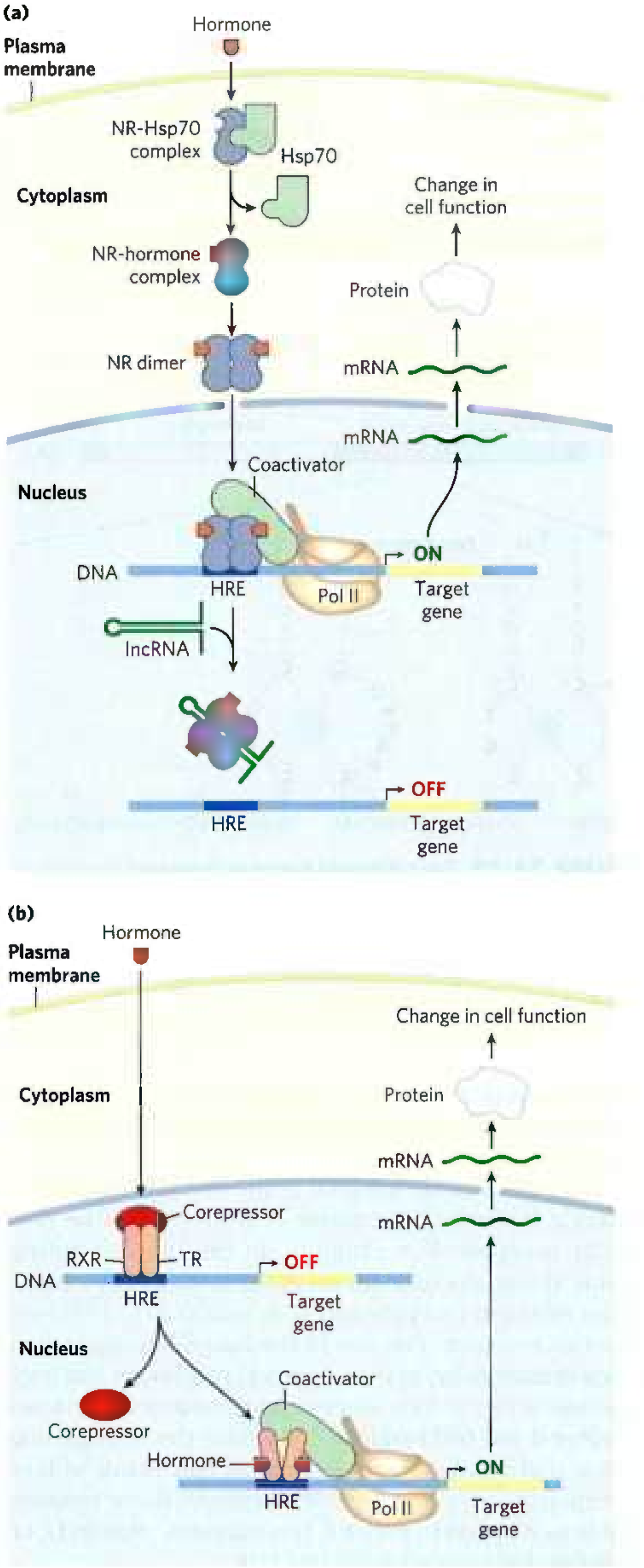

(a)
Hormone
Plasma membrane
NR-Hsp70 complex
Hsp70
Cytoplasm
Change in cell function
NR-hormone complex
Protein
NR dimer
mRNA
mRNA
Coactivator
Nucleus
ON
DNA
HRE
Pol II
Target gene
lncRNA
OFF
HRE
Target gene
(b)
Hormone
Plasma membrane
Change in cell function
Cytoplasm
Protein
mRNA
Corepressor
mRNA
RXR
TR
OFF
DNA
HRE
Target gene
Nucleus
Coactivator
Corepressor
Hormone
ON
HRE
Pol II
Target gene

FIGURE 28-34 Mechanisms of steroid hormone receptor function. There are two types of steroid-binding nuclear receptors. (a) Monomeric type I receptors (NR) are found in the cytoplasm, in a complex with the heat shock protein Hsp70. Receptors for estrogen, progesterone, androgens, and glucocorticoids are of this type. When the steroid hormone binds, the Hsp70 dissociates and the receptor dimerizes, exposing a nuclear localization signal. The dimeric receptor, with hormone bound, migrates to the nucleus, where it binds to a hormone response element (HRE) and acts as a transcription activator. The activity of the receptor can be repressed by binding to an lncRNA (such as GAS5), which competes directly with binding to the HRE. (b) Type II receptors, by contrast, are always in the nucleus, bound to an HRE in the DNA and to a corepressor that renders the receptor inactive. The thyroid hormone receptor (TR) is of this type. The hormone migrates through the cytoplasm and diffuses across the nuclear membrane. In the nucleus it binds to a heterodimer consisting of the thyroid hormone receptor and the retinoid X receptor (RXR). A conformation change leads to dissociation of the corepressor, and the receptor then functions as a transcription activator.

TABLE 28-4 Hormone Response Elements (HREs) Bound by Steroid-Type Hormone Receptors

Receptor	HRE consensus sequence bound[a]
Androgen	GG(A/T)ACAN$_2$TGTTCT
Glucocorticoid	GGTACAN$_3$TGTTCT
Retinoic acid (some)	AGGTCAN$_5$AGGTCA
Vitamin D	AGGTCAN$_3$AGGTCA
Thyroid hormone	AGGTCAN$_3$AGGTCA
RX[b]	AGGTCANAGGTCANAG GTCANAGGTCA

[a] N represents any nucleotide.

[b] Forms a dimer with the retinoic acid receptor or vitamin D receptor.

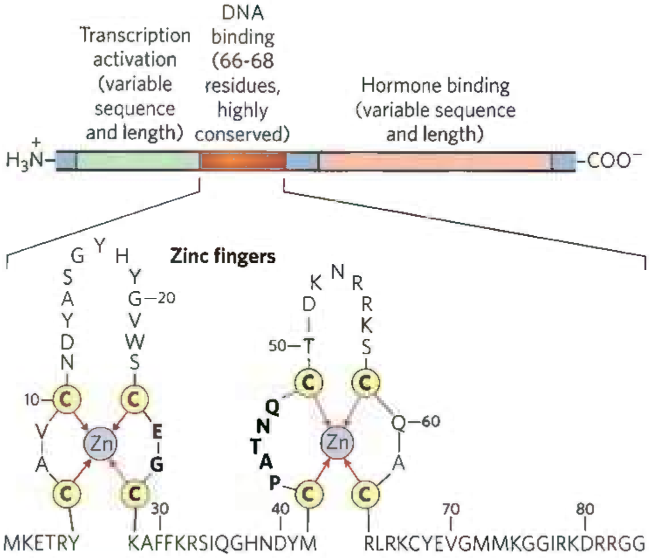

Transcription activation (variable sequence and length)
DNA binding (66-68 residues, highly conserved)
Hormone binding (variable sequence and length)
$H_3\overset{+}{N}$–
–COO⁻
Zinc fingers
Zn
Zn
10
20
30
40
50
60
70
80
MKETRY
KAFFKRSIQGHNDYM
RLRKCYEVGMMKGGIRKDRRGG

FIGURE 28-35 Typical steroid hormone receptors. These receptor proteins have a binding site for the hormone, a DNA-binding domain, and a region that activates transcription of the regulated gene. The highly conserved DNA-binding domain has two zinc fingers. The sequence shown here is that for the estrogen receptor, but the residues in bold type are common to all steroid hormone receptors.

gene depends on the exact sequence of the HRE, its position relative to the gene, and the number of HREs associated with the gene.

The ligand-binding region of the receptor protein—always at the carboxyl terminus—is specific to the particular receptor. For example, in the ligand-binding region, the glucocorticoid receptor is only 30% similar to the estrogen receptor and 17% similar to the thyroid hormone receptor. The size of the ligand-binding region varies dramatically; in the vitamin D receptor it has only 25 amino acid residues, whereas in the mineralocorticoid receptor it has 603 residues. Mutations that change one amino acid residue in these regions can result in loss of responsiveness to a specific hormone. Some humans unable to respond to cortisol, testosterone, vitamin D, or thyroxine have mutations of this type.

P2 The lncRNAs introduce another dimension to regulation by hormone receptors. An lncRNA called GAS5 (*g*rowth *a*rrest *s*pecific 5) inhibits transcriptional activation by the glucocorticoid receptor by directly competing with DNA for receptor binding. GAS5 also inhibits activity of the closely related androgen, progesterone, and mineralocorticoid receptors. In addition, GAS5 interacts with and sequesters an miRNA called miR-21, which interacts with and inhibits the activity of some regulatory proteins that act as tumor suppressors. Expression of GAS5 is suppressed in a wide range of tumors, resulting in increased expression of steroid hormones, higher levels of active miR-21, and faster tumor growth. Low GAS5 levels thus correlate with worsened outcomes for cancer patients, making this lncRNA a subject of intense ongoing investigation.

Some hormone receptors, including the human progesterone receptor, activate transcription with the aid of a different lncRNA of ~700 nucleotides that acts as a coactivator—**steroid receptor RNA activator (SRA)**. SRA is part of a ribonucleoprotein complex, but it is the RNA component that is required for transcription coactivation. The detailed set of interactions between SRA and other components of the regulatory systems for these genes remains to be worked out.

Regulation Can Result from Phosphorylation of Nuclear Transcription Factors

We noted in Chapter 12 that the effects of insulin on gene expression are mediated by a series of steps leading ultimately to the activation of a protein kinase in the nucleus that phosphorylates specific DNA-binding proteins, thereby altering their ability to act as transcription factors (see Fig. 12-22). This general mechanism mediates the effects of many nonsteroid hormones. For example, the β-adrenergic pathway that leads to elevated levels of cytosolic cAMP, which acts as a second messenger in both eukaryotes and bacteria (Fig. 28-18), also affects the transcription of a set of genes, each of which is located near a specific DNA sequence called a **cAMP response element (CRE)**. The catalytic subunit of protein kinase A, released when cAMP levels rise (see Fig. 12-6), enters the nucleus and phosphorylates a nuclear protein, the **CRE-binding protein (CREB)**. When phosphorylated, CREB binds to CREs near certain genes and acts as a transcription factor, turning on expression of these genes.

Many Eukaryotic mRNAs Are Subject to Translational Repression

P1 Regulation at the level of translation assumes a much more prominent role in eukaryotes than in bacteria and is observed in a range of cellular situations. In contrast to the tight coupling of transcription and translation in bacteria, the transcripts generated in a eukaryotic nucleus must be processed and transported to the cytoplasm before translation. This can impose a significant delay on the appearance of a protein. When a rapid increase in protein production is needed, a translationally repressed mRNA already in the cytoplasm can be activated for translation without delay. Translational regulation may play an especially important role in regulating certain very long eukaryotic genes (a few are measured in the millions of base pairs), for which transcription and mRNA processing can require many hours. Some genes are regulated at both the transcriptional and translational stages, with the latter playing a role in the fine-tuning of cellular protein levels. In some non-nucleated cells, such as reticulocytes (immature erythrocytes), transcriptional control is entirely unavailable and translational control of stored mRNAs becomes essential. As described below, translational controls can also have spatial significance during development, when the regulated translation of prepositioned mRNAs creates a local gradient of the protein product.

Eukaryotes have at least four main mechanisms of translational regulation:

1. Translation initiation factors are subject to phosphorylation by protein kinases. The phosphorylated forms are often less active and cause a general depression of translation in the cell.
2. Some proteins bind directly to mRNA and act as translational repressors, many of them binding at specific sites in the 3′ untranslated region (3′UTR). So positioned, these proteins interact with other translation initiation factors bound to the mRNA, or with the 40S ribosomal subunit, to prevent translation initiation (**Fig. 28-36**).
3. Binding proteins, present in eukaryotes from yeast to mammals, disrupt the interaction between eIF4E and eIF4G (see Fig. 27-27). The mammalian versions are known as 4E-BPs (eIF4E binding proteins). When cell growth is slow, these proteins limit translation by binding to the site on eIF4E that normally interacts with eIF4G. When cell growth resumes or increases in response to growth factors or other stimuli, the binding proteins are inactivated by protein kinase–dependent phosphorylation.
4. RNA-mediated regulation of gene expression often occurs at the level of translational repression, often by the binding of ncRNAs to mRNAs.

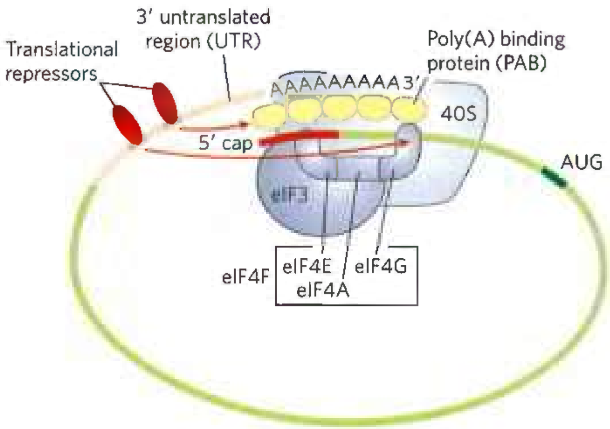


FIGURE 28-36 Translational regulation of eukaryotic mRNA. One of the most important mechanisms for translational regulation in eukaryotes is the binding of translational repressors (RNA-binding proteins) to specific sites in the 3′ untranslated region (3′UTR) of the mRNA. These proteins interact with eukaryotic initiation factors or with the ribosome to prevent or slow translation.

The variety of translational regulation mechanisms provides flexibility, allowing focused repression of a few mRNAs or global regulation of all cellular translation.

Translational regulation has been particularly well studied in reticulocytes. One such mechanism in these cells involves eIF2, the initiation factor that binds to the initiator tRNA and conveys it to the ribosome; when Met-tRNA has bound to the P site, the factor eIF2B binds to eIF2, recycling it with the aid of GTP binding and hydrolysis. The maturation of reticulocytes includes destruction of the cell nucleus, leaving behind a plasma membrane packed with hemoglobin. Messenger RNAs deposited in the cytoplasm before the loss of the nucleus allow for the replacement of hemoglobin. When reticulocytes become deficient in iron or heme, the translation of globin mRNAs is repressed. A protein kinase called **HCR (hemin-controlled repressor)** is then activated, catalyzing the phosphorylation of eIF2. When phosphorylated, eIF2 forms a stable complex with eIF2B that sequesters the eIF2, making it unavailable for participation in translation. In this way, the reticulocyte coordinates the synthesis of globin with the availability of heme.

Posttranscriptional Gene Silencing Is Mediated by RNA Interference

In higher eukaryotes, including nematodes, fruit flies, plants, and mammals, microRNAs (miRNAs) mediate the silencing of many genes. In a phenomenon first described and explained by Craig Mello and Andrew Fire, the RNAs function by interacting with mRNAs, often in the 3′UTR, resulting in either degradation of the mRNA or inhibition of translation. In either case, the mRNA, and thus the gene that produces it, is silenced. This form of gene regulation controls developmental timing in at least some organisms. It is also used as a mechanism to protect against invading RNA viruses (particularly important in plants, which lack an immune system) and to control the activity of transposons. In addition, small RNA molecules may play a critical (as yet undefined) role in the formation of heterochromatin.

Many miRNAs are present only transiently during development, and these are sometimes referred to as **small temporal RNAs (stRNAs)**. Thousands of different miRNAs have been identified in higher eukaryotes, and they may affect the regulation of a third of mammalian genes. They are transcribed as precursor RNAs ~70 nucleotides long, with internally complementary sequences that form hairpinlike structures. Details of the pathway for processing of miRNAs were described in Fig. 26-26). The precursors are cleaved by endonucleases such as Drosha and Dicer to form short duplexes of 20 to 25 nucleotides. One strand of the processed miRNA is transferred to the target mRNA (or to a viral or transposon RNA), leading to inhibition of translation or degradation of the mRNA (**Fig. 28-37a**). Some miRNAs bind to and affect a single mRNA and thus affect expression of only one gene. Others interact with multiple mRNAs and form the mechanistic core of regulons that coordinate the expression of multiple genes.

This gene regulation mechanism has an interesting and very useful practical side. If an investigator introduces into an organism a duplex RNA molecule corresponding in sequence to virtually any mRNA, Dicer cleaves the duplex into short segments, called **small interfering RNAs (siRNAs)**. These bind to the mRNA and silence it (Fig. 28-37b). The process is known as **RNA interference (RNAi)**. In plants, almost any gene can be effectively shut down in this way. Nematodes can readily ingest entire functional RNAs, and simply introducing the duplex RNA into the worm's diet produces very effective suppression of the target gene. The technique is an important tool in the ongoing efforts to study gene function, because it can disrupt gene function without creating a mutant organism. The procedure can be applied to humans as well. Laboratory-produced siRNAs have been used to block HIV and poliovirus infections in cultured human cells for a week or so at a time. The wider application of RNAi-based pharmaceuticals was initially stymied by the difficulty inherent in delivering RNAi molecules to their required target, given the many nucleases that degrade RNA in human tissues. With recent advances in delivery methods, there are now more than a dozen RNAi pharmaceuticals in advanced clinical trials to treat a range of conditions, from familial amyloidotic polyneuropathy to viral infections and cancer.

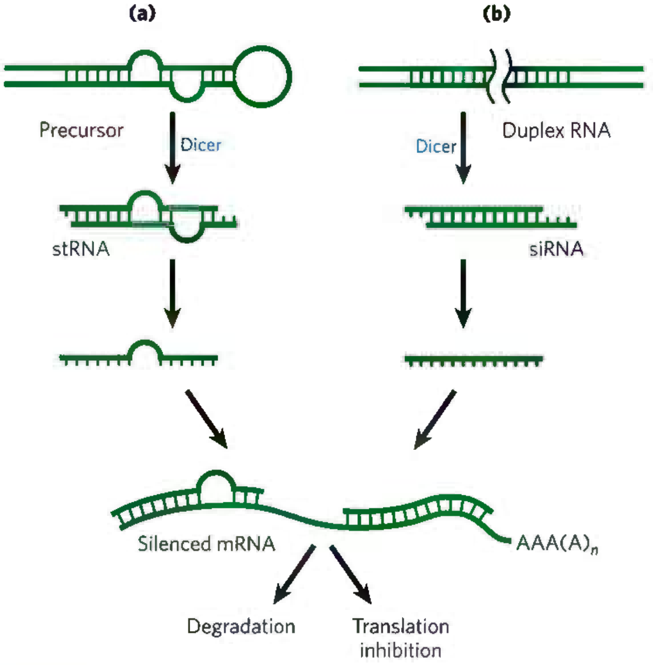


FIGURE 28-37 Gene silencing by RNA interference. (a) Small temporal RNAs (stRNAs, a class of miRNAs) are generated by Dicer-mediated cleavage of longer precursors that fold to create duplex regions. The stRNAs then bind to mRNAs, leading to degradation of mRNA or inhibition of translation. (b) Double-stranded RNAs designed to interact with a particular target and to function as Dicer substrates can be constructed and introduced into a cell. Dicer processes the duplex RNAs into small interfering RNAs (siRNAs), which interact with the target mRNA. Again, either the mRNA is degraded or translation is inhibited.

RNA-Mediated Regulation of Gene Expression Takes Many Forms in Eukaryotes

All RNAs (regardless of their length) that do not encode proteins, including rRNAs and tRNAs, come under the general designation of ncRNAs. Mammalian genomes encode more ncRNAs than coding mRNAs. The ncRNAs in eukaryotes include miRNAs, described above; snRNAs, involved in RNA splicing (see Fig. 26-16); snoRNAs, involved in rRNA modification (see Fig. 26-24); and lncRNAs, already encountered in this chapter. Not surprisingly, additional functional classes of ncRNAs are still being discovered. P2 Here we describe a few more examples of ncRNAs that participate in gene regulation, which are designated lncRNAs when their length exceeds 200 nucleotides.

Heat shock factor 1 (HSF1) is an activator protein that, in nonstressed cells, exists as a monomer bound by the chaperone Hsp90. Under stress conditions, HSF1 is released from Hsp90 and trimerizes. The HSF1 trimer binds to DNA and activates transcription of genes encoding products required to deal with the stress. An lncRNA called HSR1 (heat shock RNA 1; ~600 nucleotides) stimulates HSF1 trimerization and DNA binding. HSR1 does not act alone; it functions in a complex with the translation elongation factor eEF1A.

Additional RNAs affect transcription in a variety of ways. A 331 nucleotide lncRNA called 7SK, abundant in mammals, binds to the Pol II transcription elongation factor pTEFb (see Table 26-2) and represses transcript elongation. The ncRNA B2 (~178 nucleotides) binds directly to Pol II during heat shock and represses transcription. The B2-bound Pol II assembles into stable PICs, but transcription is blocked. The mechanism that allows HSF1-responsive genes to be expressed in the presence of B2 remains to be worked out.

The recognized roles of ncRNAs in gene expression and in many other cellular processes are rapidly expanding. At the same time, the study of the biochemistry of gene regulation is becoming much less protein-centric.

Development Is Controlled by Cascades of Regulatory Proteins

For sheer complexity and intricacy of coordination, the patterns of gene regulation that bring about development of a zygote into a multicellular animal or plant have no peer. Development requires transitions in morphology and protein composition that depend on tightly coordinated changes in expression of the genome. More genes are expressed during early development than in any other part of the life cycle. For example, in the sea urchin, an oocyte has about 18,500 *different* mRNAs, compared with about 6,000 different mRNAs in the cells of a typical differentiated tissue. The mRNAs in the oocyte give rise to a cascade of events that regulate the expression of many genes across both space and time.

Several organisms have emerged as important model systems for the study of development, because they are easy to maintain in a laboratory and have relatively short generation times. These include nematodes, fruit flies, zebra fish, mice, and the plant *Arabidopsis*. Here, we provide a brief discussion of the development of fruit flies. Our understanding of the molecular events during development of *Drosophila melanogaster* is particularly well advanced and can be used to illustrate patterns and principles of general significance.

The life cycle of the fruit fly includes complete metamorphosis during its progression from an embryo to an adult (**Fig. 28-38**). Among the most important characteristics of the embryo are its **polarity** (the anterior and posterior parts of the animal are readily distinguished, as are its dorsal and ventral surfaces) and its **metamerism** (the embryo body is made up of serially repeating segments, each with characteristic features). During development, these segments become organized into a head, thorax, and abdomen. Each segment of the adult thorax has a different set of appendages. Development of this complex pattern is under genetic control, and a variety of pattern-regulating genes have been discovered that greatly affect the organization of the body.

The *Drosophila* egg, along with 15 nurse cells, is surrounded by a layer of follicle cells (**Fig. 28-39**). As the egg cell forms (before fertilization), mRNAs and proteins originating in the nurse and follicle cells are deposited in the egg cell, where some play a critical role in development. Once a fertilized egg is laid, its nucleus divides and the nuclear descendants continue to divide in synchrony every 6 to 10 min. Plasma membranes are not formed around the nuclei, which are distributed within the egg cytoplasm, forming a syncytium. Between the eighth and eleventh rounds of nuclear division, the nuclei migrate to the outer layer of the egg, forming a monolayer of nuclei surrounding the common yolk-rich cytoplasm; this is the syncytial blastoderm. After a few additional divisions, membrane invaginations surround the nuclei to create a layer of cells that form the cellular blastoderm. At this stage, the mitotic cycles in the various cells lose their synchrony. The developmental fate of the cells is determined by the mRNAs and proteins originally deposited in the egg by the nurse and follicle cells.

Proteins that, through changes in local concentration or activity, cause the surrounding tissue to take up a particular shape or structure are sometimes referred to as **morphogens**; they are the products of pattern-regulating genes. As defined by Christiane Nüsslein-Volhard, Edward B. Lewis, and Eric F. Wieschaus, three major classes of pattern-regulating genes—maternal, segmentation, and homeotic genes—function in successive stages of development to specify the basic features of the *Drosophila* embryo body. **Maternal genes** are expressed in the unfertilized egg, and the resulting **maternal mRNAs** remain dormant until fertilization.

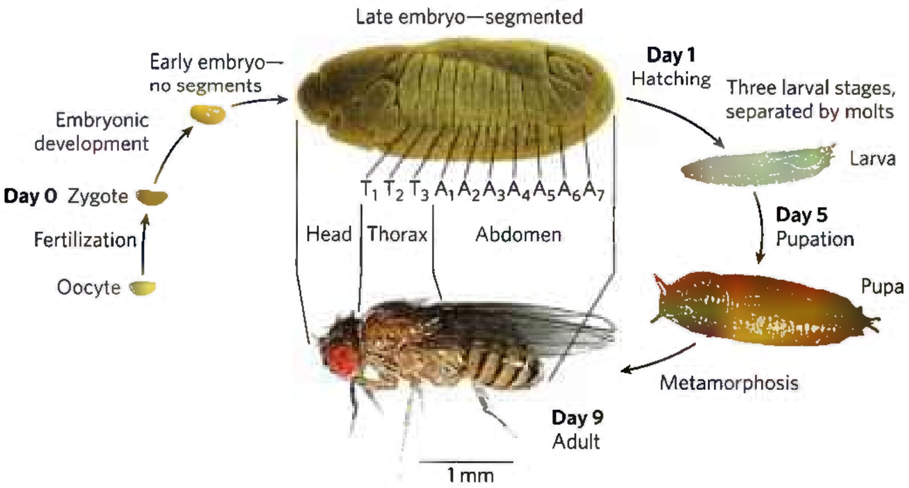


FIGURE 28-38 Life cycle of the fruit fly *Drosophila melanogaster*. *Drosophila* undergoes a complete metamorphosis, which means that the adult insect is radically different in form from its immature stages, a transformation that requires extensive alterations during development. By the late embryonic stage, segments have formed, each containing specialized structures from which the various appendages and other features of the adult fly will develop. [Late embryo: Courtesy of F. R. Turner, Department of Biology, University of Indiana, Bloomington. Other photos: Courtesy of Prof. Dr. Christian Klambt, Westfälische Wilhelms-Universität Münster, Institut für Neuro- und Verhaltensbiologie.]

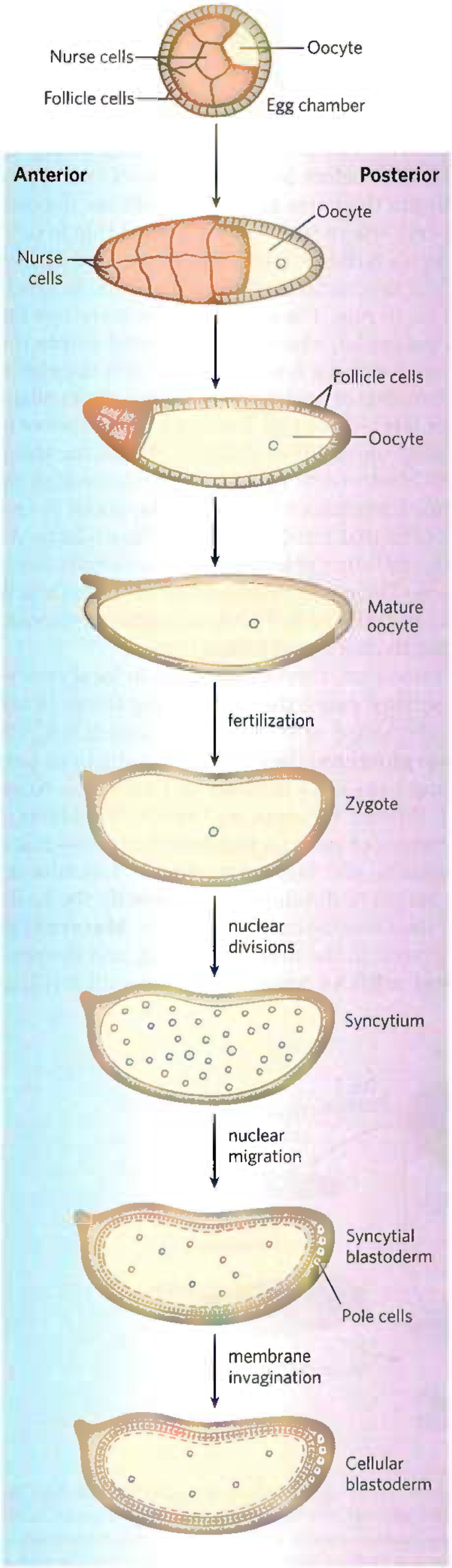


FIGURE 28-39 Early development in *Drosophila*. During development of the egg, maternal mRNAs and proteins are deposited in the developing oocyte (unfertilized egg cell) by nurse cells and follicle cells. After fertilization, the nuclei of the egg divide in synchrony within the common cytoplasm (syncytium), then migrate to the periphery. Membrane invaginations surround the nuclei to create a monolayer of cells at the periphery; this is the cellular blastoderm stage. During the early nuclear divisions, several nuclei at the far posterior become pole cells, which later become the germ-line cells.

These provide most of the proteins needed in very early development, until the cellular blastoderm is formed. Some of the proteins encoded by maternal mRNAs direct the spatial organization of the developing embryo at early stages, establishing its polarity. **Segmentation genes**, transcribed after fertilization, direct the formation of the proper number of body segments. At least three subclasses of segmentation genes act at successive stages: **gap genes** divide the developing embryo into several broad regions; **pair-rule genes**, together with **segment polarity genes**, define 14 stripes that become the 14 segments of a normal embryo. **Homeotic genes** are expressed still later; they specify which organs and appendages will develop in particular body segments.

If all cells divided to produce two identical daughter cells, multicellular organisms would never be more than a ball of identical cells. A key event in very early development is establishment of mRNA and protein gradients along the body axes, producing asymmetric cell divisions and different cell fates. Some maternal mRNAs have protein products that diffuse through the cytoplasm to create an asymmetric distribution in the egg. Different cells in the cellular blastoderm therefore inherit different amounts of these proteins, setting the cells on different developmental paths. An example is the *bicoid* gene. The *bicoid* gene product is a major anterior morphogen. The mRNA from the *bicoid* gene is synthesized by nurse cells and deposited in the unfertilized egg near its anterior pole. Translated soon after fertilization, the Bicoid protein diffuses through the cell to create, by the seventh nuclear division, a concentration gradient radiating out from the anterior pole (**Fig. 28-40**). The Bicoid protein contains a homeodomain (p. 1062), encoded by a gene sequence motif called a homeobox and found in many proteins involved in regulating development. Bicoid is multifunctional—a transcription factor that activates the expression of several segmentation genes and also a translational repressor that inactivates certain mRNAs. The amount of Bicoid protein in various parts of the embryo increases or decreases the expression of other genes in a threshold-dependent manner. As its concentration varies along its gradient, interactions of the *bicoid* gene product with proteins and RNAs encoded by the *nanos, pumilio, caudal, hunchback*, and other regulatory genes also vary to produce different effects along the axis of the developing organism. This results in different developmental fates of cells in the blastoderm, depending on their location.

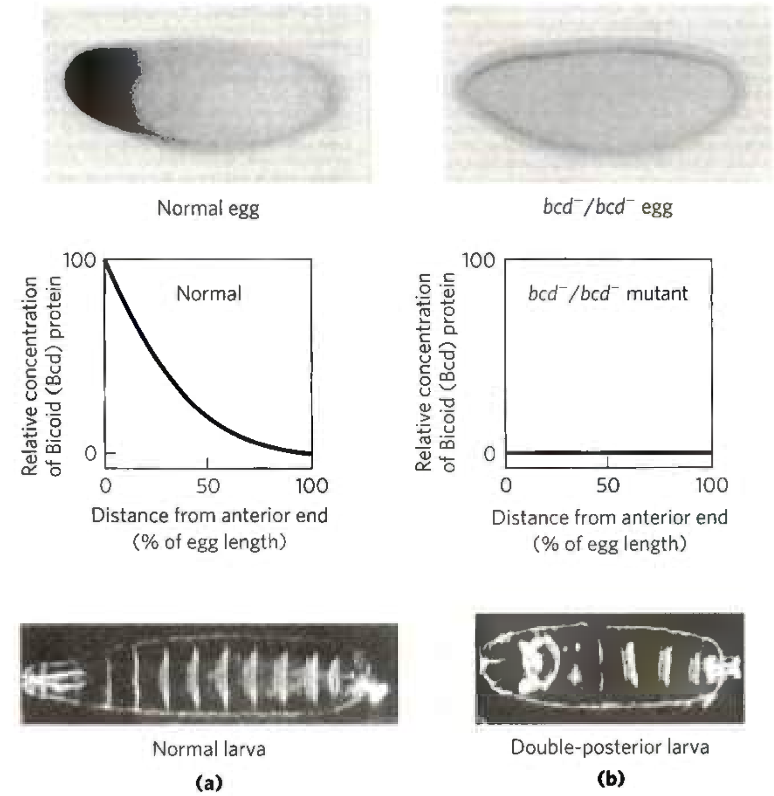


FIGURE 28-40 Distribution of a maternal gene product in a *Drosophila* egg. (a) Micrograph of an immunologically stained egg (top), showing distribution of the *bicoid* (*bcd*) gene product. The graph shows stain intensity along the length of the egg. This distribution is essential for normal development of the anterior structures in the larva (bottom). (b) If the *bcd* gene is not expressed by the mother (bcd^-/bcd^- mutant) and thus no *bicoid* mRNA is deposited in the egg, the resulting larva has two posteriors (and soon dies). [Republished with permission of Elsevier, from "The bicoid protein determines position in the *Drosophila* embryo in a concentration-dependent manner" by Wolfgang Driever and Christiane Nüsslein-Volhard, *Cell* 54:83–93, July 1, 1988; permission conveyed through Copyright Clearance Center, Inc.]

Humans do not resemble fruit flies, but the genes and mechanisms involved in development are nevertheless highly conserved. This can be seen in the gene clusters encoding the homeotic or ***Hox* genes**, the latter term derived from homeobox. *Drosophila* has one such cluster, while humans have four (**Fig. 28-41**), with the genes within the clusters remarkably similar from nematodes to humans.

The many regulatory genes in these three classes direct the development of an adult fly, with a head, thorax, and abdomen, with the proper number of segments, and with the correct appendages on each segment. Although embryogenesis takes about a day to complete, all these genes are activated during the first four hours. Some mRNAs and proteins are present for only a few minutes at specific points during this period. Some of the genes code for transcription factors that affect the expression of other genes in a kind of developmental cascade. Regulation at the level of translation also occurs, and many of the regulatory genes encode translational repressors, most of which bind to the 3′UTR of the mRNA (Fig. 28-36). P1 Because many mRNAs are deposited in the egg long before their translation is required, translational repression provides an especially important avenue for regulation in developmental pathways.

Many of the principles of development outlined above apply to other eukaryotes, from nematodes to humans. Some of the regulatory proteins are conserved. For example, the products of the homeobox-containing genes *HOXA7* in mouse and *antennapedia* in fruit fly differ in only one amino acid residue. Of course, although the molecular regulatory mechanisms may be similar, many of the ultimate developmental events are not conserved (humans do not have wings or antennae). The different outcomes are brought about by differences in the downstream target genes controlled by the *Hox* genes. The discovery of structural determinants with identifiable molecular functions is the first step in understanding the molecular events underlying development. As more genes and their protein products are discovered, the biochemical side of this vast puzzle will be elucidated in increasingly rich detail.

Stem Cells Have Developmental Potential That Can Be Controlled

If we can understand development, and the mechanisms of gene regulation behind it, we can control it. An adult human has many different types of tissues. Many of the cells are terminally differentiated and no longer divide. If an organ malfunctions due to disease, or a limb is lost

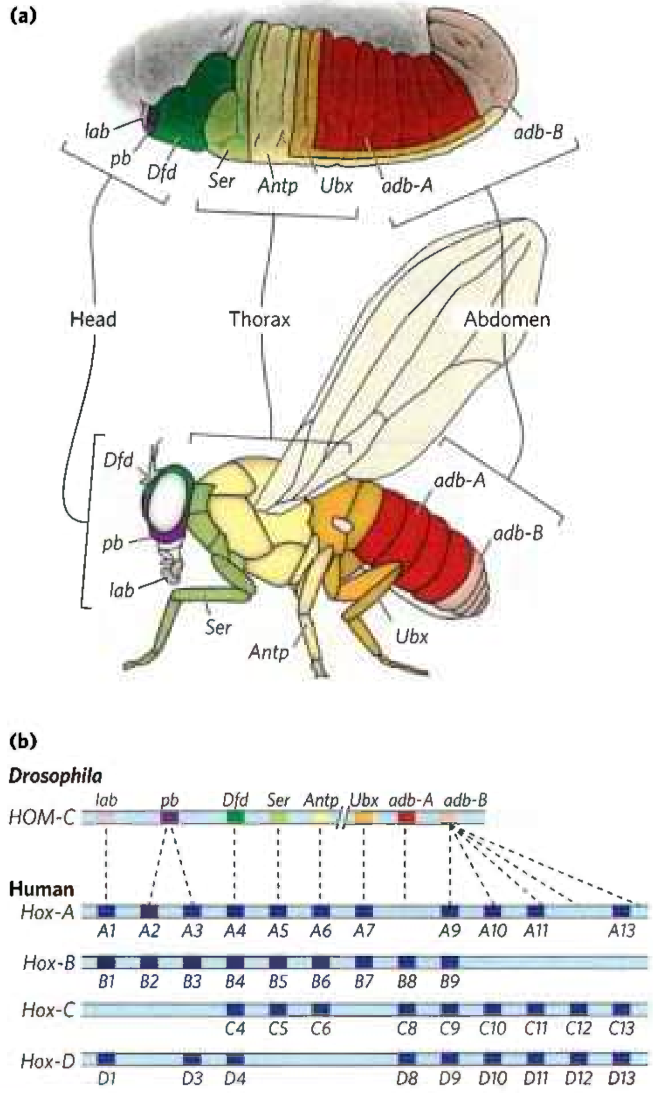

(a)
lab
pb
Dfd
Ser
Antp
Ubx
adb-A
adb-B
Head
Thorax
Abdomen
Dfd
pb
lab
Ser
Antp
Ubx
adb-A
adb-B
(b)
Drosophila
HOM-C
lab
pb
Dfd
Ser
Antp
Ubx
adb-A
adb-B
Human
Hox-A
A1 A2 A3 A4 A5 A6 A7 A9 A10 A11 A13
Hox-B
B1 B2 B3 B4 B5 B6 B7 B8 B9
Hox-C
C4 C5 C6 C8 C9 C10 C11 C12 C13
Hox-D
D1 D3 D4 D8 D9 D10 D11 D12 D13

FIGURE 28-41 The *Hox* gene clusters and their effects on development. (a) Each *Hox* gene in the fruit fly is responsible for the development of structures in a defined part of the body and is expressed in defined regions of the embryo, as labeled. (b) *Drosophila* has one *Hox* gene cluster; the human genome has four. Many of these genes are highly conserved in multicellular animals. Evolutionary relationships, as indicated by sequence alignments, between genes in the fruit fly *Hox* gene cluster and those in the mammalian *Hox* gene clusters are shown by dashed lines. Similar relationships among the four sets of mammalian *Hox* genes are indicated by vertical alignment. [(a) Information from F. R. Turner, University of Indiana, Department of Biology.]

in an accident, the tissues are not readily replaced. Most cells, because of the regulatory processes in place, or even because of the loss of some or all of the genomic DNA, are not easily reprogrammed. Medical science has made organ transplants possible, but organ donors are a limited resource and organ rejection remains a major medical problem. If humans could regenerate their own organs or limbs or nervous tissue, rejection would no longer be an issue. Cures for kidney failure or neurodegenerative disorders could become reality.

The key to tissue regeneration lies in **stem cells**—cells that have retained the capacity to differentiate into various tissues. In humans, after an egg is fertilized, the first few cell divisions create a ball of **totipotent** cells, called the morula, that have the capacity to differentiate individually into any tissue or even into a complete organism (**Fig. 28-42**). Continued cell division produces a hollow ball, the blastocyst. The outer cells of the blastocyst eventually form the placenta. The inner layers form the germ layers of the developing fetus—the ectoderm, mesoderm, and endoderm. These

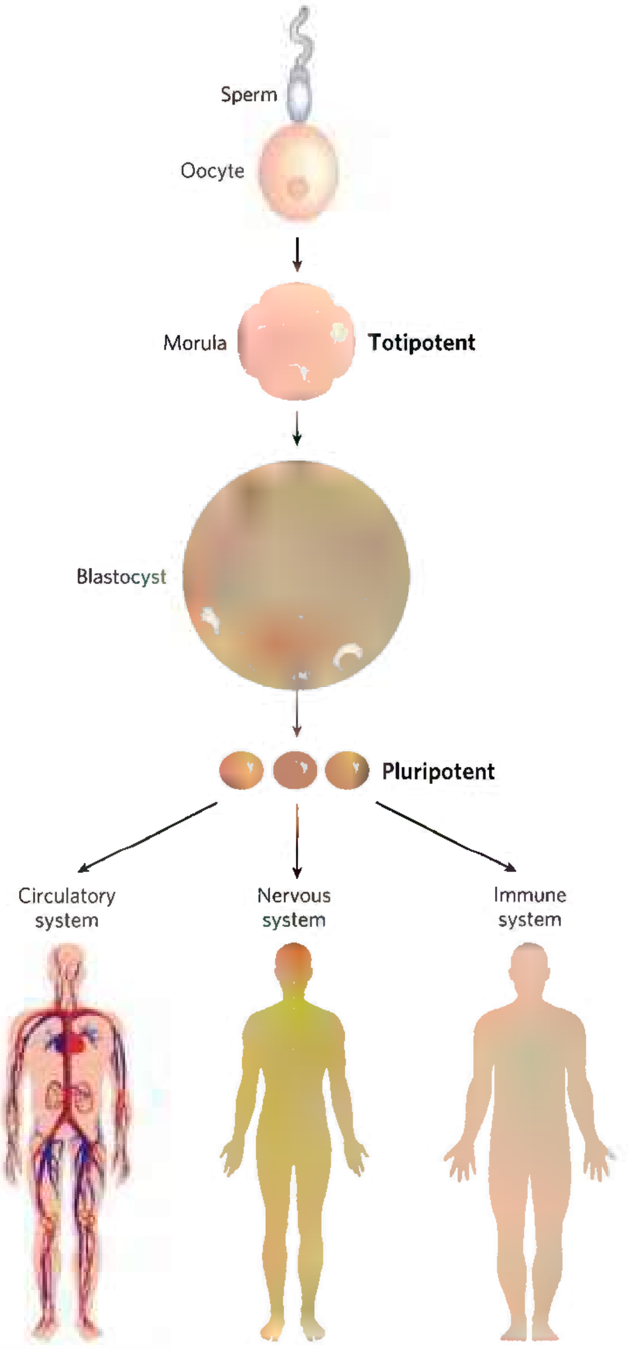

Sperm
Oocyte
Morula
Totipotent
Blastocyst
Pluripotent
Circulatory system
Nervous system
Immune system

FIGURE 28-42 Totipotent and pluripotent stem cells. Cells at the morula stage are totipotent and have the capacity to differentiate into a complete organism. The source of pluripotent embryonic stem cells is the cells in the cavity of the blastocyst. Pluripotent cells give rise to many tissue types but cannot form complete organisms.

cells are **pluripotent**: they can give rise to cells of all three germ layers and can differentiate into many types of tissues. However, they cannot differentiate into a complete organism. Some of these cells are **unipotent**: they can develop into only one type of cell and/or tissue. It is the pluripotent cells of the blastocyst, the **embryonic stem cells**, that are currently used in embryonic stem cell research.

Stem cells have two functions: to replenish themselves and, at the same time, provide cells that can differentiate. These tasks are accomplished in multiple ways (**Fig. 28-43a**). All or parts of the stem cell population can, in principle, be involved in replenishment, differentiation, or both.

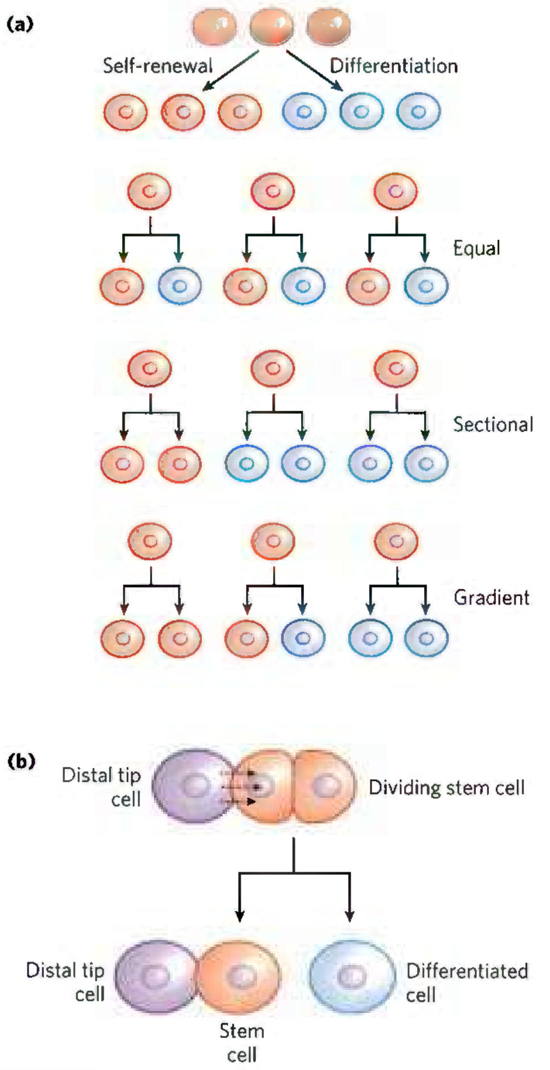


FIGURE 28-43 Stem cell proliferation versus differentiation and development. Stem cells must strike a balance between self-renewal and differentiation. (a) Some possible cell division patterns that allow the replenishment of stem cells and production of some differentiated cells. Each cell may produce one stem cell and one differentiated cell, or two differentiated cells, or two stem cells in defined parts of the tissue or culture. Or a gradient of growth conditions can be established, with cell fates differing from one end of the gradient to the other. (b) Establishing a developmental niche through stem cell contact with a cell or group of cells. Molecular signals provided by the niche cells (in this case, in plants, a distal tip cell) help orient the mitotic spindle for stem cell division and ensure that one daughter cell retains stem cell properties.

Other types of stem cells can potentially be used for medical benefit. In the adult organism, **adult stem cells**, as products of additional differentiation, have a more limited potential for further development than do embryonic stem cells. For example, the hematopoietic stem cells of bone marrow can give rise to many types of blood cells and also to cells with the capacity to regenerate bone. They are referred to as **multipotent**. However, these cells cannot differentiate into a liver or kidney or neuron. Adult stem cells are often said to have a **niche**, a microenvironment that promotes stem cell maintenance while allowing differentiation of some daughter cells as replacements for cells in the tissue they serve (Fig. 28-43b). Hematopoietic stem cells in the bone marrow occupy a niche in which signaling from neighboring cells and other cues maintain the stem cell lineage. At the same time, some daughter cells differentiate to provide needed blood cells. Understanding the niche in which stem cells operate, and the signals the niche provides, is essential in efforts to harness the potential of stem cells for tissue regeneration. The identification and culturing of pluripotent stem cells from human blastocysts was reported by James Thomson and colleagues in 1998. This advance led to the long-term availability of established cell lines for research.

All stem cells present problems for human medical applications. Adult stem cells have a limited capacity to regenerate tissues, are generally present in small numbers, and are hard to isolate from an adult human. Embryonic stem cells have much greater differentiation potential and can be cultured to generate large numbers of cells, but their use is accompanied by ethical concerns related to the necessary destruction of human embryos. Identifying a source of plentiful and medically useful stem cells that does not raise such concerns remains a major goal of medical research.

Our ability to culture stem cells (i.e., maintain them in an undifferentiated state), and to manipulate them to grow and differentiate into particular tissues, is very much a function of our understanding of developmental biology.

Thus far, mouse and human embryonic stem cells have been used for most research. Although both types of stem cells are pluripotent, they require very different culture conditions, optimized to allow cell division indefinitely without differentiation. Mouse embryonic stem cells are grown on a layer of gelatin and require the presence of leukemia inhibitory factor (LIF). Human embryonic stem cells are grown on a feeder layer of mouse embryonic fibroblasts and require basic fibroblast growth factor (bFGF, or FGF2). The use of a feeder cell layer implies that the mouse cells are providing a diffusible product or some surface signal, not yet known, that is needed by human stem cells to either promote cell division or prevent differentiation.

A significant advance, reported in 2007, centers on success in reversing differentiation. In effect, skin

BOX 28-1

Of Fins, Wings, Beaks, and Things

South America has several species of seed-eating finches, commonly called grassquits. About 3 million years ago, a small group of grassquits, of a single species, took flight from the continent's Pacific coast. Perhaps driven by a storm, they lost sight of land and traveled nearly 1,000 km. Small birds such as these might easily have perished on such a journey, but the smallest of chances brought this group to a newly formed volcanic island in an archipelago later to be known as the Galápagos. It was a virgin landscape with untapped plant and insect food sources, and the newly arrived finches survived. Over the years, new islands formed and were colonized by new plants and insects — and by the finches. The birds exploited the new resources on the islands, and groups of birds gradually specialized and diverged into new species. By the time Charles Darwin stepped onto the islands in 1835, many different finch species were to be found on the various islands of the archipelago, feeding on seeds, fruits, insects, pollen, or even blood.

The diversity of living creatures was a source of wonder for humans long before scientists sought to understand its origins. The extraordinary insight handed down to us by Darwin, inspired in part by his encounter with the Galápagos finches, provided a broad explanation for the existence of organisms with a vast array of appearances and characteristics. It also gave rise to many questions about the mechanisms underlying evolution. Answers to those questions have started to appear, first through the study of genomes and nucleic acid metabolism in the last half of the twentieth century, and more recently through an emerging field nicknamed evo-devo — a blend of evolutionary and developmental biology.

In its modern synthesis, the theory of evolution has two main elements: mutations in a population generate genetic diversity; natural selection then acts on this diversity to favor individuals with more useful genomic tools and to disfavor others. Mutations occur at significant rates in every individual's genome, in every cell (see Section 8.3). Advantageous mutations in single-celled organisms or in the germ line of multicellular organisms can be inherited, and they are more likely to be inherited (that is, passed on to greater numbers of offspring) if they confer an advantage. It is a straightforward scheme. But many have wondered whether it is enough to explain, say, the many different beak shapes in the Galápagos finches or the diversity of size and shape among mammals. Until recent decades, there were several widely held assumptions about the evolutionary process: that many mutations and new genes would be needed to bring about a new physical structure, that more-complex organisms would have larger genomes, and that very different species would have few genes in common. All of these assumptions were wrong.

Modern genomics has revealed that the human genome contains fewer genes than expected — not many more than the fruit fly genome and fewer than some amphibian genomes. The genomes of every mammal, from mouse to human, are surprisingly similar in the number, types, and chromosomal arrangement of genes. Meanwhile, evo-devo is telling us how complex and very different creatures can evolve within these genomic realities.

In the late nineteenth century, English biologist William Bateson studied animals with homeotic mutations — creatures with body parts growing in the wrong location. Bateson used his observations to challenge the Darwinian notion that evolutionary change would have to be gradual. Recent studies of the genes that control organismal development have put an exclamation point on Bateson's ideas. Subtle changes in regulatory patterns during development, reflecting just one or a few mutations, can result in startling physical changes and fuel surprisingly rapid evolution.

The Galápagos finches provide a wonderful example of the link between evolution and development. There are at least 14 (some specialists list 15) species of Galápagos finches, distinguished in large measure by their beak structure. The ground finches, for example, have broad, heavy beaks adapted to crushing large, hard seeds. The cactus finches have longer, slender beaks ideal for probing cactus fruits and flowers (Fig. 1). Clifford Tabin and colleagues carefully surveyed a set of genes expressed during avian craniofacial development. They identified a single gene, *Bmp4*, whose expression level correlated with formation of the more robust beaks of

cells — first from mice, then from humans — have been reprogrammed to take on the characteristics of pluripotent stem cells. The reprogramming involves manipulations to get the cells to express at least four transcription factors, Oct4, Sox2, Nanog, and Lin28, all of which are known to help maintain the stem cell–like state. Gradual improvements in this technology may make the harvesting of embryonic stem cells unnecessary and provide a source of stem cells that is genetically matched to a prospective patient.

Our discussion of developmental regulation and stem cells brings us full circle, back to a biochemical beginning. Evolution appropriately provides the first and last words of this book. If evolution is to generate the kind of changes in an organism that would render it a different species, it is the developmental program that must be affected. Developmental and evolutionary processes are closely allied, each informing the other (**Box 28-1**). The continuing study of biochemistry has everything to do with enriching the future of humanity and understanding our origins.

the ground finches. More-robust beaks were also formed in chicken embryos when high levels of Bmp4 were artificially expressed in the appropriate tissues, confirming the importance of *Bmp4*. In a similar study, the formation of long, slender beaks was linked to the expression of calmodulin (see Fig. 12-17) in particular tissues at appropriate developmental stages. Thus, major changes in the shape and function of the beak can be brought about by subtle changes in the expression of just two genes involved in developmental regulation. Very few mutations are required, and the needed mutations affect regulation. New genes are *not* required.

The system of regulatory genes that guides development is remarkably conserved among all vertebrates. Elevated expression of *Bmp4* in the right tissue at the right time leads to more-robust jaw parts in zebrafish. The same gene plays a key role in tooth development in mammals. The development of eyes is triggered by the expression of a single gene, *Pax6*, in fruit flies and in mammals. The mouse *Pax6* gene will trigger the development of fruit fly eyes in the fruit fly, and the fruit fly *Pax6* gene will trigger the development of mouse eyes in the mouse. In each organism, these genes are part of the much larger regulatory cascade that ultimately creates the correct structures in the correct locations in each organism. The cascade is ancient; for example, the *Hox* genes (described in the text) have been part of the developmental program of multicellular eukaryotes for more than 500 million years. Subtle changes in the cascade can have large effects on development, and thus on the ultimate appearance, of the organism. These same subtle changes can fuel remarkably rapid evolution. For example, the 400 to 500 described species of cichlids (spiny-finned fish) in Lake Malawi and Lake Victoria on the African continent are all derived from one or a few populations that colonized each lake in the past 100,000 to 200,000 years. The Galápagos finches simply followed a path of evolution and change that living creatures have been traveling for billions of years.

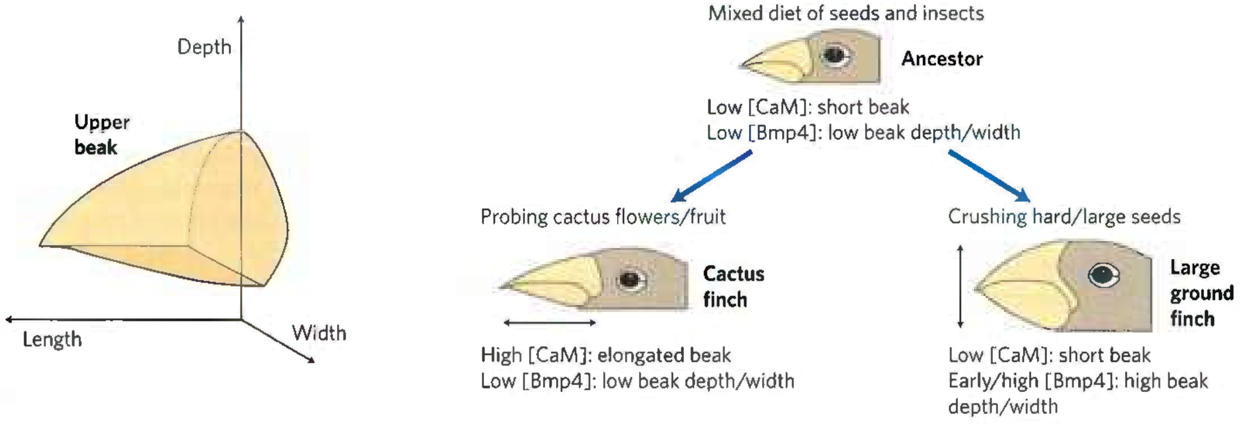

Depth
Upper beak
Length
Width
Mixed diet of seeds and insects
Ancestor
Low [CaM]: short beak
Low [Bmp4]: low beak depth/width
Probing cactus flowers/fruit
Cactus finch
High [CaM]: elongated beak
Low [Bmp4]: low beak depth/width
Crushing hard/large seeds
Large ground finch
Low [CaM]: short beak
Early/high [Bmp4]: high beak depth/width

FIGURE 1 Evolution of new beak structures to exploit new food sources. In the Galápagos finches, the different beak structures of the cactus finch and the large ground finch, which feed on different, specialized food sources, were produced to a large extent by a few mutations that altered the timing and level of expression of just two genes: those encoding calmodulin (CaM) and Bmp4. [Information from A. Abzhanov et al., *Nature* 442:563, 2006, Fig. 4.]

SUMMARY 28.3 *Regulation of Gene Expression in Eukaryotes*

- In eukaryotes, large changes in chromatin structure accompany the expression of a gene. Transcriptionally inactive heterochromatin is opened up by chromatin remodeling proteins. These eject, replace, or modify nucleosomes to allow other proteins, mainly RNA polymerase components and regulators, to access sites required to initiate transcription.
- In eukaryotes, positive regulation is more common than negative regulation.
- Promoters for Pol II typically have a TATA box and Inr sequence, as well as multiple binding sites for transcription activators. The latter sites, sometimes located hundreds or thousands of base pairs away from the TATA box, are called upstream activator sequences in yeast and enhancers in higher eukaryotes. To regulate transcriptional activity generally requires large complexes

of proteins. These include basal transcription factors, activators, coactivators, architectural regulators, and the enzymes that modify and remodel chromatin. The effects of transcription activators on Pol II are facilitated by coactivator protein complexes such as Mediator.

- The well-studied yeast genes involved in galactose metabolism provide examples of both positive and negative regulation in a eukaryote.
- The modular structures of the activators have distinct activation and DNA-binding domains.
- Hormones affect the regulation of gene expression in one of two ways. Steroid hormones interact directly with intracellular receptors that are DNA-binding regulatory proteins; binding of the hormone has either positive or negative effects on the transcription of targeted genes.
- Nonsteroid hormones bind to cell surface receptors, triggering a signaling pathway that can lead to phosphorylation of a regulatory protein, affecting its activity.
- Translational regulation is particularly important in eukaryotes. Modulating the translation of an mRNA stored in the cytoplasm affords a more rapid response to cellular challenges than de novo assembly of transcription complexes and mRNA synthesis.
- MicroRNAs (miRNAs) are involved in gene silencing during development and as an antiviral defense. The pathway for processing miRNAs from larger precursors has been harnessed by researchers to develop the gene-silencing technology called RNA interference, or RNAi.
- Regulation mediated by ncRNAs plays an important role in eukaryotic gene expression, with known mechanisms including interactions with proteins, mRNA, and other ncRNAs.
- Development of a multicellular organism presents the most complex regulatory challenge. The fate of cells in the early embryo is determined by establishment of anterior-posterior and dorsal-ventral gradients of proteins that act as transcription activators or translational repressors, regulating the genes required for development of structures appropriate to a particular part of the organism. Sets of regulatory genes operate in temporal and spatial succession, transforming given areas of an egg cell into predictable structures in the adult organism.
- The differentiation of stem cells into functional tissues can be controlled by extracellular signals and conditions.

KEY TERMS

Terms in bold are defined in the glossary.

induction	1054
repression	1054
housekeeping genes	1056
specificity factor	1056
repressor	1056
activator	1056
noncoding RNA (ncRNA)	1056
long noncoding RNA (lncRNA)	1056
operator	1057
negative regulation	1057
positive regulation	1057
architectural regulator	1057
operon	1058
helix-turn-helix	1061
zinc finger	1061
homeodomain	1062
homeobox	1062
RNA recognition motif (RRM)	1062
leucine zipper	1063
basic helix-loop-helix	1064
combinatorial control	1065
cAMP receptor protein (CRP)	1066
regulon	1067
transcription attenuation	1068
translational repressor	1070
stringent response	1071
riboswitch	1072
phase variation	1073
chromatin remodeling	1075
SWI/SNF	1075
histone acetyltransferases (HATs)	1076
enhancers	1078
upstream activator sequences (UASs)	1078
transcription activators	1078
coactivators	1078
basal transcription factors	1078
preinitiation complex (PIC)	1078
high mobility group (HMG)	1079
Mediator	1079
TATA-binding protein (TBP)	1080
hormone response element (HRE)	1083
RNA interference (RNAi)	1086
polarity	1087
metamerism	1087
maternal genes	1087
maternal mRNAs	1087
segmentation genes	1088
gap genes	1088
pair-rule genes	1088
segment polarity genes	1088
homeotic genes	1088
totipotent	1090
pluripotent	1091
unipotent	1091
embryonic stem cells	1091

PROBLEMS

1. Effect of mRNA and Protein Stability on Regulation *E. coli* cells are growing in a medium with glucose as the sole carbon source. After the sudden addition of tryptophan, the cells continue to grow and divide every 30 min. Describe (qualitatively) how the amount of tryptophan synthase activity in the cells changes with time under each condition:

(a) The *trp* mRNA is stable (degrades slowly over many hours).

(b) The *trp* mRNA degrades rapidly, but tryptophan synthase is stable.

(c) The *trp* mRNA and tryptophan synthase both degrade rapidly.

2. The Lactose Operon A researcher engineers a *lac* operon on a plasmid but inactivates all parts of the *lac* operator (*lacO*) and the *lac* promoter, replacing them with the binding site for the LexA repressor (which acts in the SOS response) and a promoter regulated by LexA. She then introduces the plasmid into *E. coli* cells that have a *lac* operon with an inactive *lacZ* gene. Under what conditions will these transformed cells produce β-galactosidase?

3. Negative Regulation Describe the probable effects on gene expression in the *lac* operon of each mutation:

(a) Mutation in the *lac* operator that deletes most of O_1

(b) Mutation in the *lacI* gene that eliminates binding of repressor to operator

(c) Mutation in the promoter near position –10 that increases its similarity to the *E. coli* consensus sequence
(d) Mutation in the *lacI* gene that eliminates binding of repressor to lactose
(e) Mutation in the promoter near position –10 that decreases its similarity to the *E. coli* consensus sequence

4. Specific DNA Binding by Regulatory Proteins A typical bacterial repressor protein discriminates between its specific DNA-binding site (operator) and nonspecific DNA by a factor of 10^4 to 10^6. About 10 molecules of repressor per cell are sufficient to ensure a high level of repression. Assume that a very similar repressor existed in a human cell, with a similar specificity for its binding site. How many copies of the repressor would a human cell require to elicit a level of repression similar to that in the bacterial cell? (Hint: The *E. coli* genome contains about 4.6 million bp; the human haploid genome has about 3.2 billion bp.)

5. Repressor Concentration in *E. coli* The dissociation constant for a particular repressor-operator complex is very low, about 10^{-13} M. An *E. coli* cell (volume 2×10^{-12} mL) contains 10 copies of the repressor. Calculate the cellular concentration of the repressor protein. How does this value compare with the dissociation constant of the repressor-operator complex? What is the significance of this answer?

6. Catabolite Repression *E. coli* cells are growing in a medium that contains lactose but no glucose. Indicate whether each of the following changes or conditions would increase, decrease, or not change the expression of the *lac* operon. It may be helpful to draw a model depicting what is happening in each situation.
(a) Addition of a high concentration of glucose
(b) A mutation that prevents dissociation of the Lac repressor from the operator
(c) A mutation that completely inactivates β-galactosidase
(d) A mutation that completely inactivates galactoside permease
(e) A mutation that prevents binding of CRP to its binding site near the *lac* promoter

7. Transcription Attenuation How would each manipulation of the leader region of the *trp* mRNA affect transcription of the *E. coli trp* operon?
(a) Increasing the distance (number of bases) between the leader peptide gene and sequence 2
(b) Increasing the distance between sequences 2 and 3
(c) Removing sequence 4
(d) Changing the two Trp codons in the leader peptide gene to His codons
(e) Eliminating the ribosome-binding site for the gene that encodes the leader peptide
(f) Changing several nucleotides in sequence 3 so that it can base-pair with sequence 4 but not with sequence 2

8. Repressors and Repression How would a mutation in the *lexA* gene that prevents autocatalytic cleavage of the LexA protein affect the SOS response in *E. coli*?

9. Regulation by Recombination In the phase variation system of *Salmonella*, what would happen to the cell if the Hin recombinase became more active and promoted recombination (DNA inversion) several times in each cell generation?

10. Initiation of Transcription in Eukaryotes A biochemist discovers a new RNA polymerase activity in crude extracts of cells derived from an exotic fungus. The RNA polymerase initiates transcription only from a single, highly specialized promoter. As the biochemist purifies the polymerase, its activity declines, and the purified enzyme is completely inactive unless he adds crude extract to the reaction mixture. Suggest an explanation for these observations.

11. Functional Domains in Regulatory Proteins A biochemist replaces the DNA-binding domain of the yeast Gal4 protein with the DNA-binding domain from the Lac repressor and finds that the engineered protein no longer regulates transcription of the *GAL* genes in yeast. Draw a diagram of the different functional domains you would expect to find in the Gal4 protein and in the engineered protein. Why does the engineered protein no longer regulate transcription of the *GAL* genes? What might be done to the DNA-binding site recognized by this chimeric protein to make it functional in activating transcription of *GAL* genes?

12. Nucleosome Modification during Transcriptional Activation To prepare genomic regions for transcription, cells acetylate and methylate certain histones in the resident nucleosomes at specific locations. Once transcription is no longer needed, cells need to reverse these modifications. In mammals, peptidylarginine deiminases (PADIs) reverse the methylation of Arg residues in histones. The reaction promoted by these enzymes does not yield unmethylated arginine. Instead, it produces citrulline residues in the histone. What is the other product of the reaction? Suggest a mechanism for this reaction.

13. Gene Repression in Eukaryotes Explain why repression of a eukaryotic gene by an RNA might be more efficient than repression by a protein repressor.

14. Inheritance Mechanisms in Development A *Drosophila* egg that is *bcd*$^-$/*bcd*$^-$ may develop normally, but the adult fruit fly will not be able to produce viable offspring. Explain.

DATA ANALYSIS PROBLEM

15. Engineering a Genetic Toggle Switch in *E. coli* Gene regulation is often described as an "on or off" phenomenon: a gene is either fully expressed or not expressed at all. In fact, repression and activation of a gene involve ligand-binding reactions, so genes can show intermediate levels of expression when intermediate levels of regulatory molecules are present. For example, for the *E. coli lac* operon, consider the binding equilibrium of the Lac repressor, operator DNA, and inducer (see Fig. 28-8). Although this is a complex, cooperative process, it can be approximately modeled by the following reaction (R is repressor; IPTG is the inducer isopropyl-β-D-thiogalactoside):

$$\mathrm{R} + \mathrm{IPTG} \xrightleftharpoons{K_\mathrm{d} = 10^{-4}\,\mathrm{M}} \mathrm{R \cdot IPTG}$$

Free repressor, R, binds to the operator and prevents transcription of the *lac* operon; the R • IPTG complex does not bind to the operator, and thus transcription of the *lac* operon can proceed.
(a) Using Equation 5-8, we can calculate the relative expression level of the proteins of the *lac* operon as a function of [IPTG]. Use this calculation to determine over what range of [IPTG] the expression level would vary from 10% to 90%.
(b) Describe qualitatively the level of *lac* operon proteins present in an *E. coli* cell before, during, and after induction with IPTG. You do not need to give the amounts at exact times—just indicate the general trends.

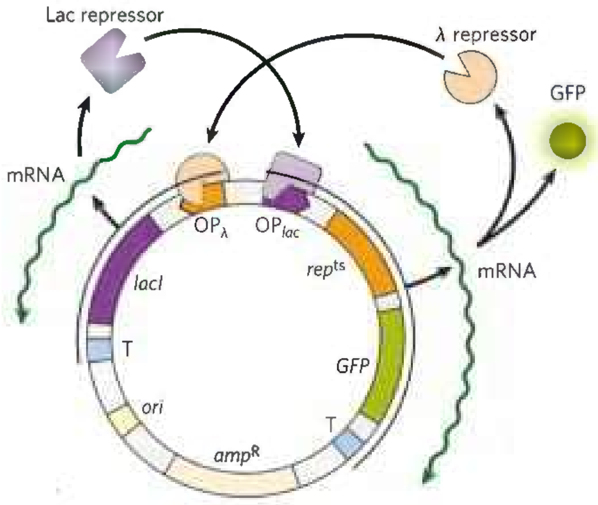
Gardner, Cantor, and Collins (2000) set out to make a "genetic toggle switch"—a gene-regulatory system with two key characteristics, A and B, of a light switch. (A) *It has only two states:* it is either fully on or fully off; it is not a dimmer switch. In biochemical terms, the target gene or gene system (operon) is either fully expressed or not expressed at all; it cannot be expressed at an intermediate level. (B) *Both states are stable:* although you must use a finger to flip the light switch from one state to the other, once you have flipped it and removed your finger, the switch stays in that state. In biochemical terms, exposure to an inducer or some other signal changes the expression state of the gene or operon, and it remains in that state once the signal is removed.

(c) Explain how the *lac* operon lacks both characteristics A and B.

To make their "toggle switch," Gardner and coworkers constructed a plasmid from the following components:

ori	An origin of replication
amp^R	A gene conferring resistance to the antibiotic ampicillin
OP_{lac}	The operator-promoter region of the *E. coli lac* operon
OP_λ	The operator-promoter region of λ phage
lacI	The gene encoding the *lac* repressor protein, LacI. In the absence of IPTG, this protein strongly represses OP_{lac}; in the presence of IPTG, it allows full expression from OP_{lac}.
rep^{ts}	The gene encoding a temperature-sensitive mutant λ repressor protein, rep^{ts}. At 37 °C, this protein strongly represses OP_λ; at 42 °C, it allows full expression from OP_λ.
GFP	The gene for green fluorescent protein (GFP), a highly fluorescent reporter protein (see Fig. 9-16)
T	Transcription terminator

The investigators arranged these components, as shown in the following figure, so that the two promoters were reciprocally repressed: OP_{lac} controlled expression of rep^{ts}, and OP_λ controlled expression of *lacI*. The state of this system was reported by the expression level of *GFP*, which was also under the control of OP_{lac}.



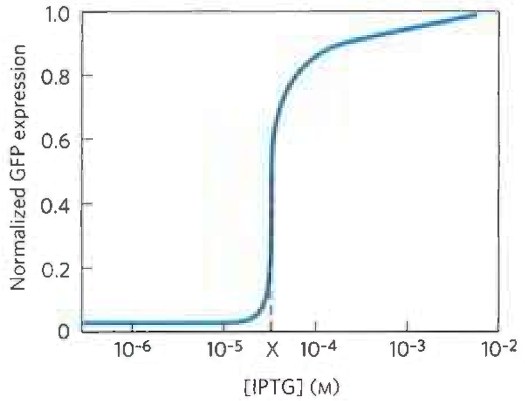
(d) The constructed system has two states: GFP-on (high level of expression) and GFP-off (low level of expression). For each state, describe which proteins are present and which promoters are being expressed.

(e) Treatment with IPTG would be expected to toggle the system from one state to the other. From which state to which? Explain your reasoning.

(f) Treatment with heat (42 °C) would be expected to toggle the system from one state to the other. From which state to which? Explain your reasoning.

(g) Why would this plasmid be expected to have characteristics A and B as described above?

To confirm that their construct did indeed exhibit these characteristics, Gardner and colleagues first showed that, once switched, the GFP expression level (high or low) was stable for long periods of time (characteristic B). Next, they measured the GFP level at different concentrations of the inducer IPTG, with the following results.



They noticed that the average GFP expression level was intermediate at concentration X of IPTG. However, when they measured the GFP expression level *in individual cells* at [IPTG] = X, they found either a high level or a low level of GFP—no cells showed an intermediate level.

(h) Explain how this finding demonstrates that the system has characteristic A. What is happening to cause the bimodal distribution of expression levels at [IPTG] = X?

Reference

Gardner, T.S., C.R. Cantor, and J.J. Collins. 2000. Construction of a genetic toggle switch in *Escherichia coli. Nature* 403:339–342.

Abbreviated Solutions to Problems

Fuller solutions to all chapter problems are published in the *Absolute Ultimate Guide to Lehninger Principles of Biochemistry.* For all numerical problems, answers are expressed with the correct number of significant figures.

Chapter 1

1. (a) Diameter of magnified cell = 500 mm **(b)** 36,000 mitochondria **(c)** 3.9×10^{10} glucose molecules

2. (a) 10% **(b)** 5% **(c)** 1.6 mm; 800 times longer than the cell; DNA must be tightly coiled

3. Collect the supernatant from the high-speed centrifugation and centrifuge at a very high speed (150,000 *g*) for 3 hours. The ribosomes will be in the pellet.

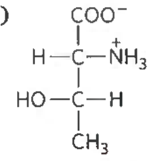
4. (a) Metabolic rate is limited by diffusion, which is limited by surface area. **(b)** 12 μm^{-1} for the bacterium **(c)** Surface-to-volume ratio 300 times higher in the bacterium.

5. 2×10^6 s (about 23 days)

6. The vitamin molecules from the two sources are identical; the body cannot distinguish the source; only associated impurities might vary with the source.

7. (a) COO^-, carboxyl; NH_3, amino; OH, hydroxyl; CH_3, methyl

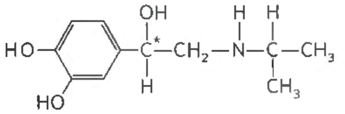
(b)



D-Threonine

(c) 2

8.



The two enantiomers have different interactions with a chiral biological "receptor" (a protein).

9. (a) Fatty acids are more nonpolar than amino acids, which makes them separable on the basis of solubility. The larger size and longer shape of fatty acids could allow separation by some types of chromatography. **(b)** The charge on the phosphate groups of nucleotides could be used to separate them from glucose. The larger size and shape could allow separation by some types of chromatography.

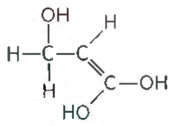
10. Carbon atoms can form linear chains, branched chains, and cyclic structures. It is improbable that silicon could serve as the central organizing element for life, especially in an O_2-containing atmosphere such as that of Earth. Long chains of silicon atoms are not readily synthesized; the polymeric macromolecules necessary for more complex functions would not readily form. Oxygen disrupts bonds between silicon atoms, and silicon-oxygen bonds are extremely stable and difficult to break, preventing the breaking and making of bonds that are essential to life processes.

11. (a) (*R*)-enantiomer: A is COO^-; B is H; C is CH_3. (*S*)-enantiomer: A is CH_3; B is H; C is COO^-. **(b)** It is unnecessary to make enantiomerically pure (*S*)-ibuprofen available because the isomerase converts the less effective enantiomer to the effective enantiomer, but does not catalyze the reverse reaction.

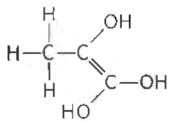
12. (a) 3 Phosphoric acid groups; α-D-ribose; guanine **(b)** Tyrosine; 2 glycines; phenylalanine; methionine **(c)** Choline; phosphoric acid; glycerol; oleic acid; palmitic acid

13. (a) CH_2O; $C_3H_6O_3$

(b)



1



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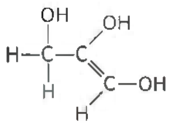

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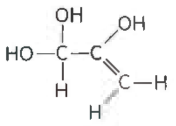

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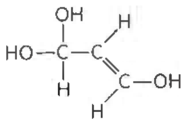

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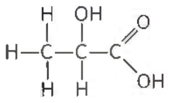

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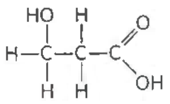

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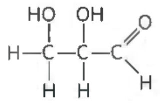

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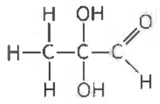

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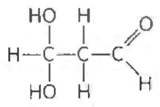

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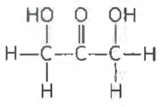

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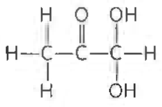
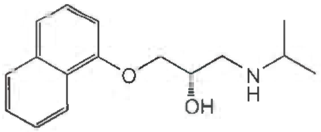


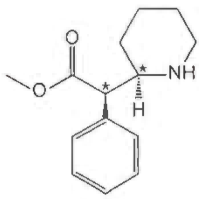
12

(c) X contains a chiral center; eliminates all but **6** and **8**. **(d)** X contains an acidic functional group; eliminates **8**; structure **6** is consistent with all data. **(e)** Structure **6**; we cannot distinguish between the two possible enantiomers.

14. (a) The carbon bearing the hydroxyl group is the chiral carbon. **(b)** The structure shows the (*R*) isomer of propranolol. **(c)** (*S*)-Propranolol has this structure:



15. (a) The chiral carbons are indicated with asterisks. **(b)** The structure shows the (*S,S*) isomer of methylphenidate. **(c)** (*R,R*)-methylphenidate has this structure:



16. Spores are alive because they can transition from a state of being metabolically inert under harsh environmental conditions to an actively growing state when conditions improve. During this transition, spores take in water, which is essential to many biochemical reactions. Germination does not seem to depend on ATP.

17. **(a)** $\Delta G°$ is negative and relatively large. **(b)** There is not enough thermal energy in the wood to overcome the activation energy. **(c)** A match supplies thermal energy to overcome the activation energy barrier. **(d)** The enzyme lowers the activation energy enough for the reaction to occur at room temperature.

18. If this mutation occurs in a coding sequence, it may cause an amino acid substitution during protein synthesis. The consequence to the cell could be anywhere on the spectrum from beneficial, to neutral, to fatal.

19. The resulting protein may fold incorrectly and may not attain its native conformation. Alternatively, the shape of the region that binds with its partner may change, preventing complementary fit. A mutation that malforms an enzyme's active site can destroy the enzyme's catalytic activity.

20. **(a)** The copy begins with already-formed domains with biological activity, so it does not have to evolve from scratch. The copy can undergo mutation without harm to the cell, since the original gene encodes the original product. Alternatively, the copy could undergo mutations that create a lethal product, even though the original gene remains intact. The daughter cell's newly acquired function may be positive or negative. **(b)** Each time a duplicate gene undergoes mutation is an opportunity for the cell to acquire a new, positive trait without risking the loss of function provided by the original gene, which may remain unchanged.

21. Yes, when environmental conditions improve, such as temperature moderation and the availability of water, the animal can rehydrate and recover. That is, biochemical mechanisms exist for restoring the normal state.

22. Mutations may have made DNA repair more efficient. Resistant cells may have developed or increased the ability to synthesize a compound that destroys free radicals. Gene duplication may have provided backup for genes damaged by radiation.

23. **(a)** The enzyme that catalyzes the reaction is a membrane-bound enzyme because the preparation containing only membranes had the largest amount of labeled product. **(b)** The enzyme generated the largest amount of product at pH 7. **(c)** Enzyme activity is slightly higher at pH 8 than at pH 6. **(d)** Magnesium (Mg^{2+}) is effective at activating the system over a range of concentrations. At low concentrations, manganese (Mn^{2+}) is effective at activating the system, but it may inhibit the reaction at higher concentrations. The system shows minimal response to calcium ions (Ca^{2+}). **(e)** The reaction requires CTP, but not GTP, UTP, or ATP. However, the addition of ATP may increase the rate of lecithin synthesis. The ATP from lot 116 was likely contaminated with CTP. **(f)** Phosphocholine + membrane-bound enzyme + Mg^{2+} + CTP $\rightleftharpoons$ lecithin

Chapter 2

1. Weaker; ionic attractive force is proportional to the *inverse* of the dielectric constant, and a hydrophobic "solvent" such as the environment inside the protein has a lower dielectric constant than a polar solvent such as water.

2. Biomolecular interactions generally need to be reversible; weak interactions allow reversibility.

3. Ethanol is polar; ethane is not. The ethanol —OH group can hydrogen-bond with water.

4. **(a)** 4.76 **(b)** 9.19 **(c)** 4.0 **(d)** 4.82

5. **(a)** 1.51×10^{-4} M **(b)** 3.02×10^{-7} M **(c)** 7.76×10^{-12} M

6. 1.1

7. **(a)** $HCl \rightleftharpoons H^+ + Cl^-$ **(b)** 3.3 **(c)** $NaOH \rightleftharpoons Na^+ + OH^-$ **(d)** 9.8

8. 1.1

9. 1.7 nmol of acetylcholine

10. 0.1 M hydrofluoric acid

11. **(a)** strong **(b)** weak **(c)** strong **(d)** strong **(e)** weak **(f)** weak

12. 3.3 mL

13. **(a)** $H_2PO_4^-$ **(b)** HCO_3^- **(c)** CH_3COO^- **(d)** CH_3NH_2

14. **(a)** 5.06 **(b)** 4.28 **(c)** 5.46 **(d)** 4.76 **(e)** 3.76

15. quinoline ion: 0.1 M HCl; *m*-cresol: 0.1 M NaOH; 2-(methylthio)pyridine ion: 0.1 M HCl

16. **(d)** Bicarbonate, a weak base, titrates —OH to —O$^-$, making the compound more polar and more water-soluble.

17. Stomach; the neutral form of aspirin present at the lower pH is less polar and passes through the membrane more easily.

18. 8.8

19. 7.4

20. **(a)** pH 8.6 to 10.6 **(b)** 4/5 **(c)** 10 mL **(d)** pH = $pK_a - 2$

21. 8.9

22. 2.4

23. 6.9

24. 1.4

25. $NaH_2PO_4 \cdot H_2O$, 5.8 g/L; Na_2HPO_4, 8.2 g/L

26. $[A^-]/[HA] = 0.10$

27. Mix 150 mL of 0.10 M sodium acetate and 850 mL of 0.10 M acetic acid.

28. **(a)** pH 3 **(b)** pH 5 **(c)** pH 9 **(d)** pH 9 **(e)** pH 3 **(f)** pH 5. The total buffering region spans approximately 1 pH unit on either side of the pK_a value.

29. **(a)** 4.6 **(b)** 0.1 pH unit **(c)** 4 pH units

30. 4.3

31. 0.13 M acetate and 0.07 M acetic acid

32. 1.8

33. 7

34. **(a)** fully protonated **(b)** zwitterionic **(c)** zwitterionic **(d)** zwitterionic **(e)** fully deprotonated. When the pH is lower than both pK_a values, both the α-amino group and the α-carboxyl group are protonated. When the pH is greater than both pK_a values, neither the α-amino group nor the α-carboxyl group is protonated. When the pH is between the two pK_a values, the α-amino group is protonated, whereas the α-carboxyl group is unprotonated.

35. **(a)** Blood pH is controlled by the carbon dioxide–bicarbonate buffer system, $CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$. During *hypoventilation*, pCO_2 increases in the air space of the lungs and arterial blood, driving the equilibrium to the right, raising $[H^+]$ and lowering blood pH. **(b)** During *hyperventilation*, $[CO_2]$ decreases in the lungs and arterial blood, reducing $[H^+]$ and increasing pH above the normal 7.4 value. **(c)** Lactate is a moderately strong acid, completely dissociating under physiological conditions and thus lowering the pH of blood and muscle tissue. Hyperventilation removes H^+, raising the pH of blood and tissues in anticipation of the acid buildup.

36. 7.4

37. Dissolving more CO_2 in the blood increases $[H^+]$ in blood and extracellular fluids, lowering pH: $CO_2(aq) + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$

38. **(a)** $CH_3—OH < CH_3—CH_2—OH < HO—CH_2CH_2—OH < HO—CH_2CH_2CH_2—OH$ **(b)** number of hydrogen bonds that the compound can form and length of the carbon chain

39. The average bond duration decreases.

40. **(a)** H_2S forms hydrogen bonds with H_2O but not with itself. **(b)** H_2S has a lower boiling point than H_2O. **(c)** No, H_2S is a less polar solvent than H_2O.

41. sodium acetate > sodium propionate > glycine > L-phenylalanine > sodium octanoate

42. $CH_3—(CHOH)—CH_2—CHOH—CH_2—OH > CH_3—(CH_2)_5—OH > CH_3—(CH_2)_{10}—OH$

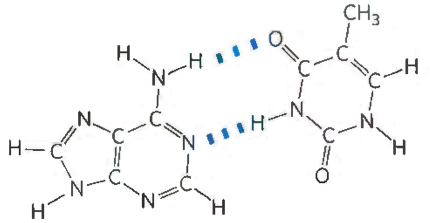
43. (a) HOOC—$(CH_2)_4$—COOH, –2; CH_3—$(CH_2)_4$—COOH, –1; HOOC—$(CH_2)_2$—COOH, –2
(b) HOOC—$(CH_2)_2$—COOH > HOOC—$(CH_2)_4$—COOH > CH_3—$(CH_2)_4$—COOH

44. (a) No, because an effluent of pH 1 would harm the trout and other life in the stream. **(b)** The pH scale runs from very acidic to very alkaline, with the point of neutrality (which is best for living creatures, including trout) midway between 0 and 14. He is proposing to jump out of the frying pan into the fire!

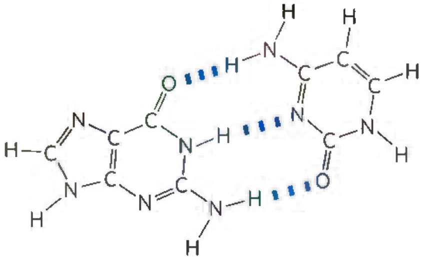
45. pH = 7.6; osmolarity = 0.313 osm/L

46.

(a) Adenine



(b) Guanine



47. (a) Use the substance in its surfactant form to emulsify the spilled oil, collect the emulsified oil, then switch to the nonsurfactant form. The oil and water will separate, and the oil can be collected for further use. **(b)** The equilibrium lies strongly to the right. The stronger acid (lower pK_a), H_2CO_3, donates a proton to the conjugate base of the weaker acid (higher pK_a), amidine. **(c)** The strength of a surfactant depends on the hydrophilicity of its head groups: the more hydrophilic, the more powerful the surfactant. The amidinium form of s-surf is much more hydrophilic than the amidine form, so it is a more powerful surfactant. **(d)** *Point A:* amidinium; the CO_2 has had plenty of time to react with the amidine to produce the amidinium form. *Point B:* amidine; Ar has removed CO_2 from the solution, leaving the amidine form. **(e)** The conductivity rises as uncharged amidine reacts with CO_2 to produce the charged amidinium form. **(f)** The conductivity falls as Ar removes CO_2, shifting the equilibrium to the uncharged amidine form. **(g)** Treat s-surf with CO_2 to produce the surfactant amidinium form, and use this to emulsify the spill. Treat the emulsion with Ar to remove the CO_2 and produce the nonsurfactant amidine form. The oil will separate from the water and can be recovered.

Chapter 3

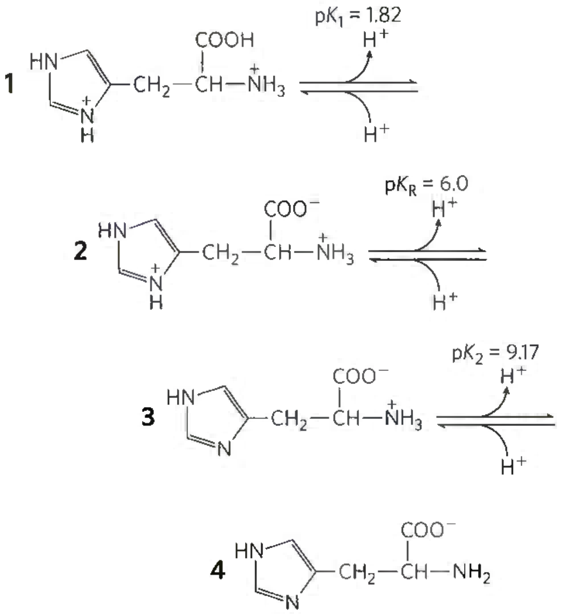
1. The constituents are Glu, Cys, and Gly. The Glu links to the Cys via its γ-carboxyl group.

2. It is L-ornithine, because the amino group occupies the same relative position as the hydroxyl group in L-glyceraldehyde.

3. (a) II **(b)** IV **(c)** I **(d)** III **(e)** II **(f)** II **(g)** IV **(h)** III **(i)** V **(j)** III **(k)** II and IV

4. (a) pI > pK_a of the α-carboxyl group and pI < pK_a of the α-amino group, so both groups are charged (ionized). **(b)** 1 in 2.19×10^7. The pI of alanine is 6.01. From Table 3-1 and the Henderson-Hasselbalch equation, 1 in 4,680 carboxyl groups and 1 in 4,680 amino groups are uncharged. The fraction of alanine molecules with both groups uncharged is 1 in $4{,}680^2$.

5. (a)

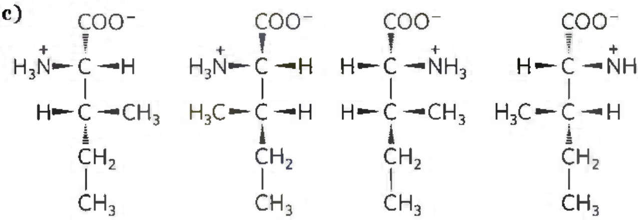


(b), (c)

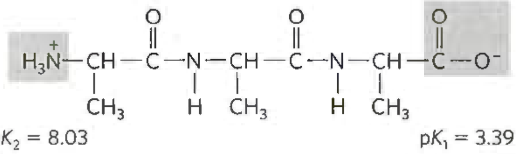
pH	Structure identified in (a)	Net charge	Migrates toward
1	**1**	+2	Cathode
4	**2**	+1	Cathode
8	**3**	0	Does not migrate
12	**4**	–1	Anode

6. (a) Glutamate **(b)** Methionine **(c)** Aspartate **(d)** Glycine **(e)** Serine

7. (a) 2 **(b)** 4
(c)



8. (a) Structure at pH 7:



(b) Electrostatic interaction between the carboxylate anion and the protonated amino group of the alanine zwitterion favorably affects ionization of the carboxyl group. This favorable electrostatic interaction decreases as the length of the poly(Ala) increases, resulting in an increase in pK_1. **(c)** Ionization of the protonated amino group destroys the favorable electrostatic interaction noted in (b). With increasing distance between the charged groups, removal of the proton from the amino group in poly(Ala) becomes easier and thus pK_2 is lower. The intramolecular effects of the amide (peptide bond) linkages keep pK_a values lower than they would be for an alkyl-substituted amine.

9. One H comes from the α-amino group of one amino acid, and an OH is removed from the α-carboxyl group of the amino acid to which the first is joined.

10. 75,000

11. (a) 10,300. The elements of water are lost when a peptide bond forms, so the molecular weight of a Cys residue is not the same as the molecular weight of free cysteine. **(b)** 21

12. The protein has four subunits, with molecular masses of 160, 90, 90, and 60 kDa. The two 90 kDa subunits (possibly identical) are linked by one or more disulfide bonds.

13. (a) at pH 3, +2; at pH 8, 0; at pH 11, –1 **(b)** pI = 7.8

14. Lys, His, Arg; negatively charged phosphate groups in DNA interact with positively charged side groups in histones.

15. (a) $(\text{Glu})_{20}$ **(b)** $(\text{Lys–Val})_3$ **(c)** $(\text{Asn–Ser–His})_5$ **(d)** $(\text{Asn–Ser–His})_5$

16. (a) Specific activity after step 1 is 6.8 units/mg; step 2, 13 units/mg; step 3, 14 units/mg; step 4, 700 units/mg; step 5, 3,500 units/mg; step 6, 5,000 units/mg. **(b)** Step 4 **(c)** Step 3 **(d)** Yes. Specific activity increased only modestly in step 6; SDS polyacrylamide gel electrophoresis.

17. (a) [NaCl] = 0.5 mM **(b)** [NaCl] = 8 μm

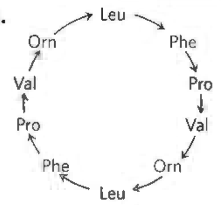
18. B elutes first, A second, C last.

19. The chymotrypsin protein has three distinct polypeptide chains linked by disulfide bonds. They move on the gel as separate species once the disulfide bonds are broken to form the three peptides in lane 2.

20. (a) Amino terminus **(b)** Tyr–Gly–Gly–Phe–Leu

21. Phosphorylation of serine would alter the mass by 80.

22.



The arrows correspond to the orientation of the peptide bonds, —CO → NH—.

23. 75%, 93%. If the efficiency of each amino acid addition is x, then the percentage of full-length peptides with the correct sequence after the addition of seven amino acids will be x^7, as there are *seven* peptide bonds.

24. (a) Y (Tyr) at position 1, F (Phe) at position 7, and R (Arg) at position 9. **(b)** Positions 4 and 9; K (Lys) is more common at 4, R (Arg) is invariant at 9. **(c)** Positions 5 and 10; E (Glu) is more common at both positions. **(d)** Position 2; S (Ser).

25. (a) Anion-exchange chromatography: peptide 2; cation-exchange chromatography: peptide 1; size-exclusion chromatography: peptide 2 **(b)** peptide 3

26. (a) Any linear polypeptide chain has only two kinds of free amino groups: a single α-amino group at the amino terminus, and an ε-amino group on each Lys residue. These amino groups react with FDNB to form a DNP–amino acid derivative. Insulin gave two different α-amino-DNP derivatives, suggesting that it has two amino termini and thus two polypeptide chains—one with an amino-terminal Gly and the other with an amino-terminal Phe. Because the DNP-lysine product is ε-DNP-lysine, the Lys is not at an amino terminus. **(b)** Yes. The A chain has amino-terminal Gly, the B chain has amino-terminal Phe, and (nonterminal) residue 29 in the B chain is Lys. **(c)** Phe–Val–Asp–Glu–. Peptide B1 shows that the amino-terminal residue is Phe. Peptide B2 also includes Val, but since no DNP-Val is formed, Val is not at the amino terminus; it must be on the carboxyl side of Phe. Thus the sequence of B2 is DNP-Phe–Val. Similarly, the sequence of B3 must be DNP-Phe–Val–Asp, and the sequence of the A chain must begin Phe–Val–Asp–Glu–. **(d)** No. The known amino-terminal sequence of the A chain is Phe–Val–Asn–Gln–. The Asn and Gln appear in Sanger's analysis as Asp and Glu because the vigorous hydrolysis in step 7 hydrolyzed the amide bonds in Asn and Gln (as well as the peptide bonds), forming Asp and Glu. Sanger et al. could not distinguish Asp from Asn or Glu from Gln at this stage in their analysis. **(e)** The sequence exactly matches that in Fig. 3-24. Each peptide in the table gives specific information about which Asx residues are Asn or Asp and which Glx residues are Glu or Gln.

Ac1: residues 20–21. This is the only Cys–Asx sequence in the A chain; there is ~1 amido group in this peptide, so it must be Cys–Asn:

N–Gly–Ile–Val–Glx–Glx–Cys–Cys–Ala–Ser–Val–
1 5 10
Cys–Ser–Leu–Tyr–Glx–Leu–Glx–Asx–Tyr–Cys–**Asn**–*C*
15 20

Ap15: residues 14–15–16. This is the only Tyr–Glx–Leu sequence in the A chain; there is ~1 amido group, so the peptide must be Tyr–Gln–Leu:

N–Gly–Ile–Val–Glx–Glx–Cys–Cys–Ala–Ser–Val–
1 5 10
Cys–Ser–Leu–Tyr–**Gln**–Leu–Glx–Asx–Tyr–Cys–Asn–*C*
15 20

Ap14: residues 14–15–16–17. There is ~1 amido group, and we already know that residue 15 is Gln, so residue 17 must be Glu:

N–Gly–Ile–Val–Glx–Glx–Cys–Cys–Ala–Ser–Val–
1 5 10
Cys–Ser–Leu–Tyr–Gln–Leu–**Glu**–Asx–Tyr–Cys–Asn–*C*
15 20

Ap3: residues 18–19–20–21. There are ~2 amido groups, and we know that residue 21 is Asn, so residue 18 must be Asn:

N–Gly–Ile–Val–Glx–Glx–Cys–Cys–Ala–Ser–Val–
1 5 10
Cys–Ser–Leu–Tyr–Gln–Leu–Glu–**Asn**–Tyr–Cys–Asn–*C*
15 20

Ap1: residues 17–18–19–20–21, which is consistent with residues 18 and 21 being Asn.

Ap5pa1: residues 1–2–3–4. There are ~0 amido groups, so residue 4 must be Glu:

N–Gly–Ile–Val–**Glu**–Glx–Cys–Cys–Ala–Ser–Val–
1 5 10
Cys–Ser–Leu–Tyr–Gln–Leu–Glu–Asn–Tyr–Cys–Asn–*C*
15 20

Ap5: residues 1 through 13. There is ~1 amido group, and we know that residue 4 is Glu, so residue 5 must be Gln:

N–Gly–Ile–Val–Glu–**Gln**–Cys–Cys–Ala–Ser–Val–
1 5 10
Cys–Ser–Leu–Tyr–Gln–Leu–Glu–Asn–Tyr–Cys–Asn–*C*
15 20

Chapter 4

1. (a) Shorter bonds have a higher bond order (are multiple rather than single) and are stronger. The peptide C—N bond is stronger than a single bond and is midway between a single bond and a double bond in character. **(b)** Rotation about the peptide bond is difficult at physiological temperatures because of its partial double-bond character.

2. (a) The principal structural units in the wool fiber polypeptide (α-keratin) are successive turns of the α helix, at 5.4 Å intervals; coiled coils produce the 5.2 Å spacing. Steaming and stretching the fiber yields an extended polypeptide chain with the β conformation, with a distance between adjacent R groups of about

7.0 Å. As the polypeptide reassumes an α-helical structure, the fiber shortens. **(b)** Wool shrinks in the presence of moist heat, as polypeptide chains are converted from an extended β conformation to the native α-helix conformation. The structure of silk—β sheets, with their small, closely packed amino acid side chains—is more stable than that of wool.

3. ~42 peptide bonds per second

4. At pH > 6, the carboxyl groups of poly(Glu) are deprotonated; repulsion among negatively charged carboxylate groups leads to unfolding. Similarly, at pH 7, the amino groups of poly(Lys) are protonated; repulsion among these positively charged groups also leads to unfolding.

5. (a) Disulfide bonds are covalent bonds, which are much stronger than the noncovalent interactions that stabilize most proteins. They cross-link protein chains, increasing their stiffness, mechanical strength, and hardness. **(b)** Cystine residues (disulfide bonds) prevent the complete unfolding of the protein.

6. ϕ = (f) and ψ = (e)

7. (a) Bends are most likely at residues 7 and 19; Pro residues in the cis configuration accommodate turns well. **(b)** The Cys residues at positions 13 and 24 can form disulfide bonds. **(c)** External surface: polar and charged residues (Asp, Gln, Lys); interior: nonpolar and aliphatic residues (Ala, Ile); Thr, though polar, has a hydropathy index near zero and thus can be found either on the external surface or in the interior of the protein.

8. (a) At pH 6.0, the amino acid residues are in the correct protonation state to form an ion pair. At pH 2.0, both Asp and His are predominantly protonated, and at pH 10.0, they are both predominantly deprotonated. **(b)** Burial of a charged amino acid residue will destabilize the protein and shift the thermal denaturation curve to lower temperatures. **(c)** Lesser. At pH 10.0, a greater fraction of the Lys side chain will be deprotonated and uncharged, facilitating its burial in a hydrophobic environment.

9. 30 amino acid residues; 0.87

10. For many proteins, the amino acid sequence dictates the formation of a unique, folded structure. However, the reverse is not true. Many different amino acid sequences can give rise to similar folded structures. For example, the relative orientation of the charged amino acid residues in an ion pair can be switched while still preserving the overall location of the interaction.

11. Protein (a), a β barrel, is described by Ramachandran plot (c), which shows most of the allowable conformations in the upper left quadrant where the bond angles characteristic of the β conformation are concentrated. Protein (b), a series of α helices, is described by plot (d), where most of the allowable conformations are in the lower left quadrant.

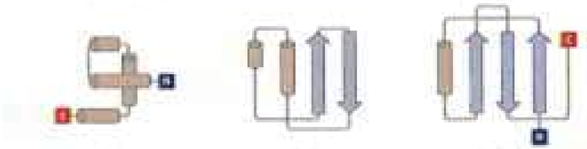
12. (a) The number of moles of DNP-valine formed per mole of protein equals the number of amino termini and thus the number of polypeptide chains. **(b)** 4 **(c)** Different chains would probably run as discrete bands on an SDS polyacrylamide gel.

13. (a) Aromatic residues seem to play an important role in stabilizing amyloid fibrils. Thus, molecules with aromatic substituents may inhibit amyloid formation by interfering with the stacking or association of the aromatic side chains. **(b)** Amyloid forms in the pancreas in association with type 2 diabetes, and forms in the brain in Alzheimer disease. Although the amyloid fibrils in the two diseases involve different proteins, the fundamental structure of the amyloid is similar and is similarly stabilized in both, so they are potential targets for similar drugs designed to disrupt this structure.

14. Although a protein may have only one unique folded structure, many different unfolded structures may exist. The different folding pathways and structures used by the product of other disease alleles of CFTR may not be corrected by lumacaftor.

15. (a) x-ray crystallography **(b)** cryo-EM **(c)** NMR **(d)** NMR or CD

16. (a) 2QYC is B; 2BNH is C; 2Q5R is E or M; 1XU9 is H; 3H7X is I; 1OU5 is E or M; 2WCD is O. **(b)** dimer, α/β; monomer, α/β; dimer, α/β; tetramer, α/β; trimer, all α; dimer, α/β; 24-mer or double dodecamer (12-mer), all α. **(c)** BIOCHEM



(a) (b) (c)

17. (a) NFκB transcription factor, also called RelA transforming factor **(b)** No. You will obtain similar results, but with additional related proteins listed. **(c)** The protein has two subunits. There are multiple variants of the subunits, with the best characterized being 50, 52, or 65 kDa. These pair with each other to form a variety of homodimers and heterodimers. The structures of a number of different variants can be found in the PDB. **(d)** The NFκB transcription factor is a dimeric protein that binds specific DNA sequences, enhancing transcription of nearby genes. One such gene is the immunoglobulin κ (kappa) light chain, from which the transcription factor gets its name.

18. (a) Aba is a suitable replacement because Aba and Cys have side chains that are approximately the same size and are similarly hydrophobic. However, Aba cannot form disulfide bonds, so it will not be a suitable replacement if these are required. **(b)** There are many important differences between the synthesized protein and HIV protease produced by a human cell, any of which could result in an inactive synthetic enzyme. (1) Although Aba and Cys have a similar size and hydrophobicity, Aba may not be similar enough for the protein to fold properly. (2) HIV protease may require disulfide bonds for proper functioning. (3) Many proteins synthesized by ribosomes fold while being produced; the protein in this study folded only after the chain was complete. (4) Proteins synthesized by ribosomes may interact with the ribosomes as they fold; this is not possible for the protein in the study. (5) Cytosol is a more complex solution than the buffer used in the study; some proteins may require specific, unknown proteins for proper folding. (6) Proteins synthesized in cells often require chaperones for proper folding; these are not present in the study buffer. (7) In cells, HIV protease is synthesized as part of a larger chain that is then proteolytically processed; the protein in the study was synthesized as a single molecule. **(c)** Because the enzyme *is* functional when Aba is substituted for Cys, disulfide bonds do not play an important role in the structure of HIV protease. **(d)** *Model 1:* It would fold like the L-protease. *For:* The covalent structure is the same (except for chirality), so it should fold like the L-protease. *Against:* Chirality is not a trivial detail; three-dimensional shape is a key feature of biological molecules. The synthetic enzyme will not fold like the L-protease. *Model 2:* It would fold to the mirror image of the L-protease. *For:* Because the individual components are mirror images of those in the biological protein, it will fold in the mirror-image shape. *Against:* The interactions involved in protein folding are very complex, so the synthetic protein will most likely fold in another form. *Model 3:* It would fold to something else. *For:* The interactions involved in protein folding are very complex, so the synthetic protein will most likely fold in another form. *Against:* Because the individual components are mirror images of those in the biological protein, it will fold in the mirror-image shape. **(e)** *Model 2.* The enzyme is active, but with the enantiomeric form of the biological substrate, and it is inhibited by the enantiomeric form of the biological inhibitor. This is consistent with the D-protease being the mirror image of the L-protease. **(f)** Evans blue is achiral; it binds to both forms of the enzyme. **(g)** No. Because proteases contain only L-amino acids and recognize only L-peptides, chymotrypsin would not digest the D-protease. **(h)** Not necessarily. Depending on the individual enzyme, any of the problems listed in (b) could result in an inactive enzyme.

Chapter 5

1. Protein B has a higher affinity for ligand X; its lower K_d indicates that protein B will be half-saturated at a much lower concentration of X than will protein A. Protein A has $K_a = 3.3 \times 10^6 \text{M}^{-1}$; protein B has $K_a = 2.5 \times 10^7 \text{M}^{-1}$.

2. (a) $n_H < 1.0$ can mean that the protein exhibits negative cooperativity, where the binding of one ligand to the protein decreases its affinity for other ligand molecules. **(b), (c)** There are some instances where $n_H < 1.0$ occurs without true negative cooperativity. For example, $n_H < 1.0$ could also occur if a single polypeptide contained multiple binding sites that had a different affinity for the ligand. If a protein preparation contains a heterogeneous mixture of the protein where some molecules are partially denatured, the measured binding affinity would be artificially decreased, resulting in $n_H < 1.0$. **(d)** If a protein has multiple ligand binding sites that all have the same binding affinity and do not affect one another, no cooperativity, positive or negative, will be observed.

3. $k_d = 8.9 \times 10^{-5} \text{s}^{-1}$

4. (a) 33 nM, **(b)** 0.15 μM, **(c)** 1.9 μM

5. (a) $K_d = 4.0$ nM (shortcut: the K_d is equivalent to the ligand concentration where $Y = 0.5$). **(b)** The rat receptor has the highest affinity, as it has the lowest K_d.

6. Tight binding of CO to a few binding sites in a hemoglobin tetramer tends to force the entire protein into the R state. O_2 can still bind to the unoccupied sites, but it will bind tightly and not be released into the tissues.

7. The cooperative behavior of hemoglobin arises from subunit interactions.

8. (a) Shift the curve to the right. **(b)** Shift the curve to the right. **(c)** Shift the curve to the right. All of these conditions would decrease the affinity of hemoglobin for O_2.

9. (a) The observation that HbA (maternal) is about 60% saturated when the pO_2 is 4 kPa, whereas HbF (fetal) is more than 90% saturated under the same physiological conditions, indicates that HbF has a higher O_2 affinity than HbA. **(b)** The higher O_2 affinity of HbF ensures that oxygen will flow from maternal blood to fetal blood in the placenta. Fetal blood approaches full saturation where the O_2 affinity of HbA is low. **(c)** The observation that the O_2-saturation curve of HbA undergoes a larger shift on BPG binding than that of HbF suggests that HbA binds BPG more tightly than does HbF. Differential binding of BPG to the two hemoglobins may determine the difference in their O_2 affinities.

10. (a) Hb Memphis **(b)** HbS, Hb Milwaukee, Hb Providence, and possibly Hb Cowtown **(c)** Hb Providence

11. More tightly. An inability to form tetramers would limit the cooperativity of these variants, and the binding curve would become more hyperbolic. Also, the BPG-binding site would be disrupted. Oxygen binding would probably be tighter, because the default state in the absence of bound BPG is the tight-binding R state.

12. (a) 3.3×10^{-8} M **(b)** 5×10^{-8} M **(c)** 2×10^{-7} M **(d)** 4.5×10^{-7} M. Note that a rearrangement of Eqn 5-8 gives $[L] = YK_d/(1 - Y)$.

13. The epitope is likely to be a structure that is buried when G-actin polymerizes to form F-actin.

14. (a) The human immune system requires several days to mount a staged response to antigens on the surface of a pathogen. Both trypanosomes and HIV evade the immune system by altering the surface proteins to which immune system components initially bind. Thus, the host organism regularly faces new antigens and requires time to mount an immune response to each one, giving the pathogen time to replicate and spread. HIV also evades the immune system by actively infecting and destroying immune system cells—namely, helper T cells (T_H cells).

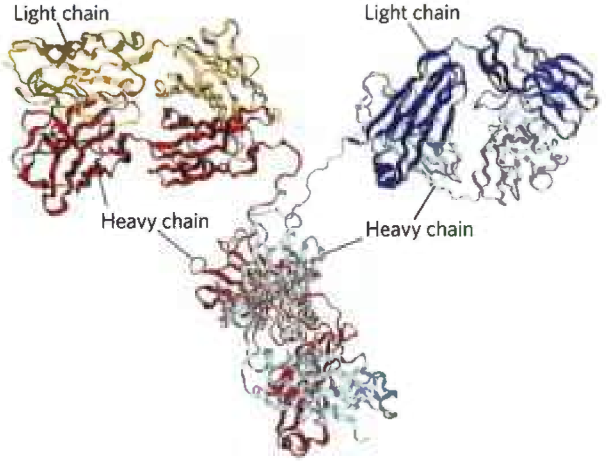
15. Binding of ATP to myosin triggers dissociation of myosin from the actin thin filament. In the absence of ATP, actin and myosin bind tightly to each other.

16. (a) 2 **(b)** 3 **(c)** 1 **(d)** 4

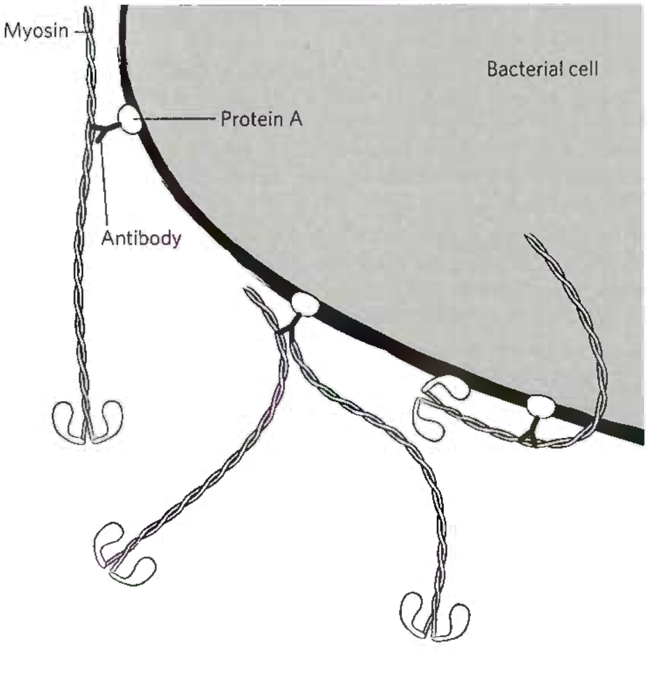
17. (a) Chain L is the light chain and chain H is the heavy chain of the Fab fragment. Chain Y is lysozyme. **(b)** β conformation structures are predominant in the variable and constant regions of the fragment. **(c)** Fab heavy-chain fragment: 218 amino acid residues; light-chain fragment: 214; lysozyme: 129. Less than 15% of the lysozyme molecule is in contact with the Fab fragment. **(d)** Residues that seem to be in contact with lysozyme include, in the H chain: Gly^{31}, Tyr^{32}, Arg^{99}, Asp^{100}, and Tyr^{101}; in the L chain: Tyr^{32}, Tyr^{49}, Tyr^{50}, and Trp^{92}. In lysozyme, residues Asn^{19}, Gly^{22}, Tyr^{23}, Ser^{24}, Lys^{116}, Gly^{117}, Thr^{118}, Asp^{119}, Gln^{121}, and Arg^{125} seem to be situated at the antigen-antibody interface. Not all of these residues are adjacent in the primary structure. Folding of the polypeptide into higher levels of structure brings nonconsecutive residues together to form the antigen-binding site.

18. (a) 2 **(b)** Instantly. Suitable antibodies are almost always present before any challenge from the virus. **(c)** >100,000,000

(d)



19. (a)



The drawing is not to scale; any given cell would have many more myosin molecules on its surface. **(b)** ATP is needed to provide the chemical energy to drive the motion (see Chapter 13). **(c)** An antibody that bound to the myosin tail, the actin-binding site, would block actin binding and prevent movement. An antibody that bound to actin would also prevent actin-myosin interaction and thus movement. **(d)** There are two possible explanations: (1) Trypsin cleaves only at Lys and Arg residues (see Table 3-6), so would not cleave at many sites in the protein. (2) Not all Arg or Lys residues are equally accessible to trypsin; the most-exposed sites would be cleaved first. **(e)** The S1 model. The hinge model predicts that bead-antibody-HMM complexes (with the hinge) would move, but bead-antibody-SHMM complexes (no hinge) would not. The S1 model predicts that because both complexes include S1, both would move. The finding that the beads move with SHMM (no hinge) is consistent only with the S1 model. **(f)** With fewer myosin molecules bound, the beads could temporarily fall off the actin as a myosin let go of it. The beads would then move more slowly, as time is required for a second myosin to bind. At higher myosin density, as one myosin lets go, another quickly binds, leading to faster motion. **(g)** Above a certain density, what limits the rate of movement is the intrinsic speed with which myosin molecules move the beads. The myosin molecules are moving at a maximum rate; adding more will not increase speed.

Chapter 6

1. The activity of the enzyme that converts sugar to starch is destroyed by heat denaturation.

2. 2.4×10^{-6} M

3. 9.5×10^{8} years

4. The enzyme-substrate complex is more stable than the enzyme alone.

5. The reaction rate can be measured by following the decrease in absorption by NADH (at 340 nm) as the reaction proceeds. Determine the K_m value; using substrate concentrations well above the K_m, measure initial rate (rate of NADH disappearance with time, measured spectrophotometrically) at several known enzyme concentrations, and plot initial rate versus concentration of enzyme. The plot should be linear, with a slope that provides a measure of LDH concentration.

6. (a), (c), (e)

7. **(a)** 1.7×10^{-3} M **(b)** 0.33, 0.67, 0.91 **(c)** The red curve corresponds to enzyme B ([X] > K_m for this enzyme); the black curve, to enzyme A.

8. **(a)** 0.2 $\mu\text{M s}^{-1}$ **(b)** 0.6 $\mu\text{M s}^{-1}$ **(c)** 0.9 $\mu\text{M s}^{-1}$

9. **(a)** 2,000 s^{-1} **(b)** Measured $V_{max} = 1\ \mu\text{M s}^{-1}$; $K_m = 2\ \mu\text{M}$

10. **(a)** 400 s^{-1} **(b)** 10 μM **(c)** $\alpha = 2, \alpha' = 3$ **(d)** Mixed inhibitor

11. **(a)** 24 nM **(b)** 4 μM (V_0 is exactly one-half V_{max}, so [X] = K_m) **(c)** 40 μM (V_0 is exactly one-half V_{max}, so [X] = 10 times K_m in the presence of inhibitor) **(d)** No. $k_{cat}/K_m = (0.33\ \text{s}^{-1})/(4 \times 10^{-6}\ \text{M}) = 8.25 \times 10^{4}\ \text{M}^{-1}\ \text{s}^{-1}$, well below the diffusion-controlled limit.

12. $V_{max} \approx 140\ \mu\text{M/min}$; $K_m \approx 1 \times 10^{-5}$ M

13. **(a)** $V_{max} = 51.5$ mM/min; $K_m = 0.59$ mM **(b)** Competitive inhibition

14. $V_{max} = 0.50\ \mu\text{mol/min}$; $K_m = 2.2$ mM

15. **(a)** A; **(b)** B

16. $2.0 \times 10^{7}\ \text{min}^{-1}$

17. **(a)** P **(b)** Q **(c)** E **(d)** B **(e)** A **(f)** F

18. **(a)** Increase **(b)** Decrease **(c)** No change **(d)** No change

19. The basic assumptions of the Michaelis-Menten equation still hold. The reaction is at steady state, and the rate is determined by $V_0 = k_2[\text{ES}]$. The equations needed to solve for [ES] are

$$[\text{E}_t] = [\text{E}] + [\text{ES}] + [\text{EI}] \quad \text{and} \quad [\text{EI}] = \frac{[\text{E}][\text{I}]}{K_I}$$

[E] can be obtained by rearranging Eqn 6-19. The rest follows the pattern of the Michaelis-Menten equation derivation in the text.

20. 29,000. The calculation assumes that there is only one essential Cys residue per enzyme molecule.

21. Activity of the prostate enzyme equals total phosphatase activity in a blood sample minus phosphatase activity in the presence of enough tartrate to completely inhibit the prostate enzyme.

22. The inhibition is mixed. Because K_m seems not to change appreciably, this could be the special case of mixed inhibition called noncompetitive.

23. Apparent $K_m = \alpha K_m/\alpha'$. The [S] at which $V_0 = V_{max}/2\alpha'$ is obtained when all terms except V_{max} on the right side of Eqn 6-30—that is, $[\text{S}]/(\alpha K_m + \alpha'[\text{S}])$—equal $\alpha'/2$. Begin with $[\text{S}]/(\alpha K_m + \alpha'[\text{S}]) = \alpha'/2$ and solve for [S].

24. An amino acid with a positively charged side chain, such as Lys, His, or Arg, could pull electron density away from Tyr.

25. At pH 5.2, Glu^{35} is protonated and neutral and Asp^{52} is unprotonated and negatively charged. The optimum activity occurs when Glu^{35} is protonated and Asp^{52} is unprotonated. Optimal activity requires that Glu^{35} be protonated and Asp^{52} be unprotonated. Activity thus declines with decreasing pH as Asp^{52} is protonated, and with increasing pH as Glu^{35} is unprotonated.

26. **(a)** Although it can be ionized, the sulfhydryl group is relatively nonpolar (see Chapter 3). Amino butyric acid has a nonpolar side chain of similar size to the Cys side chain. **(b)** Because the peptide is made up of D-amino acids, the same shape is unlikely. A mirror image shape is likely, as the D-amino acids are mirror images of their L-stereoisomers. Prior to testing, a completely inactive form is at least plausible. **(c)** The mirror image hypothesis is strongly supported. The substrates and inhibitors made up of D-amino acids are uniquely active. **(d)** No. The active site of chymotrypsin is configured to act on peptides made up of L-amino acids. **(e)** No. The folding process is complex, and sometimes requires assistance from chaperones and specialized enzymes. Some enzymes and proteins can fold spontaneously into an active protein, but others cannot.

Chapter 7

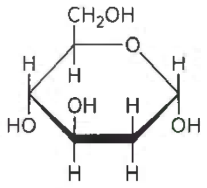
1. With reduction of the carbonyl oxygen to a hydroxyl group, the chemistry at C-1 and C-3 is the same; the glycerol molecule is not chiral.

2. Epimers differ by the configuration about only *one* carbon.
(a) D-altrose (C-2), D-glucose (C-3), D-gulose (C-4)
(b) D-idose (C-2), D-galactose (C-3), D-allose (C-4)
(c) D-arabinose (C-2), D-xylose (C-3)

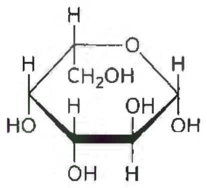
3. To convert α-D-glucose to β-D-glucose, the bond between C-1 and the hydroxyl on C-5 (as in Fig. 7-6) must be broken; to convert D-glucose to D-mannose, either the —H or —OH bond on C-2 must be broken. Conversion between chair conformations does not require bond breakage; this is the critical distinction between configuration and conformation.

4. **(a)** Both are polymers of D-glucose, but they differ in the glycosidic linkage: (β1→4) for cellulose, (α1→4) for glycogen. **(b)** Both are hexoses, but glucose is an aldohexose, fructose a ketohexose. **(c)** Both are disaccharides, but maltose has two (α1→4)-linked D-glucose units, and sucrose has (α1↔2β)-linked D-glucose and D-fructose.

5.

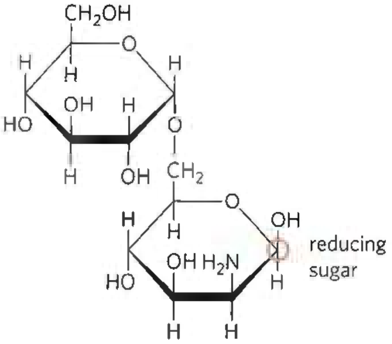


α-D-Mannose



β-L-Galactose

6.



7. An individual with a condition that increases the rate of erythrocyte destruction and turnover would be expected to exhibit less hemoglobin glycation (a lower HbA1c value) because of the shorter-than-normal exposure of the hemoglobin to glucose.

8. A hemiacetal forms when an aldose or a ketose condenses with an alcohol; a glycoside forms when a hemiacetal condenses with an alcohol (see Fig. 7-5).

9. Fructose cyclizes to either the pyranose structure or the furanose structure. Increasing the temperature shifts the equilibrium in the direction of the furanose, the less-sweet form.

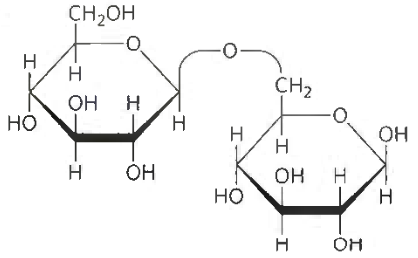
10. In 6-phosphogluconolactone, C-1 is a carboxylic acid ester; in glucose, C-1 is a hemiacetal.

11. Boiling a solution of sucrose in water hydrolyzes some of the sucrose to invert sugar. Hydrolysis is accelerated and occurs at lower temperatures with the addition of a small amount of acid (lemon juice or cream of tartar, for example).

12. Prepare a slurry of sucrose and water for the core; add a small amount of sucrase (invertase); immediately coat with chocolate.

13. Sucrose has no free anomeric carbon to undergo mutarotation.

14.



Yes; yes

15. *N*-Acetyl-*β*-D-glucosamine is a reducing sugar; its C-1 can be oxidized (p. 237). D-Gluconate is not a reducing sugar; its C-1 is already at the oxidation state of a carboxylic acid. GlcN(*α*1↔1*α*)Glc is not a reducing sugar; the anomeric carbons of both monosaccharides are involved in the glycosidic bond.

16. Native cellulose consists of glucose units linked by (*β*1→4) glycosidic bonds, which force the polymer chain into an extended conformation. Parallel series of these extended chains form intermolecular hydrogen bonds, aggregating into long, tough, insoluble fibers. Glycogen consists of glucose units linked by (*α*1→4) glycosidic bonds, which cause bends in the chain and prevent formation of long fibers. In addition, glycogen is highly branched and, because many of its hydroxyl groups are exposed to water, is highly hydrated and disperses in water.

Cellulose is a structural material in plants, consistent with its side-by-side aggregation into insoluble fibers. Glycogen is a storage fuel in animals. Highly hydrated glycogen granules with their many nonreducing ends can be rapidly hydrolyzed by glycogen phosphorylase to release glucose 1-phosphate.

17. Cellulose is several times longer; it assumes an extended conformation, whereas amylose has a helical structure.

18. 7×10^3 residues/s

19. Glycoproteins: b, c, f; proteoglycans: a, d, e

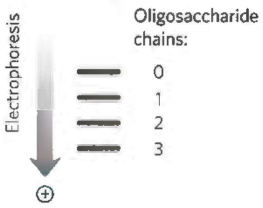
20. The ball-and-stick models of the disaccharide in Fig. 7-16 show no steric interactions, but space-filling models, showing atoms with their correct relative sizes, would show several strong steric hindrances in the high-energy conformer that are not present in the extended conformer.

21. The negative charges on chondroitin sulfate repel each other and force the molecule into an extended conformation. The polar molecule attracts many water molecules, increasing the molecular volume. In the dehydrated solid, each negative charge is counterbalanced by a positive ion, and the molecule condenses.

22. Positively charged amino acid residues would bind the highly negatively charged groups on heparin. In fact, Lys residues of antithrombin III interact with heparin.

23. The order of the hexoses (ABC, ACB, etc.), the stereochemistry at each of two anomeric carbons (*α* or *β*), and the carbon atoms involved in each glycosidic linkage ((1→4), (1→6), (3→4), etc.)

24.



25. Oligosaccharides; their subunits can be combined in more ways than the amino acid subunits of oligopeptides. Each hydroxyl group can participate in glycosidic bonds, and the configuration of each glycosidic bond can be either *α* or *β*. The polymer can be linear or branched.

26. Administer an oligosaccharide with the same structure as that recognized by ricin, or high concentrations of *N*-acetylgalactosamine itself. Ricin will bind the free oligosaccharide or acetylgalactosamine instead of the cell surface target, preventing the entry of the toxin.

27. (a) Branch-point residues yield 2,3-di-*O*-methylglucose; unbranched residues yield 2,3,6-tri-*O*-methylglucose. **(b)** 3.75%

28. (a) The tests involve trying to dissolve only part of the sample in a variety of solvents, then analyzing both dissolved and undissolved materials to see whether their compositions differ. **(b)** For a pure substance, all molecules are the same and any dissolved fraction will have the same composition as any undissolved fraction. An impure substance is a mixture of more than one compound. When the sample is treated with a particular solvent, more of one component may dissolve, leaving more of the other component(s) behind. As a result, the dissolved and undissolved fractions will have different compositions. **(c)** A quantitative assay allows researchers to be sure that none of the activity has been lost through degradation. When the structure of a molecule is being determined, it is important that the sample under analysis consist only of intact (undegraded) molecules. If the sample is contaminated with degraded material, this will give confusing and perhaps uninterpretable structural results. A qualitative assay would detect the presence of activity, even if the sample had become significantly degraded. **(d)** Results 1 and 2. Result 1 is consistent with the known structure, because type B antigen has three molecules of galactose; types A and O each have only two. Result 2 is also consistent, because type A has two amino sugars (*N*-acetylgalactosamine and *N*-acetylglucosamine); types B and O have only one (*N*-acetylglucosamine). Result 3 is *not* consistent with the known structure: for type A, the glucosamine:galactosamine ratio is 1:1; for type B, it is 1:0. **(e)** The samples were probably impure and/or partly degraded. The first two results were correct possibly because the method was only roughly quantitative and thus not as sensitive to inaccuracies in measurement. The third result is more quantitative and thus more likely to differ from predicted values because of impure or degraded samples. **(f)** An exoglycosidase. If it were an endoglycosidase, one of the products of its action on O antigen would include galactose, *N*-acetylglucosamine, or *N*-acetylgalactosamine, and at least one of those sugars would be able to inhibit the degradation. Given that the enzyme is not inhibited by any of these sugars, it must be an exoglycosidase, removing only the terminal sugar from the chain. The terminal sugar of O antigen is fucose, so fucose is the only sugar that could inhibit the degradation of O antigen. **(g)** The exoglycosidase removes *N*-acetylgalactosamine from A antigen and galactose from B antigen. Because fucose is not a product of either reaction, it will not prevent removal of these sugars, and the resulting substances will no longer be active as A antigen or as B antigen. However, the products should be active as O antigen, because degradation stops at fucose. **(h)** All the results are consistent with Fig. 10-13. (1) D-Fucose and L-galactose, which would protect against degradation, are not present in any of the antigens. (2) The terminal sugar of A antigen is *N*-acetylgalactosamine, and this sugar alone protects this antigen from degradation. (3) The terminal sugar of B antigen is galactose, which is the only sugar capable of protecting this antigen.

Chapter 8

1. N-3 and N-7

2. (5′)GCGCAATATTTTGAGAAATATTGCGC(3′); it contains a palindrome. The individual strands can form hairpin structures; the two strands can form a cruciform.

3. 9.4×10^{17}

4. (a) Deoxyadenylate, deoxy-O^6-methylguanylate, an apurinic site (or AP site or abasic site), deoxyuridylate **(b)** 5′ end at upper left and 3′ end at lower right **(c)** The tetranucleotide is DNA, as it is made up of deoxynucleotides. This is true despite the presence of a uracil base.

5. Helices in RNA hairpins assume an A conformation; helices in DNA hairpins generally assume a B conformation.

6. In eukaryotic DNA, about 5% of C residues are methylated. 5-Methylcytosine can spontaneously deaminate to form thymine; the resulting G–T pair is one of the most common mismatches in eukaryotic cells.

7. Higher

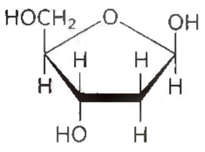
8. Without the base, the ribose ring can be opened to generate the noncyclic aldehyde form. This, and the loss of base-stacking interactions, could contribute significant flexibility to the DNA backbone.

9. CGCGCGTGCGCGCGCG

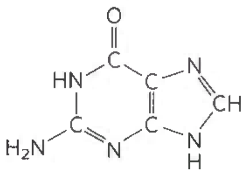
10. RNA nucleotides have a 2′-hydroxyl group on the pentose ring, and the common pyrimidine bases for RNA nucleotides are uracil and cytosine.

11. Base stacking in nucleic acids tends to reduce the absorption of UV light. Denaturation involves loss of base stacking, and UV absorption increases.

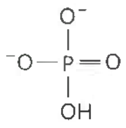
12.



Deoxyribose



Guanine



Phosphate

Solubilities: phosphate > deoxyribose > guanine. The highly polar phosphate groups and sugar moieties are on the outside of the double helix, exposed to water; the hydrophobic bases are in the interior of the helix.

13. Primer 1: CCTCGAGTCAATCGATGCTG

Primer 2: CGCGCACATCAGACGAACCA

Recall that all DNA sequences are written in the 5′→3′ direction, left to right; that DNA polymerase synthesizes DNA in the 5′→3′ direction; that the two strands of a DNA molecule are antiparallel; and that both PCR primers must target the end sequences so that their 3′ ends are oriented toward the segment to be amplified.

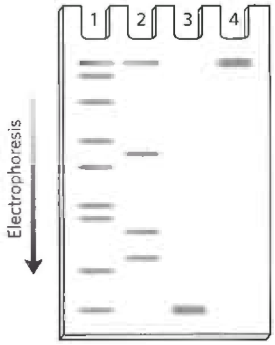
14. (a) B **(b)** C **(c)** A

15. The primers can be used to probe libraries containing long genomic clones to identify contig ends that lie close to each other. If the contigs flanking the gap are close enough, the primers can be used in PCR to directly amplify the intervening DNA separating the contigs, which can then be cloned and sequenced.

16. The 3′-H would prevent addition of any subsequent nucleotides, so the sequence for each cluster would end after the first nucleotide addition.

17. If dCTP is omitted, when the first G residue is encountered in the template, ddCTP will be added, and polymerization will halt. Only one band will be seen in the sequencing gel.

18.



19. The products are:

(5′)GCGCCAUUGC(3′)—OH

(5′)GCGCCAUUG(3′)—OH

(5′)GCGCCAUU(3′)—OH

(5′)GCGCCAU(3′)—OH

(5′)GCGCCA(3′)—OH

(5′)GCGCC(3′)—OH

(5′)GCGC(3′)—OH

(5′)GCG(3′)—OH

(5′)GC(3′)—OH

and the nucleoside 5′-phosphates

20. (a) Water is a participant in most biological reactions, including those that cause mutations. The low water content in endospores reduces the activity of mutation-causing enzymes and slows the rate of nonenzymatic depurination reactions, which are hydrolysis reactions. **(b)** UV light induces formation of cyclobutane pyrimidine dimers. Because *B. subtilis* is a soil organism, spores can be lofted to the top of the soil or into the air, where they may be subject to prolonged UV exposure.

21. DMT is a blocking group that prevents reaction of the incoming base with itself.

22. (a) Right-handed. The base at one 5′ end is adenine; at the other 5′ end, cytosine. **(b)** Left-handed **(c)** If you cannot see the structures in stereo, use a search engine to find tips online.

23. (a) It would not be easy! The data for different samples from the same organism show significant variation, and the recovery is never 100%. The numbers for C and T show much more consistency than those for A and G, so for C and T it is much easier to make the case that samples from the same organism have the same composition. But even with the less consistent values for A and G, (1) the range of values for different tissues does overlap substantially; (2) the difference between different preparations of the same tissue is about the same as the difference between samples from different tissues; and (3) in samples for which recovery is high, the numbers are more consistent. **(b)** This technique would not be sensitive enough to detect a difference between normal cells and cancerous cells. Cancer is caused by mutations, but these changes in DNA—a few base pairs out of several billion—would be too small to detect with these techniques. **(c)** The ratios of A:G and T:C vary widely among different species. For example, in the bacterium *Serratia marcescens*, both ratios are 0.4, meaning that the DNA contains mostly G and C. In *Haemophilus influenzae*, by contrast, the ratios are 1.74 and 1.54, meaning that the DNA is mostly A and T. **(d)** Conclusion 4 has three requirements. (1) A = T: The table shows an A:T ratio very close to 1 in all cases. Certainly, the variation in this ratio is substantially less than the variation in the A:G and T:C ratios. (2) G = C: Again, the G:C ratio is very close to 1, and the other ratios vary widely. (3) A + G = T + C: This is the purine:pyrimidine ratio, which also is very close to 1.

Chapter 9

1. (a) (5′) – – – G(3′) and (5′)AATTC – – – (3′)
(3′) – – – CTTAA(5′) (3′) G – – – (5′)

(b) (5′) – – – GAATT(3′) and (5′)AATTC – – – (3′)
(3′) – – – CTTAA(5′) (3′)TTAAG – – – (5′)

(c) (5′) – – – GAATTAATTC – – – (3′)
(3′) – – – CTTAATTAAG – – – (5′)

(d) (5′) – – – G(3′) and (5′)C – – – (3′)
(3′) – – – C(5′) (3′)G – – – (5′)

(e) (5′) – – – GAATTC – – – (3′)
(3′) – – – CTTAAG – – – (5′)

(f) (5′) – – – CAG(3′) and (5′)CTG – – – (3′)
(3′) – – – GTC(5′) (3′)GAC – – – (5′)

(g) (5′) – – – CAGAATTC – – – (3′)
(3′) – – – GTCTTAAG – – – (5′)

(h) Method 1: Cut the DNA with EcoRI as in (a), then treat the DNA as in (b) or (d), and then ligate a synthetic DNA fragment with the BamHI recognition sequence between the two resulting blunt ends. Method 2 (more efficient): Synthesize a DNA fragment with the structure

(5′)AATTGGATCC(3′)
(3′)CCTAGGTTAA(5′)

This would ligate efficiently to the sticky ends generated by EcoRI cleavage, would introduce a BamHI site, but would not regenerate the EcoRI site.

(i) The four fragments (with N = any nucleotide), in order of discussion in the problem, are

(5′)AATTCNNNNCTGCA(3′)
(3′)GNNNNG(5′)

(5′)AATTCNNNNGTGCA(3′)
(3′)GNNNNC(5′)

(5′)AATTGNNNNCTGCA(3′)
(3′)CNNNNG(5′)

(5′)AATTGNNNNGTGCA(3′)
(3′)CNNNNC(5′)

2. Yeast artificial chromosomes (YACs) are not stable in a cell unless they have two telomere-containing ends and a large DNA segment cloned into the chromosome. YACs less than 10,000 bp long are soon lost during continued mitosis and cell division.

3. (a) Plasmids in which the original pBR322 was regenerated without insertion of a foreign DNA fragment; these would retain resistance to ampicillin. Also, two or more molecules of pBR322 might be ligated together with or without insertion of foreign DNA.

(b) The clones in lanes 1 and 2 each have one DNA fragment inserted in different orientations. The clone in lane 3 has two DNA fragments, ligated such that the EcoRI proximal ends are joined.

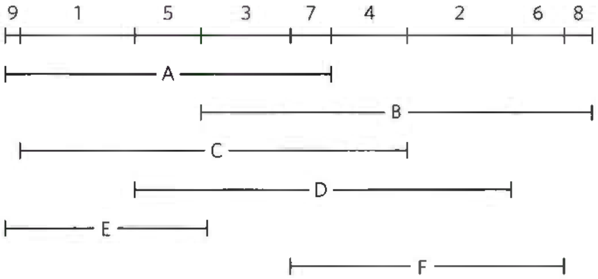
4. (5′)GAAAGTCCGCGTTATAGGCATG(3′)
(3′)ACGTCTTTCAGGCGCAATATCCGTACTTAA(5′)

5. Your test would require DNA primers, a heat-stable DNA polymerase, deoxynucleoside triphosphates, and a PCR machine (thermal cycler). The primers would be designed to amplify a DNA segment encompassing the CAG repeat. The DNA strand shown is the coding strand, oriented 5′→3′, left to right. The primer targeted to DNA to the left of the repeat would be identical to any 25-nucleotide sequence shown in the region to the left of the CAG repeat. The primer on the right side must be complementary and antiparallel to a 25-nucleotide sequence to the right of the CAG repeat. Using the primers, DNA including the CAG repeat would be amplified by PCR, and its size would be determined by comparison with size markers after electrophoresis. The length of the DNA would reflect the length of the CAG repeat, providing a simple test for the disease.

6. Design PCR primers that are complementary to the DNA in the deleted segment but would direct DNA synthesis away from each other. No PCR product is generated unless the ends of the deleted segment are joined to create a circle.

7. The two proteins likely co-localize under nutrient starvation and possibly form a protein complex.

8.



9. The production of labeled antibodies is difficult and expensive, and the labeling of every antibody to every protein target would be impractical. By labeling one antibody preparation for binding to all antibodies of a particular class, the same labeled antibody preparation can be used in many different immunofluorescence experiments.

10. Express the protein in yeast strain 1 as a fusion protein with one of the domains of Gal4p—say, the DNA-binding domain. Using yeast strain 2, make a library in which essentially every protein of the fungus is expressed as a fusion protein with the interaction domain of Gal4p. Mate strain 1 with the strain 2 library, and look for colonies that are colored due to expression of the reporter gene. These colonies will generally arise from mated cells containing a fusion protein that interacts with your target protein.

11. Reverse transcriptase is used to convert single-stranded RNA into double-stranded DNA in one of the early steps of RNA-Seq.

12. RNA-Seq detects noncoding RNAs. These have special functions, and they lack protein-coding sequences. Many RNAs encoded by eukaryotic genomes are not messenger RNAs. Instead, they are noncoding RNAs with a variety of functions. They need not possess an open reading frame as part of their sequence.

13. ATSAAG**W**DEWEGGK**V**LIHL**DG**KLQNRGALLELDIGAV

14. The pattern of haplotypes in the Aleut and Eskimo populations suggests that their ancestors' migration into the American Arctic regions was separate from the migrations that eventually populated the rest of North America and South America.

15. Interbreeding between the Denisovans and *Homo sapiens* must have occurred in Asia, sometime in the many millennia during which humans migrated from Africa to Asia and then to Australia and Melanesia.

16. The same disease condition can be caused by defects in two or more genes that are on different chromosomes.

17. (b) would act as a suitable primer pair for this transcript; **(a)** would form primer dimers because of the high number of complementary bases in the primers; **(c)** also exhibits significant self-complementarity and would have a very high melting point due to the C–G pairs; **(d)** also exhibits self-complementarity and would form stem-loops.

18. (a) DNA solutions are highly viscous because the very long molecules are tangled in solution. Shorter molecules tend to tangle less and form a less viscous solution, so decreased viscosity corresponds to shortening of the polymers—as caused by nuclease activity. **(b)** An endonuclease. An exonuclease removes single nucleotides from the 5′ end or 3′ end and would produce TCA-soluble ^{32}P-labeled nucleotides. An endonuclease cuts DNA into oligonucleotide fragments and produces little or no TCA-soluble ^{32}P-labeled material. **(c)** The 5′ end. If the phosphate were left on the 3′ end, the kinase would incorporate significant ^{32}P as it added phosphate to the 5′ end; treatment with the phosphatase would have no effect on this. In this case, samples A and B would incorporate significant amounts of ^{32}P. When the phosphate is left on the 5′ end, the kinase does not incorporate any ^{32}P: it cannot add a phosphate if one is already present. Treatment with the phosphatase removes 5′ phosphate, and the kinase then incorporates significant amounts of ^{32}P. Sample A will have little or no ^{32}P, and B will show substantial ^{32}P incorporation—as was observed. **(d)** Random breaks would produce a distribution of fragments of random size. The production of specific fragments indicates that the enzyme is site-specific. **(e)** Cleavage at the site of recognition. This produces a specific sequence at the 5′ end of the fragments. If cleavage occurred near but not within the recognition site, the sequence at the 5′ end of the fragments would be random. **(f)** The results are consistent with two recognition sequences, as shown below, cleaved where shown by the arrows:

↓
(5′) – – – GTT AAC – – – (3′)
(3′) – – – CAA TTG – – – (5′)
↑

which gives the (5′)pApApC and (3′)TpTp fragments; and

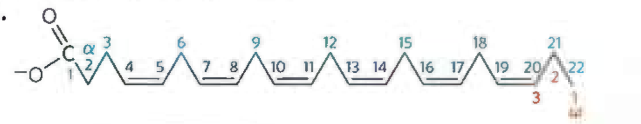
↓
(5′) – – – GTC GAC – – – (3′)
(3′) – – – CAG CTG – – – (5′)
↑

which gives the (5′)pGpApC and (3′)CpTp fragments.

Chapter 10

1. The term "lipid" does not specify a particular chemical structure. Compounds are categorized as lipids based on their greater solubility in organic solvents than in water.

2.



20:6($\Delta^{4,7,10,13,16,19}$) Docosahexaenoic acid (DHA)

3. (a) The number of cis double bonds. Each cis double bond causes a bend in the hydrocarbon chain, lowering the melting temperature. **(b)** Six different triacylglycerols can be constructed, in order of increasing melting points:

OOO < OOP = OPO < PPO = POP < PPP

(c) where O = oleic and P = palmitic acid. The greater the content of saturated fatty acid, the higher the melting point. **(d)** Branched-chain fatty acids increase the fluidity of membranes because they decrease the extent of membrane lipid packing.

4. It reduces double bonds, which increases the melting point of lipids containing the fatty acids.

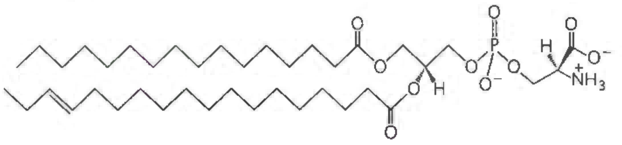
5. Long, saturated acyl chains, nearly solid at air temperatures, form a hydrophobic layer in which a polar compound such as H_2O cannot dissolve or diffuse.

6. Spearmint is (*R*)-carvone; caraway is (*S*)-carvone.

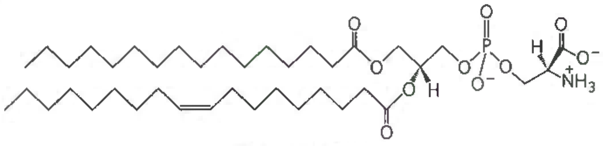
7. The ^{18}O label appears in the fatty acid salts.

8. *Hydrophobic units:* **(a)** 2 fatty acids **(b)**, **(c)**, and **(d)** 1 fatty acid and the hydrocarbon chain of sphingosine **(e)** the steroid nucleus and acyl side chain. *Hydrophilic units:* **(a)** phosphoethanolamine **(b)** phosphocholine **(c)** D-galactose **(d)** several sugar molecules **(e)** alcohol group (—OH)

9. Serine



10.



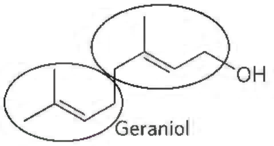
Phosphatidylserine

11. The part of the membrane lipid that determines blood type is the oligosaccharide in the head group of the membrane sphingolipids (see Fig. 10-13). This same oligosaccharide is attached to certain membrane glycoproteins, which also serve as points of recognition by the antibodies that distinguish blood groups.

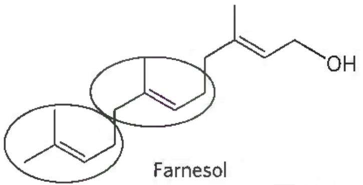
12. (a) The free —OH group on C-2 and the phosphocholine head group on C-3 are hydrophilic; the fatty acid on C-1 of lysolecithin is hydrophobic. **(b)** Certain steroids, such as prednisone, inhibit the action of phospholipase A_2, inhibiting the release of arachidonic acid from C-2. Arachidonic acid is converted to a variety of eicosanoids, some of which cause inflammation and pain. **(c)** Phospholipase A_2 releases arachidonic acid, a precursor of other eicosanoids with vital protective functions in the body; it also breaks down dietary glycerophospholipids.

13. Diacylglycerol is hydrophobic and remains in the membrane. Inositol 1,4,5-trisphosphate is highly polar, very soluble in water, and more readily diffusible in the cytosol. Both are second messengers.

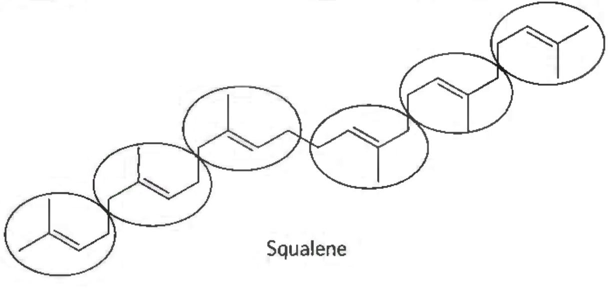
14.



Geraniol



Farnesol



Squalene

15. (a) Glycerol and the sodium salts of palmitic and stearic acids **(b)** D-Glycerol 3-phosphocholine and the sodium salts of palmitic and oleic acids

16. Solubility in water: monoacylglycerol > diacylglycerol > triacylglycerol

17. First eluted to last eluted: cholesteryl palmitate and triacylglycerol; cholesterol and *n*-tetradecanol; phosphatidylcholine and phosphatidylethanolamine; sphingomyelin; phosphatidylserine and palmitate. The lipids elute from the silica gel column in order of polarity. The least polar lipid will elute first and the most polar lipid will elute last.

18. (a) Subject acid hydrolysates of each compound to chromatography (GC or silica gel TLC), and compare the result with known standards. *Sphingomyelin hydrolysate:* sphingosine, fatty acids, phosphocholine, choline, and phosphate; *cerebroside hydrolysate:* sphingosine, fatty acids, sugars, but no phosphate. **(b)** Strong alkaline hydrolysis of sphingomyelin yields sphingosine; phosphatidylcholine yields glycerol. Detect hydrolysate components on thin-layer chromatograms by comparing with standards or by their differential reaction with FDNB (only sphingosine reacts to form a colored product). Treatment with phospholipase A_1 or A_2 releases free fatty acids from phosphatidylcholine, but not from sphingomyelin.

19. (a) Sphingosine (4.78); linoleic acid (5.88); stearic acid (6.33); cholesterol (7.68) **(b)** Log *P* describes the lipophilicity of the drug, crucial for determining how to formulate the drug for transport through aqueous compartments of the body such as the gut and the bloodstream. Log *P* also determines the likelihood of a drug being absorbed by fats and fatty tissues, which can alter its effectiveness, half-life, and potential toxicity.

20. (a) β barrel **(b)** Phe, Trp, Tyr, Leu. All are hydrophobic or have nonpolar R groups. **(c)** The polar head group can hydrogen bond with water; the hydrocarbon tail cannot. Hydrophobic portions of the residues of the pocket protect the tail from contact with water as it moves through the bloodstream.

21. (a) GM1 and globoside. Both glucose and galactose are hexoses, so "hexose" in the molar ratio refers to glucose + galactose. The ratios for the four gangliosides are GM1, 1:3:1:1; GM2, 1:2:1:1; GM3, 1:2:0:1; globoside, 1:3:1:0. **(b)** Yes. The ratio matches GM2, the ganglioside expected to build up in Tay-Sachs disease (see Box 10-1, Fig. 1). **(c)** This analysis is similar to that used by Sanger to determine the amino acid sequence of insulin. The analysis of each fragment reveals only its *composition*, not its *sequence*, but because each fragment is formed by sequential removal of one sugar, we can draw conclusions about sequence. The structure of the normal asialoganglioside is ceramide–glucose–galactose–galactosamine–galactose, consistent with Box 10-1 (excluding Neu5Ac, removed before hydrolysis). **(d)** The Tay-Sachs asialoganglioside is ceramide–glucose–galactose–galactosamine, consistent with Box 10-1. **(e)** The structure of the normal asialoganglioside, GM1, is *ceramide–glucose* [2 —OH involved in glycosidic links; 1 —OH involved in ring structure; 3 —OH (2, 3, 6) free for methylation]–*galactose* [2 —OH in links; 1 —OH in ring; 3 —OH (2, 4, 6) free for methylation]–*galactosamine* [2 —OH in links; 1 —OH in ring; 1 —NH_2 instead of —OH; 2 —OH (4, 6) free for methylation]–*galactose* [1 —OH in link; 1 —OH in ring; 4 —OH (2, 3, 4, 6) free for methylation]. **(f)** Two key pieces of information are missing: What are the linkages between the sugars? Where is Neu5Ac attached?

Chapter 11

1. The area per molecule would be calculated from the known amount (number of molecules) of lipid used and the area occupied by a monolayer when it begins to resist compression (when the required force increases dramatically, as shown in the plot of force versus area).

2. (a) Lipids that form bilayers are amphipathic molecules: they contain a hydrophilic region and a hydrophobic region. To minimize the hydrophobic area exposed to the water surface, these lipids form two-dimensional sheets, with the hydrophilic regions exposed to water and the hydrophobic regions buried in the interior of the sheet. Furthermore, to avoid exposing the hydrophobic edges of the sheet to water, lipid bilayers close on themselves. **(b)** These sheets form the closed membrane surfaces that envelop cells and compartments within cells (organelles).

3. 2 nm. Two palmitates placed end to end span about 4 nm, approximately the thickness of a typical bilayer.

4. Integral proteins are firmly embedded in the lipid bilayer and can be released only by treating membranes with a detergent or nonpolar solvent. Peripheral membrane proteins are more easily released, by changes in pH, metal ion concentration, or protein-denaturing reagents like urea. Amphitropic membrane proteins are loosely and reversibly associated with membranes and move between membrane and cytosol as part of their function.

5. Salt extraction indicates a peripheral location, and inaccessibility to protease in intact cells indicates an internal location. Protein X is likely to be a peripheral protein on the cytosolic face of the membrane.

6. Construct a hydropathy plot; hydrophobic regions of 20 or more residues suggest transmembrane segments. Determine whether the protein in intact erythrocytes reacts with a membrane-impermeant reagent specific for primary amines; if it does, the transporter's amino terminus is on the outside of the cell.

7. ~4%; estimated by calculating the surface area of the cell and of 10,000 transporter molecules

8. ~22. To estimate the fraction of membrane surface covered by phospholipids, you would need to know (or estimate) the average cross-sectional area of a phospholipid molecule in a bilayer (e.g., from an experiment such as that described in Problem 1 in this chapter) and the average cross-sectional area of a 50 kDa protein.

9. Rate of diffusion would decrease. Movement of individual lipids in bilayers occurs much faster at 37 °C, when the lipids are in the "fluid" phase, than at 10 °C, when they are in the "solid" phase. This effect is more pronounced than the usual decrease in Brownian motion with decreased temperature.

10. Interactions among membrane lipids are due to the hydrophobic effect, noncovalent and reversible, allowing membranes to spontaneously reseal.

11. The temperature of body tissues at the extremities is lower than that of tissues closer to the center of the body. If lipid is to remain fluid at this lower temperature, it must contain a higher proportion of unsaturated fatty acids; unsaturated fatty acids lower the melting point of lipid mixtures.

12. There is a very high energetic barrier to taking the polar head of a membrane lipid through the hydrocarbon core. Higher temperatures might make it more likely to occur, as would the presence of a catalyst, such as a flippase, a floppase, or a scramblase protein.

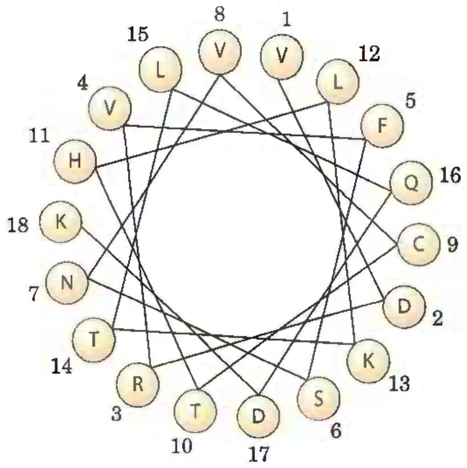
13. The energetic cost of moving the highly polar, sometimes charged, head group through the hydrophobic interior of the bilayer is prohibitive.

14. Scramblases catalyze the transport of membrane lipids from one membrane leaflet to the other. The reaction is ATP-independent, driven by a transbilayer lipid gradient. Scramblases cannot create an asymmetric distribution of lipids across the bilayer. Flippases catalyze the ATP-dependent transport of aminophospholipids (phosphatidylserine and phosphatidylethanolamine) from the extracellular or lumenal leaflet of a membrane to the cytosolic leaflet.

15. At pH 7, tryptophan bears both a positive charge and a negative charge, but indole is uncharged. The movement of the less polar indole through the hydrophobic core of the bilayer is energetically more favorable.

16. The transporter has a K_t greater than 0.2 mM and it is a cotransporter, a symporter with Na^+.

17.



The amino acids with the greatest hydropathy index (V, L, F, and C) are clustered on one side of the helix. This amphipathic helix is likely to dip into the lipid bilayer along its hydrophobic surface while exposing its other surface to the aqueous phase. Alternatively, a group of helices may cluster with their polar surfaces in contact with one another and their hydrophobic surfaces facing the lipid bilayer.

18. 0.60 mol

19. Valinomycin is an ionophore that will carry K^+ across the plasma membrane, deflating the membrane potential that is normally achieved by the unequal pumping of Na^+ and K^+ by the Na^+K^+ ATPase.

20. 13 kJ/mol

21. Most of the O_2 consumed by a tissue is for oxidative phosphorylation, the source of most of the ATP. Therefore, about two-thirds of the ATP synthesized by the kidney is used for pumping K^+ and Na^+.

22. Under normal conditions, the Na^+Ca^{2+} exchanger pumps Ca^{2+} out as it allows Na^+ to move inward. The excess of Na^+ on the outside is created by the Na^+K^+ ATPase. When that enzyme is blocked with digoxin, the Na^+ gradient is depleted, and with it, the driving force for Ca^{2+} exit. So Ca^{2+} would flow down its gradient, entering the cell. This (increased Ca^{2+} concentration) is generally lethal for the cell.

23. No. The symporter may carry more than one equivalent of Na^+ for each mole of glucose transported.

24. Treat a suspension of cells with unlabeled NEM in the presence of excess lactose, remove the lactose, then add radiolabeled NEM. Use SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the M_r of the radiolabeled band (the transporter).

25. ATP-dependence signifies active transport; $[Na^+]$-independence suggests primary transport.

26. The leucine transporter is specific for the L isomer, but the binding site can accommodate either L-leucine or L-valine. Reduction of V_{max} in the absence of Na^+ indicates that leucine (or valine) is transported by symport with Na^+. By depleting the Na^+ gradient, ouabain would inhibit L-leucine uptake.

27. The K^+ channel has a "pore" that allows K^+ to diffuse through the channel, stabilized by its interaction with the carbonyl oxygens of the amino acids that line the pore. Na^+ is smaller than K^+, so it is not sterically hindered from passage through the pore, but Na^+ is *too small* to interact with the carbonyl oxygens, so it is not stabilized by that interaction.

28. V_{max} increases; K_t is unaffected.

29. **(a)** Glycophorin A: 1 transmembrane segment; myoglobin: no segments long enough to cross a membrane (not a membrane protein); aquaporin: 6 transmembrane segments (may be a membrane channel or a receptor protein) **(b)** The 15 residue window provides a better signal-to-noise ratio. **(c)** A narrower window reduces the impact of "edge effects" when a transmembrane sequence occurs near either end of the protein.

30. **(a)** The rise per residue for an α helix (Chapter 4) is about 1.5 Å = 0.15 nm. To span a 4 nm bilayer, an α helix must contain about 27 residues; thus, for seven spans, about 190 residues are required. A protein of M_r 64,000 has about 580 residues. **(b)** A hydropathy plot is used to locate transmembrane regions. **(c)** Because about half of this portion of the receptor consists of charged residues, it probably represents an intracellular loop that connects two adjacent membrane-spanning regions of the protein. **(d)** Because this helix is composed mostly of hydrophobic residues, this portion of the receptor is probably one of the membrane-spanning regions of the protein.

31. **(a)** *Model A:* supported. The two dark lines are either the protein layers or the phospholipid heads, and the clear space is either the bilayer or the hydrophobic core, respectively. *Model B:* not supported. This model requires a more-or-less uniformly stained band surrounding the cell. *Model C:* supported, with one reservation. The two dark lines are the phospholipid heads; the clear zone is the tails. This assumes that the membrane proteins are not visible, because they do not stain with osmium or do not happen to be in the sections viewed. **(b)** *Model A:* supported. A "naked" bilayer (4.5 nm) + two layers of protein (2 nm) sums to 6.5 nm, which is within the observed range of thickness. *Model B:* neither. This model makes no predictions about membrane thickness. *Model C:* unclear. The result is hard to reconcile with this model, which predicts a membrane as thick as, or slightly thicker than (due to the projecting ends of embedded proteins), a "naked" bilayer. The model is supported only if the smallest values for membrane thickness are correct or if a substantial amount of protein projects from the bilayer. **(c)** *Model A:* unclear. The result is hard to reconcile with this model. If the proteins are bound to the membrane by ionic interactions, the model predicts that the proteins contain a high proportion of charged amino acids, in contrast to what was observed. Also, because the protein layer must be very thin (see (b)), there would not be much room for a hydrophobic protein core, so hydrophobic residues would be exposed to the solvent. *Model B:* supported. The proteins have a mixture of hydrophobic residues (interacting with lipids) and charged residues (interacting with water). *Model C:* supported. The proteins have a mixture of hydrophobic residues (anchoring in the membrane) and charged residues (interacting with water). **(d)** *Model A:* unclear. The result is hard to reconcile with this model, which predicts a ratio of exactly 2.0; this would be hard to achieve under physiologically relevant pressures. *Model B:* neither. This model makes no predictions about amount of lipid in the membrane. *Model C:* supported. Some membrane surface area is taken up with proteins, so the ratio would be less than 2.0, as was observed under more physiologically relevant conditions. **(e)** *Model A:* unclear. The model predicts proteins in extended conformations rather than globular conformations, so is supported only if one assumes that proteins layered on the surfaces include helical segments. *Model B:* supported. The model predicts mostly globular proteins (containing some helical segments). *Model C:* supported. The model predicts mostly globular proteins. **(f)** *Model A:* unclear. The phosphorylamine head groups are protected by the protein layer, but only if the proteins completely cover the surface will the phospholipids be completely protected from phospholipase. *Model B:* supported. Most head groups are accessible to phospholipase. *Model C:* supported. All head groups are accessible to phospholipase. **(g)** *Model A:* not supported. Proteins are entirely accessible to trypsin digestion, and virtually all will undergo multiple cleavage, with no protected hydrophobic segments. *Model B:* not supported. Virtually all proteins are in the bilayer and inaccessible to trypsin. *Model C:* supported. Segments of protein that penetrate or span the bilayer are protected from trypsin; those exposed at the surfaces will be cleaved. The trypsin-resistant portions have a high proportion of hydrophobic residues.

Chapter 12

1. X is cAMP; its production is stimulated by epinephrine. **(a)** Centrifugation sediments adenylyl cyclase (which catalyzes cAMP formation) in the particulate fraction. **(b)** Added cAMP stimulates glycogen phosphorylase. **(c)** cAMP is heat-stable; it can be prepared by treating ATP with barium hydroxide.

2. Unlike cAMP, dibutyryl cAMP passes readily through the plasma membrane.

3. (a) It increases [cAMP]. **(b)** cAMP regulates Na^+ permeability. **(c)** Replace lost body fluids and electrolytes.

4. (a) The mutation makes R unable to bind and inhibit C, so C is constantly active. **(b)** The mutation prevents cAMP binding to R, leaving C inhibited by bound R.

5. Albuterol raises [cAMP], leading to relaxation and dilation of the bronchi and bronchioles. Because β-adrenergic receptors control many other processes, this drug would have undesirable side effects. To minimize these effects, find an agonist specific for the subtype of β-adrenergic receptors found in bronchial smooth muscle.

6. Hormone degradation; hydrolysis of GTP bound to a G protein; degradation, metabolism, or sequestration of second messenger; receptor desensitization; removal of receptor from the cell surface.

7. Fuse CFP to β-arrestin and fuse YFP to the cytoplasmic domain of the β-adrenergic receptor, or vice versa. In either case, illuminate at 433 nm and observe fluorescence at both 476 nm and 527 nm. If the interaction occurs, emitted light intensity will decrease at 476 nm and increase at 527 nm upon addition of epinephrine to cells expressing the fusion proteins. If the interaction does not occur, the wavelength of emitted light will remain at 476 nm. Some reasons why this might fail: the fusion proteins (1) are inactive or otherwise unable to interact, (2) are not translocated to their normal subcellular location, or (3) are not stable to proteolytic breakdown.

8. Vasopressin acts by elevating cytosolic $[Ca^{2+}]$ to 10^{-6} M, activating protein kinase C. EGTA injection blocks vasopressin action but should not affect the response to glucagon, which uses cAMP, *not* Ca^{2+}, as second messenger.

9. Amplify: (a), (b), (e), (f). Terminate: (c), (d), (f). (f) can contribute to both.

10. IRS1, Grb2, Sos, Ras, Raf, MEK, ERK

11. A mutation in *ras* that inactivated the Ras GTPase activity would create a protein that, once activated by the binding of GTP, would continue to give, through Raf, the insulin-response signal.

12. *Shared properties of Ras and G_s:* Both bind either GDP or GTP; both are activated by GTP; both, when active, activate a downstream enzyme; both have intrinsic GTPase activity that shuts them off after a short period of activation. *Differences:* Ras is a small, monomeric protein; G_s is heterotrimeric. *Functional difference between G_s and G_i:* G_s activates adenylyl cyclase; G_i inhibits it.

13. *Kinase (factor in parentheses):* PKA (cAMP); PKG (cGMP); PKC (Ca^{2+}, DAG); Ca^{2+}/CaM kinase (Ca^{2+}, CaM); cyclin-dependent kinase (cyclin); receptor Tyr kinase (ligand for the receptor, such as insulin); MAPK (Raf); Raf (Ras); glycogen phosphorylase kinase (PKA).

14. G_s remains in its activated form when the nonhydrolyzable analog is bound. The analog therefore prolongs the effect of epinephrine on the injected cell.

15. Individuals with Oguchi disease might have a defect in rhodopsin kinase or in arrestin.

16. Rod cells would no longer show any change in membrane potential in response to light. This experiment has been done. Illumination did activate PDE, but the enzyme could not significantly reduce the 8-Br-cGMP level, which remained well above that needed to keep the gated ion channels open. Thus, light had no impact on membrane potential.

17. Insulin increases glycogen synthesis.

18. Nearly every component of the β-adrenergic and insulin receptor signaling pathways communicates signal activation by some connection through an IDR. The activation loop of protein kinases is an IDR, and the carboxyl-terminal end of most of the protein kinases in those pathways is an IDR. AKAPs and other scaffold proteins serve as anchors to hold pathway components in proximity. Phosphorylation/dephosphorylation of IDRs serves as a switch for the ability of the target proteins to associate.

19. (b), (c), (e), (d), (a)

20. (a) On exposure to heat, TRPV1 channels open, causing an influx of Na^+ and Ca^{2+} into the sensory neuron. This depolarizes the neuron, triggering an action potential. When the action potential reaches the axon terminus, neurotransmitter is released, signaling to the nervous system that heat has been sensed. **(b)** Capsaicin mimics the effects of heat by opening TRPV1 at low temperatures, leading to the false sensation of heat. The extremely low EC_{50} indicates that even very small amounts of capsaicin will have dramatic sensory effects. **(c)** At low levels, menthol should open the TRPM8 channel, leading to a sensation of cool; at high levels, both TRPM8 and TRPV3 open, leading to a mixed sensation of cool and heat, such as you may have experienced with very strong peppermints.

21. (a) These mutations might lead to permanent activation of the PGE_2 receptor, leading to unregulated cell division and tumor formation. **(b)** The viral gene might encode a constitutively active form of the receptor, causing a constant signal for cell division and thus tumor formation. **(c)** E1A protein might bind to pRb and prevent E2F from binding, so E2F is constantly active and cells divide uncontrollably. **(d)** Lung cells do not normally respond to PGE_2 because they do not express the PGE_2 receptor; mutations resulting in a constitutively active PGE_2 receptor would not affect lung cells.

22. A normal tumor suppressor gene encodes a protein that restrains cell division. A mutant form of the protein fails to suppress cell division, but if either of the two alleles in an individual encodes a normal protein, normal function will continue. A normal oncogene encodes a regulator protein that triggers cell division, but only when an appropriate signal (growth factor) is present. The mutant version of the oncogene product constantly sends the signal to divide, whether or not growth factors are present.

23. In a child who develops multiple tumors in both eyes, every retinal cell had a defective copy of the *Rb* gene at birth. Early in the child's life, several cells independently underwent a second mutation that damaged the one good *Rb* allele, producing a tumor. A child who develops a single tumor had, at birth, two good copies of the *Rb* gene in every cell; mutation in both *Rb* alleles in one cell (extremely rare) caused the single tumor.

24. Two cells expressing the same surface receptor may have access to different complements of target proteins for protein phosphorylation and therefore have different responses to the same signal.

25. (a) The data favor the cell-based model, which predicts different receptors present on different cells. **(b)** This experiment addresses the issue of the independence of different taste sensations. Even though the receptors for sweet and/or umami are missing, the animals' other taste sensations are normal; thus, pleasant and unpleasant taste sensations are independent. **(c)** Yes. Loss of either T1R1 or T1R3 subunits abolishes umami taste sensation. **(d)** Both models. With either model, removing one receptor would abolish that taste sensation. **(e)** Yes. Loss of either the T1R2 or T1R3 subunits almost completely abolishes the sweet taste sensation; complete elimination of sweet taste requires deletion of both subunits. **(f)** At very high sucrose concentrations, T1R2 and, to a lesser extent, T1R3 receptors, as homodimers, can detect sweet taste. **(g)** The results are consistent with either model of taste encoding, but they do strengthen the researchers' conclusions. Ligand binding can be completely separated from taste sensation. If the ligand for the receptor in "sweet-tasting cells" binds a molecule, mice prefer that molecule as a sweet compound.

Chapter 13

1. Consider the developing chick as the system; the nutrients, egg shell, and outside world are the surroundings. Transformation of the single cell into a chick drastically reduces the entropy of the system. Initially, the parts of the egg outside the embryo (the surroundings) contain complex fuel molecules (a low-entropy condition). During incubation, some of these complex molecules are converted to large numbers of CO_2 and H_2O molecules (high entropy). This increase in the entropy of the surroundings is larger than the decrease in entropy of the chick (the system).

2. (a) –4.8 kJ/mol **(b)** 7.56 kJ/mol **(c)** –13.7 kJ/mol

3. (a) 262 **(b)** 608 **(c)** 0.30

4. $K'_{eq} = 21$; $\Delta G'^{\circ} = -7.6$ kJ/mol

5. –31 kJ/mol

6. (a) –1.68 kJ/mol **(b)** –4.4 kJ/mol **(c)** At a given temperature, the value of $\Delta G'^{\circ}$ for any reaction is fixed and is defined for standard conditions (here, both fructose 6-phosphate and glucose 6-phosphate at 1 M). In contrast, ΔG is a variable that can be calculated for any set of reactant and product concentrations.

7. $K'_{eq} \approx 1$; $\Delta G'^{\circ} \approx 0$

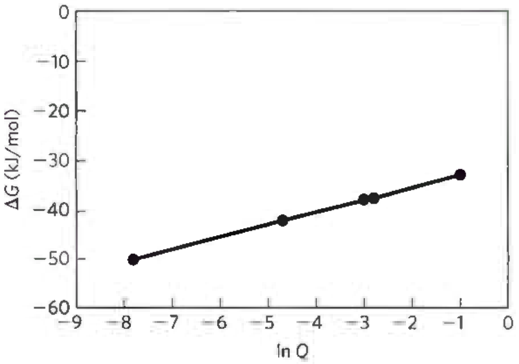
8. Less. The overall equation for ATP hydrolysis can be approximated as

$$ATP^{4-} + H_2O \rightarrow ADP^{3-} + HPO_4^{2-} + H^+$$

(This is only an approximation, because the ionized species shown here are the major, but not the only, forms present.) Under standard conditions ($[ATP] = [ADP] = [P_i] = 1$ M), the concentration of water is 55 M and does not change during the reaction. Because H^+ ions are produced in the reaction, at a higher $[H^+]$ (pH 5.0) the equilibrium would be shifted to the left and less free energy would be released.

9. 10

10.



ΔG for ATP hydrolysis is lower when [ATP]/[ADP] is low ($\ll 1$) than when [ATP]/[ADP] is high. Less energy is available to the cell from a given [ATP] when the [ATP]/[ADP] ratio falls and more is available when it rises.

11. (a) 4.74×10^{-3} M^{-1}; [glucose 6-phosphate] = 1.1×10^{-7} M. No. The cellular [glucose 6-phosphate] is much greater than this, favoring the reverse reaction. **(b)** 11 M. No. The maximum solubility of glucose is less than 1 M. **(c)** 651 ($\Delta G'^{\circ} = -16.7$ kJ/mol); [glucose] = 1.5×10^{-7} M. Yes. This reaction path can occur with a concentration of glucose that is readily soluble and does not produce a large osmotic force. **(d)** No. This would require such high $[P_i]$ that the phosphate salts of divalent cations would precipitate. **(e)** By directly transferring the phosphoryl group from ATP to glucose, the phosphoryl group transfer potential ("tendency" or "pressure") of ATP is utilized without generating high concentrations of intermediates. The essential part of this transfer is, of course, the enzymatic catalysis.

12. (a) –12.5 kJ/mol **(b)** –14.6 kJ/mol

13. (a) 3.16×10^{-4} **(b)** 68.7 **(c)** 7.39×10^{4}

14. –13 kJ/mol

15. 46.7 kJ/mol

16. Isomerization moves the carbonyl group from C-1 to C-2, setting up a carbon–carbon bond cleavage between C-3 and C-4. Without isomerization, bond cleavage would occur between C-2 and C-3, generating one two-carbon compound and one four-carbon compound.

17. The mechanism is the same as that of the alcohol dehydrogenase reaction (see Fig. 14-12).

18. The first step is the reverse of an aldol condensation (see the aldolase mechanism, Fig. 14-5); the second step is an aldol condensation (see Fig. 13-4).

19. (a) Oxidation-reduction, dehydrogenase with NAD cofactor; NADH + H^+ also produced **(b)** Isomerization, isomerase **(c)** Internal rearrangement, isomerase **(d)** Phosphoryl group transfer, kinase and ATP; ADP produced **(e)** Hydrolysis, protease or peptidase and H_2O **(f)** Oxidation-reduction, dehydrogenase with NAD cofactor; NADH + H^+ also produced **(g)** Oxidation-reduction, dehydrogenase with NAD cofactor and H_2O; NADH + H^+ also produced

20. ATP; the products of phosphoarginine hydrolysis are stabilized by resonance forms not available in the intact molecule.

21. Yes. If [ADP] and [polyphosphate] are kept high, and [ATP] is kept low, the actual free-energy change would be negative.

22. (a) 46 kJ/mol **(b)** 46 kg; 68% **(c)** ATP is synthesized as it is needed, then broken down to ADP and P_i; its concentration is maintained in a steady state.

23. The ATP system is in a dynamic steady state; [ATP] remains constant because the rate of ATP consumption equals its rate of synthesis. ATP consumption involves release of the terminal (γ) phosphoryl group; synthesis of ATP from ADP involves replacement of this group. Hence the terminal phosphoryl undergoes rapid turnover. In contrast, the central (β) phosphoryl undergoes only relatively slow turnover.

24. (a) 1.7 kJ/mol **(b)** Inorganic pyrophosphatase catalyzes the hydrolysis of pyrophosphate and drives the net reaction toward the synthesis of acetyl-CoA.

25. Although all the options are possible in principle, the production of AMP and PP_i in the reaction tells us that AMP is the activating group.

26. 36 kJ/mol

27. (d) (a) (c) (b)

28. (a) NAD^+/NADH **(b)** Pyruvate/lactate **(c)** Lactate formation **(d)** –26.1 kJ/mol **(e)** 3.63×10^{4}

29. (a) Initially, electrons will be given up by lactate (converting it to pyruvate) and will flow to fumarate, converting it to succinate. **(b)** –42 kJ/mol **(c)** The four reactants have reached their equilibrium concentrations; $\Delta G = 0$.

30. (a) 1.14 V **(b)** –220 kJ/mol **(c)** ~4

31. (a) –0.35 V **(b)** –0.320 V **(c)** –0.29 V

32. In order of increasing tendency: (a), (d), (b), (c)

33. (c) and (d)

34. (a) 0.0293 **(b)** 308 **(c)** Q is much lower than K'_{eq}, indicating that the PFK-1 reaction is far from equilibrium in cells; this reaction is slower than the subsequent reactions in glycolysis. Flux through the glycolytic pathway is largely determined by the activity of PFK-1.

35. (a) 1.4×10^{-9} M **(b)** The physiological concentration (0.023 mM) is 16,000 times the equilibrium concentration; this reaction does not reach equilibrium in the cell. Many reactions in the cell are not at equilibrium.

36. Malate synthase is saturated with the substrate acetyl-CoA; its concentration is almost 10^2 greater than K_m for acetyl-CoA. But we are not given the concentration or K_m of its other substrate (glyoxylate). If [glyoxylate] is below the K_m for glyoxylate, the reaction rate is limited by [glyoxylate], and malate synthase is not operating at V_{max}.

37. (a) The lowest-energy, highest-entropy state occurs when the dye concentration is the same in both cells. If a "fish trap" gap junction allowed unidirectional transport, more of the dye would end up in the oligodendrocyte and less in the astrocyte. This would be a higher-energy, lower-entropy state than the starting state, violating the second law of thermodynamics. The model proposed by Robinson et al. requires an impossible spontaneous change from a lower-energy state to a higher-energy state without an energy input—again, thermodynamically impossible. **(b)** Molecules, unlike fish, do not exhibit *directed behavior*; they move randomly by Brownian motion. Diffusion results in *net* movement of molecules from a region of higher concentration to a region of lower concentration simply because it is more likely that a molecule on the high-concentration side will enter the connecting channel. The narrower end, like the rate-limiting step of a metabolic pathway, limits the rate at which molecules pass through; random motion of the molecules is less likely to move them through the smaller cross section. The wide end of the channel does *not* act like a funnel for molecules because the narrow end limits the rate of movement equally in both directions. When the concentrations on both sides are equal, the rates of movement in both directions are equal and there will be no change in concentration. **(c)** Fish exhibit *nonrandom behavior*. Fish behavior favors forward movement and avoids crowding and narrow places. Fish that enter the large opening of the channel tend to move forward but then are unlikely to enter the small opening because of their preferred behavior. **(d)** Here are two of many possible explanations: (1) *The dye could bind to a molecule in the oligodendrocyte.* Binding effectively removes the dye from the bulk solvent, yet it remains visible in the fluorescence microscope. (2) *The dye could be sequestered in a subcellular organelle of the oligodendrocyte*, either actively pumped in at the expense of ATP or drawn in by its attraction to other molecules in that organelle.

Chapter 14

1. At equilibrium,

$K_{eq} = 7.8 \times 10^2$ = [ADP][glucose 6-phosphate]/[ATP][glucose]

In living cells, [ADP][glucose 6-phosphate]/[ATP][glucose] = (0.5 mM)(1 mM)/(5 mM)(2 mM) = 0.05. The reaction is therefore *far* from equilibrium: the cellular concentrations of the products (glucose 6-phosphate and ADP) are much lower than expected at equilibrium, and those of the reactants are much higher. The reaction therefore tends strongly to go to the right.

2. Net equation: Glucose + 2ATP → 2 glyceraldehyde 3-phosphate + 2ADP; $\Delta G'^\circ = 2.1$ kJ/mol

3. Net equation: 2 Glyceraldehyde 3-phosphate + 4ADP + 2P_i → 2 lactate + 4ATP + 2H_2O; $\Delta G'^\circ = -114$ kJ/mol

4. –8.6 kJ/mol

5. C-1. This experiment demonstrates the reversibility of the aldolase reaction. The C-1 of glyceraldehyde 3-phosphate is equivalent to C-4 of fructose 1,6-bisphosphate (see Fig. 14-6). The starting glyceraldehyde 3-phosphate must have been labeled at C-1. The C-3 of dihydroxyacetone phosphate becomes labeled through the triose phosphate isomerase reaction, thus giving rise to fructose 1,6-bisphosphate labeled at C-3.

6. No. There would be no anaerobic production of ATP; aerobic ATP production would be diminished only slightly.

7. No. Lactate dehydrogenase is required to recycle NAD^+ from the NADH formed during the oxidation of glyceraldehyde 3-phosphate.

8. The transformation of glucose to lactate occurs when myocytes are low in oxygen, and it provides a means of generating ATP under O_2-deficient conditions. Because lactate can be oxidized to pyruvate, glucose is not wasted; pyruvate is oxidized by aerobic reactions when O_2 becomes plentiful. This metabolic flexibility gives the organism a greater capacity to adapt to its environment.

9. The cell rapidly removes the 1,3-bisphosphoglycerate in a favorable subsequent step, catalyzed by phosphoglycerate kinase.

10. (a) 3-Phosphoglycerate would be the product. **(b)** In the presence of arsenate, there is no net ATP synthesis under anaerobic conditions.

11. (a) Ethanol fermentation requires 2 mol of P_i per mole of glucose. **(b)** Ethanol is the reduced product formed during reoxidation of NADH to NAD^+, and CO_2 is the byproduct of the conversion of pyruvate to ethanol. Yes. Pyruvate must be converted to ethanol to produce a continuous supply of NAD^+ for the oxidation of glyceraldehyde 3-phosphate. Fructose 1,6-bisphosphate accumulates; it is formed as an intermediate in glycolysis. **(c)** Arsenate replaces P_i in the glyceraldehyde 3-phosphate dehydrogenase reaction to yield an acyl arsenate, which spontaneously hydrolyzes. This prevents formation of ATP, but 3-phosphoglycerate continues through the pathway.

12. Dietary niacin is used to synthesize NAD^+. Oxidations carried out by NAD^+ are part of cyclic processes, with NAD^+ as electron carrier (reducing agent); one molecule of NAD^+ can oxidize many thousands of molecules of glucose, and thus the dietary requirement for the precursor vitamin (niacin) is relatively small.

13. Dihydroxyacetone phosphate + NADH + H^+ → glycerol 3-phosphate + NAD^+ (catalyzed by a dehydrogenase)

14. *Galactokinase deficiency:* galactose (less toxic); *transferase deficiency:* galactose 1-phosphate (more toxic)

15. Consumption of alcohol forces competition for NAD^+ between ethanol metabolism and gluconeogenesis. The problem is compounded by strenuous exercise and lack of food, because at these times the level of blood glucose is already low.

16. (a) The rapid increase in glycolysis; the rise in pyruvate and NADH results in a rise in lactate. **(b)** Lactate is transformed to glucose via pyruvate. This is a slower process because formation of pyruvate is limited by NAD^+ availability, the lactate dehydrogenase equilibrium is in favor of lactate, and conversion of pyruvate to glucose is energy-requiring. **(c)** The equilibrium for the lactate dehydrogenase reaction is in favor of lactate formation.

17. Lactate is transformed to glucose in the liver by gluconeogenesis (see Fig. 14-16). A defect in FBPase-1 would prevent entry of lactate into the gluconeogenic pathway in hepatocytes, causing lactate to accumulate in the blood.

18. In the absence of O_2, the ATP needs are met by anaerobic glucose metabolism (fermentation to lactate). Because aerobic oxidation of glucose produces far more ATP than does fermentation, less glucose is needed to produce the same amount of ATP.

19. (a) There are two binding sites for ATP: a catalytic site and a regulatory site. Binding of ATP to a regulatory site inhibits PFK-1, by reducing V_{max} or increasing K_m for ATP at the catalytic site. **(b)** Glycolytic flux is reduced when ATP is plentiful. **(c)** The graph indicates that increased [ADP] suppresses the inhibition by ATP. Because the adenine nucleotide pool is fairly constant, consumption of ATP leads to an increase in [ADP]. The data show that the activity of PFK-1 may be regulated by the [ATP]/[ADP] ratio.

20. The phosphate group of glucose 6-phosphate is completely ionized at pH 7, giving the molecule an overall negative charge. Because membranes are generally impermeable to electrically charged molecules, glucose 6-phosphate cannot pass from the bloodstream into cells and hence cannot enter the glycolytic pathway and generate ATP. (This is why glucose, once phosphorylated, cannot escape from the cell.)

21. $CH_3CHO + NADH + H^+ \rightleftharpoons CH_3CH_2OH + NAD^+ \quad K'_{eq} = 1.45 \times 10^4$

22. (a) $^{14}CH_3CH_2OH$ **(b)** [3-^{14}C]glucose or [4-^{14}C]glucose

When aldolase splits glucose into two trioses phosphates, C-3 and C-4 of glucose become C-1 of the glyceraldehyde 3-phosphate that proceeds through glycolysis.

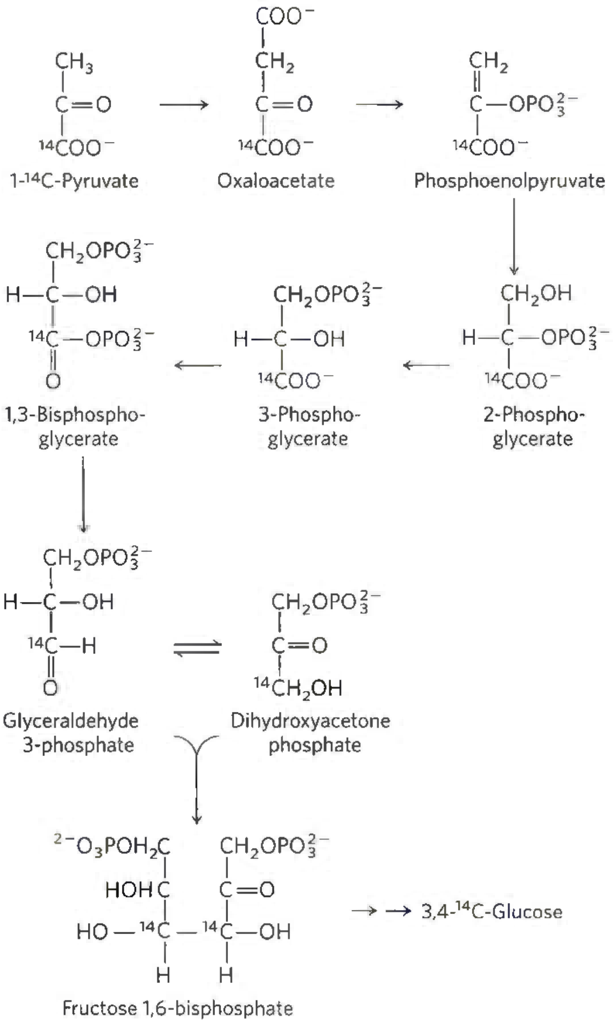
23. Fermentation releases energy, some conserved in the form of ATP but much of it dissipated as heat. Unless the fermenter contents are cooled, the temperature would become high enough to kill the microorganisms.

24. Soybeans and wheat contain starch, a polymer of glucose. The microorganisms break down starch to glucose, glucose to pyruvate via glycolysis, and—because the process is carried out in the absence of O_2 (i.e., it is a fermentation)—pyruvate to lactate and ethanol. If O_2 were present, pyruvate would be oxidized to acetyl-CoA, then to CO_2 and H_2O. Some of the acetyl-CoA, however, would also be hydrolyzed to acetic acid (vinegar) in the presence of oxygen.

25. (a), **(b)**, and **(d)** are glucogenic; **(c)** and **(e)** are not.

26. (a) In the pyruvate carboxylase reaction, $^{14}CO_2$ is added to pyruvate, but PEP carboxykinase removes the *same* CO_2 in the next step. Thus, ^{14}C is not (initially) incorporated into glucose.

(b)



27. 4 ATP equivalents per glucose molecule

28. Gluconeogenesis would be highly endergonic, and it would be impossible to separately regulate gluconeogenesis and glycolysis.

29. The cell "spends" 1 ATP and 1 GTP in converting pyruvate to PEP.

30. The proteins are degraded to amino acids and used for gluconeogenesis.

31. Succinate transforms to oxaloacetate, which passes into the cytosol and is converted to PEP by PEP carboxykinase. Two moles of PEP are then required to produce a mole of glucose by the route outlined in Fig. 14-16.

32. If the catabolic and anabolic pathways of glucose metabolism are operating simultaneously, unproductive cycling of ADP and ATP occurs, with extra O_2 consumption.

33. At the very least, accumulation of ribose 5-phosphate would tend to force this reaction in the reverse direction by mass action (see Eqn 13-4). It might also affect other metabolic reactions that involve ribose 5-phosphate as a substrate or product—such as the pathways of nucleotide synthesis.

34. (a) Ethanol tolerance is likely to involve many more genes, and thus the engineering would be a much more involved project. **(b)** L-Arabinose isomerase (the *araA* enzyme) converts an aldose to a ketose by moving the carbonyl of a nonphosphorylated sugar from C-1 to C-2. No analogous enzyme is discussed in this chapter; all the enzymes described here act on phosphorylated sugars. An enzyme that carries out a similar transformation with phosphorylated sugars is phosphohexose isomerase. L-Ribulokinase (*araB*) phosphorylates a sugar at C-5 by transferring the γ phosphate from ATP. Many such reactions are described in this chapter, including the hexokinase reaction. L-Ribulose 5-phosphate epimerase (*araD*) switches the —H and —OH groups on a chiral carbon of a sugar. No analogous reaction is described in the chapter, but it is described in Chapter 20 (see Fig. 20-31). **(c)** The three *ara* enzymes would convert arabinose to xylulose 5-phosphate by the following pathway: Arabinose $\xrightarrow{\text{L-arabinose isomerase}}$ L-ribulose $\xrightarrow{\text{L-ribulokinase}}$ 5-phosphate $\xrightarrow{\text{epimerase}}$ xylulose 5-phosphate. **(d)** The arabinose is converted to xylulose 5-phosphate as in (c), which enters the pathway in Fig. 14-31a; the glucose 6-phosphate product is then fermented to ethanol and CO_2. **(e)** 6 molecules of arabinose + 6 molecules of ATP are converted to 6 molecules of xylulose 5-phosphate, which feed into the pathway in Fig. 14-31a to yield 5 molecules of glucose 6-phosphate, each of which is fermented to yield 3 ATP (they enter as glucose 6-phosphate, not glucose)—15 ATP in all. Overall, you would expect a yield of 15 ATP − 6 ATP = 9 ATP from the 6 arabinose molecules. The other products are 10 molecules of ethanol and 10 molecules of CO_2. **(f)** Given the lower ATP yield, for an amount of growth (i.e., of available ATP) equivalent to growth without the added genes, the engineered *Z. mobilis* must ferment more arabinose, and thus it produces more ethanol. **(g)** One way to allow the use of xylose would be to add the genes for two enzymes: an analog of the *araD* enzyme that converts xylose to ribose by switching the —H and —OH on C-3, and an analog of the *araB* enzyme that phosphorylates ribose at C-5. The resulting ribose 5-phosphate would feed into the existing pathway.

Chapter 15

1. 11 s

2. (a) *In muscle:* Glycogen breakdown supplies energy (ATP) via glycolysis. Glycogen phosphorylase catalyzes the conversion of stored glycogen to glucose 1-phosphate, which is converted to glucose 6-phosphate, an intermediate in glycolysis. During strenuous activity, skeletal muscle requires large quantities of glucose 6-phosphate. *In the liver:* Glycogen breakdown maintains a steady level of blood glucose between meals (glucose 6-phosphate is converted to free glucose). **(b)** In actively working muscle, ATP flux requirements are very high and glucose 1-phosphate must be produced rapidly, requiring a high V_{max}.

3. (a) 3.5/1 **(b)**, **(c)** The value of this ratio in the cell (>100:1) indicates that [glucose 1-phosphate] is far below the equilibrium value. The rate at which glucose 1-phosphate is removed (through entry into glycolysis) is greater than its rate of production (by the glycogen phosphorylase reaction), so metabolite flow is from glycogen to glucose 1-phosphate. The glycogen phosphorylase reaction is probably the regulatory step in glycogen breakdown.

4. (a) Increases **(b)** Decreases **(c)** Increases

5. *Resting:* [ATP] high; [AMP] low; [acetyl-CoA] and [citrate] intermediate. *Running:* [ATP] intermediate; [AMP] high; [acetyl-CoA] and [citrate] low. Glucose flux through glycolysis increases during the anaerobic sprint because (1) the ATP inhibition of glycogen phosphorylase and PFK-1 is partially relieved, (2) AMP stimulates both enzymes, and (3) lower citrate and acetyl-CoA levels relieve their inhibitory effects on PFK-1 and pyruvate kinase, respectively.

6. The migrating bird relies on the highly efficient aerobic oxidation of fats, rather than the anaerobic metabolism of glucose used by a sprinting rabbit. The bird reserves its muscle glycogen for short bursts of energy during emergencies.

7. *Case A:* (f), (3); *Case B:* (c), (3); *Case C:* (h), (4); *Case D:* (d), (6)

8. (a) (1) Adipose: fatty acid synthesis slower. (2) Muscle: glycolysis, fatty acid synthesis, and glycogen synthesis slower. (3) Liver: glycolysis faster; gluconeogenesis, glycogen synthesis, and fatty acid synthesis slower; pentose phosphate pathway unchanged. **(b)** (1) Adipose and (3) liver: fatty acid synthesis slower because lack of insulin results in inactive acetyl-CoA carboxylase, the first enzyme of fatty acid synthesis. Glycogen synthesis inhibited by cAMP-dependent phosphorylation (thus activation) of glycogen synthase. (2) Muscle: glycolysis slower because GLUT4 is inactive, so glucose uptake is inhibited. (3) Liver: glycolysis slower because the bifunctional PFK-2/FBPase-2 is converted to the form with active FBPase-2, decreasing [fructose 2,6-bisphosphate], which allosterically stimulates phosphofructokinase and inhibits FBPase-1; this also accounts for the stimulation of gluconeogenesis.

9. (a) Elevated **(b)** Elevated **(c)** Elevated

10. (a) PKA cannot be activated in response to glucagon or epinephrine, and glycogen phosphorylase is not activated. **(b)** PP1 remains active, allowing it to dephosphorylate glycogen synthase (activating it) and glycogen phosphorylase (inhibiting it). **(c)** Phosphorylase remains phosphorylated (active), increasing the breakdown of glycogen. **(d)** Gluconeogenesis cannot be stimulated when blood glucose is low, leading to dangerously low blood glucose during periods of fasting.

11. The drop in blood glucose triggers release of glucagon by the pancreas. In the liver, glucagon activates glycogen phosphorylase by stimulating its cAMP-dependent phosphorylation and stimulates gluconeogenesis by lowering [fructose 2,6-bisphosphate], thus stimulating FBPase-1.

12. (a) Reduced capacity to mobilize glycogen; lowered blood glucose between meals **(b)** Reduced capacity to lower blood glucose after a carbohydrate meal; elevated blood glucose **(c)** Reduced concentration of fructose 2,6-bisphosphate (F26BP) in liver, stimulating glycolysis and inhibiting gluconeogenesis **(d)** Reduced [F26BP], stimulating gluconeogenesis and inhibiting glycolysis **(e)** Increased uptake of fatty acids and glucose; increased oxidation of both **(f)** Increased conversion of pyruvate to acetyl-CoA; increased fatty acid synthesis

13. (a) Given that each particle contains about 55,000 glucose residues, the equivalent free glucose concentration would be $55{,}000 \times 0.01\ \mu\text{M} = 550\ \text{mM}$, or 0.55 M. This would present a serious osmotic challenge for the cell! (Body fluids have a substantially lower osmolarity.) **(b)** The lower the number of branches, the lower the number of free ends available for glycogen phosphorylase activity, and the slower the rate of glucose release. With no branches, there would be just one site for phosphorylase to act. **(c)** The outer tier of the particle would be too crowded with glucose residues for the enzyme to gain access to cleave bonds and release glucose. **(d)** The number of chains doubles in each succeeding tier: tier 1 has one chain (2^0), tier 2 has two (2^1), tier 3 has four (2^2), and so on. Thus, for t tiers, the number of chains in the outermost tier, C_A, is 2^{t-1}. **(e)** The total number of chains is $2^0 + 2^1 + 2^2 + \ldots 2^{t-1} = 2^t - 1$. Each chain contains g_c glucose molecules, so the total number of glucose molecules, G_T, is $g_c(2t - 1)$. **(f)** Glycogen phosphorylase can release all but four of the glucose residues in a chain of length g_c. Therefore, from each chain in the outer tier it can release $(g_c - 4)$ glucose molecules. Given that there are 2^{t-1} chains in the outer tier, the number of glucose molecules the enzyme can release, G_{PT}, is $(g_c - 4)(2^{t-1})$. **(g)** The volume of a sphere is $\frac{4}{3}\pi r^3$. In this case, r is the thickness of one tier times the number of tiers, or $(0.12g_c + 0.35)t$ nm. Thus $V_s = \frac{4}{3}\pi t^3(0.12g_c + 0.35)^3\ \text{nm}^3$. **(h)** You can show algebraically that the value of g_c that maximizes f is independent of t. Choosing $t = 7$:

g_c	C_A	G_T	G_{PT}	V_S	f
5	64	635	64	1,232	2,111
6	64	762	128	1,760	3,547
7	64	889	192	2,421	4,512
8	64	1,016	256	3,230	5,154
9	64	1,143	320	4,201	5,572
10	64	1,270	384	5,350	5,834
11	64	1,397	448	6,692	5,986
12	64	1,524	512	8,240	6,060
13	64	1,651	576	10,011	6,079
14	64	1,778	640	12,019	6,059
15	64	1,905	704	14,279	6,011
16	64	2,032	768	16,806	5,943

Note: The optimum value of g_c (i.e., at maximum f) is 13. In nature, g_c varies from 12 to 14, which corresponds to f values very close to the optimum. If you choose another value for t, the numbers will differ but the optimal g_c will still be 13.

Chapter 16

1. (a)

❶ *Citrate synthase:*
Acetyl-CoA + oxaloacetate + H_2O → citrate + CoA
❷ *Aconitase:*
Citrate → isocitrate
❸ *Isocitrate dehydrogenase:*
Isocitrate + NAD^+ → α-ketoglutarate + CO_2 + NADH
❹ *α-Ketoglutarate dehydrogenase:*
α-Ketoglutarate + NAD^+ + CoA → succinyl-CoA + CO_2 + NADH
❺ *Succinyl-CoA synthetase:*
Succinyl-CoA + P_i + GDP → succinate + CoA + GTP
❻ *Succinate dehydrogenase:*
Succinate + FAD → fumarate + $FADH_2$
❼ *Fumarase:*
Fumarate + H_2O → malate
❽ *Malate dehydrogenase:*
Malate + NAD^+ → oxaloacetate + NADH + H^+

(b), (c) ❶ CoA, condensation; ❷ none, isomerization; ❸ NAD^+, oxidative decarboxylation; ❹ NAD^+, CoA, and thiamine pyrophosphate, oxidative decarboxylation; ❺ CoA, substrate-level phosphorylation; ❻ FAD, oxidation; ❼ none, hydration; ❽ NAD^+, oxidation

(d) $\text{Acetyl-CoA} + 3\text{NAD}^+ + \text{FAD} + \text{GDP} + \text{P}_i + 2\text{H}_2\text{O} \rightarrow 2\text{CO}_2 + \text{CoA} + 3\text{NADH} + \text{FADH}_2 + \text{GTP} + 2\text{H}^+$

2. $\text{Glucose} + 4\text{ADP} + 4\text{P}_i + 10\text{NAD}^+ + 2\text{FAD} \rightarrow 4\text{ATP} + 10\text{NADH} + 2\text{FADH}_2 + 6\text{CO}_2$

3. (a) Oxidation; methanol → formaldehyde + [H—H]

(b) Oxidation; formaldehyde + H_2O → formate + [H—H]

(c) Reduction; CO_2 + [H—H] → formate + H^+

(d) Reduction; glycerate + H^+ + [H—H] → glyceraldehyde + H_2O

(e) Oxidation; glycerol → dihydroxyacetone + [H—H]

(f) Oxidation; $2H_2O$ + toluene → benzoate + H^+ + 3[H—H]

(g) Oxidation; succinate → fumarate + [H—H]

(h) Oxidation; pyruvate + H_2O → acetate + CO_2 + [H—H]

4. From the structural formulas, we see that the carbon-bound H/C ratio of hexanoate (11/6) is higher than that of glucose (7/6). Hexanoate is more reduced and yields more energy on complete combustion to CO_2 and H_2O.

5. (a) Oxidized; ethanol + NAD^+ → acetaldehyde + NADH + H^+

(b) Reduced; 1,3-bisphosphoglycerate + NADH + H^+ →
glyceraldehyde 3-phosphate + NAD^+ + HPO_4^{2-}

(c) Unchanged; pyruvate + H^+ → acetaldehyde + CO_2

(d) Oxidized; pyruvate + NAD^+ → acetate + CO_2 + NADH + H^+

(e) Reduced; oxaloacetate + NADH + H^+ → malate + NAD^+

(f) Unchanged; acetoacetate + H^+ → acetone + CO_2

6. *TPP:* thiazolium ring adds to α carbon of pyruvate, then stabilizes the resulting carbanion by acting as an electron sink. *Lipoic acid:* oxidizes pyruvate to level of acetate (acetyl-CoA) and activates acetate as a thioester. *CoA-SH:* activates acetate as thioester. *FAD:* oxidizes lipoic acid. *NAD^+:* oxidizes $FADH_2$.

7. Lack of TPP, caused by thiamine deficiency, inhibits pyruvate dehydrogenase; pyruvate accumulates.

8. Oxidative decarboxylation; NAD^+ or $NADP^+$; α-ketoglutarate dehydrogenase reaction

9. Oxygen consumption is a measure of the activity of the first two stages of cellular respiration: glycolysis and the citric acid cycle. The addition of oxaloacetate or malate stimulates the citric acid cycle and thus stimulates respiration. The added oxaloacetate or malate serves a catalytic role: it is regenerated in the latter part of the citric acid cycle.

10. (a) 5.6×10^{-6} **(b)** 1.1×10^{-8} M **(c)** 28 molecules

11. ADP (or GDP), P_i, CoA-SH, TPP, NAD^+; *not* lipoic acid, which is covalently attached to the isolated enzymes that use it

12. The flavin nucleotides, FMN and FAD, would not be synthesized. Because FAD is required in the citric acid cycle, flavin deficiency would strongly inhibit the cycle.

13. Oxaloacetate might be withdrawn for aspartate synthesis or for gluconeogenesis. Oxaloacetate is replenished by the anaplerotic reactions catalyzed by PEP carboxykinase, PEP carboxylase, malic enzyme, or pyruvate carboxylase (see Fig. 16-15).

14. The terminal phosphoryl group of GTP can be transferred to ADP in a reaction catalyzed by nucleoside diphosphate kinase, with an equilibrium constant of 1.0: GTP + ADP → GDP + ATP.

15. (a) $^-OOC—CH_2—CH_2—COO^-$ (succinate) **(b)** Malonate is a competitive inhibitor of succinate dehydrogenase. **(c)** A block in the citric acid cycle stops NADH formation, which stops electron transfer, which stops respiration. **(d)** A large excess of succinate (substrate) overcomes the competitive inhibition.

16. (a) Add uniformly labeled [^{14}C]glucose and check for the release of $^{14}CO_2$. **(b)** Equally distributed in C-2 and C-3 of oxaloacetate; an infinite number of turns

17. (a) C-1 **(b)** C-3 **(c)** C-3 **(d)** C-2 (methyl group) **(e)** C-4 **(f)** C-4 **(g)** Equally distributed in C-2 and C-3

18. Thiamine is required for the synthesis of TPP, a prosthetic group in the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes. A thiamine deficiency reduces the activity of these enzyme complexes and causes the observed accumulation of precursors.

19. No. For every two carbons that enter as acetate, two leave the cycle as CO_2; thus there is no net synthesis of oxaloacetate. Net synthesis of oxaloacetate occurs by the carboxylation of pyruvate, an anaplerotic reaction.

20. Yes. The citric acid cycle would be inhibited. Oxaloacetate is present at relatively low concentrations in mitochondria, and removing it for gluconeogenesis would tend to shift the equilibrium for the citrate synthase reaction toward oxaloacetate.

21. (a) Inhibition of aconitase **(b)** Fluorocitrate; competes with citrate; by a large excess of citrate **(c)** Citrate and fluorocitrate are inhibitors of PFK-1. **(d)** All catabolic processes necessary for ATP production are shut down.

22. *Glycolysis:*

Glucose + $2P_i$ + 2ADP + $2NAD^+$ →
2 pyruvate + 2ATP + 2NADH + $2H^+$ + $2H_2O$

Pyruvate carboxylase reaction:

2 Pyruvate + $2CO_2$ + 2ATP + $2H_2O$ →
2 oxaloacetate + 2ADP + $2P_i$ + $4H^+$

Malate dehydrogenase reaction:

2 Oxaloacetate + 2NADH + $2H^+$ → 2 L-malate + $2NAD^+$

This sequence recycles nicotinamide coenzymes under anaerobic conditions. The overall reaction is glucose + $2CO_2$ → 2 L-malate + $4H^+$. Four H^+ are produced per glucose, increasing the acidity and thus the tartness of the wine.

23. Pyruvate + ATP + CO_2 + H_2O → oxaloacetate + ADP + P_i + H^+
Pyruvate + CoA + NAD^+ → acetyl-CoA + CO_2 + NADH + H^+
Oxaloacetate + acetyl-CoA → citrate + CoA
Citrate → isocitrate
Isocitrate + NAD^+ → α-ketoglutarate + CO_2 + NADH + H^+
Net reaction: 2 Pyruvate + ATP + $2NAD^+$ + H_2O →
α-ketoglutarate + CO_2 + ADP + P_i + 2NADH + $3H^+$

24. The cycle participates in catabolic and anabolic processes. For example, it generates ATP by substrate oxidation, but also provides precursors for amino acid synthesis (see Fig. 16-15).

25. (a) Decreases **(b)** Increases **(c)** Decreases

26. (a) Citrate is produced through the action of citrate synthase on oxaloacetate and acetyl-CoA. Citrate synthase can be used for net synthesis of citrate when (1) there is a continuous influx of new oxaloacetate and acetyl-CoA and (2) isocitrate synthesis is restricted, as in a culture medium low in Fe^{3+}. Aconitase requires Fe^{3+}, so an Fe^{3+}-restricted medium restricts the synthesis of aconitase.

(b) Sucrose + H_2O → glucose + fructose

Glucose + $2P_i$ + 2ADP + $2NAD^+$ →
2 pyruvate + 2ATP + 2NADH + $2H^+$ + $2H_2O$

Fructose + $2P_i$ + 2ADP + $2NAD^+$ →
2 pyruvate + 2ATP + 2NADH + $2H^+$ + $2H_2O$

2 Pyruvate + $2NAD^+$ + 2CoA →
2 acetyl-CoA + 2NADH + $2H^+$ + $2CO_2$

2 Pyruvate + $2CO_2$ + 2ATP + $2H_2O$ →
2 oxaloacetate + 2ADP + $2P_i$ + $4H^+$

2 Acetyl-CoA + 2 oxaloacetate + $2H_2O$ → 2 citrate + 2CoA

The overall reaction is

Sucrose + H_2O + $2P_i$ + 2ADP + $6NAD^+$ →
2 citrate + 2ATP + 6NADH + $10H^+$

(c) The overall reaction consumes NAD^+. Because the cellular pool of this oxidized coenzyme is limited, it must be regenerated from NADH by the electron-transfer chain, with consumption of O_2. Consequently, the overall conversion of sucrose to citric acid is an aerobic process and requires molecular oxygen.

27. Succinyl-CoA is an intermediate of the citric acid cycle; its accumulation signals reduced flux through the cycle, calling for reduced entry of acetyl-CoA into the cycle. Citrate synthase, by regulating the primary oxidative pathway of the cell, regulates the supply of NADH and thus the flow of electrons from NADH to O_2.

28. Fatty acid catabolism increases [acetyl-CoA], which stimulates pyruvate carboxylase. The resulting increase in [oxaloacetate] stimulates acetyl-CoA consumption by the citric acid cycle, and [citrate] rises, inhibiting glycolysis at the level of PFK-1. In addition, increased [acetyl-CoA] inhibits the pyruvate dehydrogenase complex, slowing the utilization of pyruvate from glycolysis.

29. Oxygen is needed to recycle NAD$^+$ from the NADH produced by the oxidative reactions of the citric acid cycle. Reoxidation of NADH occurs during mitochondrial oxidative phosphorylation.

30. Increased [NADH]/[NAD$^+$] inhibits the citric acid cycle by mass action at the three NAD$^+$-reducing steps; high [NADH] shifts the equilibrium toward NAD$^+$.

31. Toward citrate; ΔG for the citrate synthase reaction under these conditions is about −8 kJ/mol.

32. Steps ④ and ⑤ are essential in the reoxidation of the enzyme's reduced lipoamide cofactor.

33. Many answers are possible. A genetic defect in *MPC1* or *MPC2* switches pyruvate catabolism from the oxidative path (through acetyl-CoA and the citric acid cycle) to the anaerobic reduction of pyruvate to lactate, with a much-increased use of glucose for the glycolytic production of ATP. The increase in cytosolic lactate concentration would acidify that part of the cell. Citric acid cycle activity would slow or would draw on substrates other than glycolytic pyruvate, such as fatty acids from adipose tissue. Blood levels of pyruvate and lactate would rise and blood pH would drop, producing acidosis. Muscle would fatigue easily.

34. The citric acid cycle is so central to metabolism that a serious defect in any cycle enzyme would probably be lethal to the embryo.

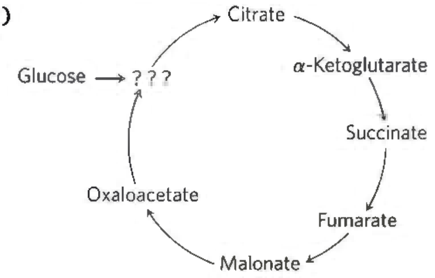
35. (a) The only reaction in muscle tissue that consumes significant amounts of oxygen is cellular respiration, so O_2 consumption is a good proxy for respiration. **(b)** Freshly prepared muscle tissue contains some residual glucose; O_2 consumption is due to oxidation of this glucose. **(c)** Yes. Because the amount of O_2 consumed increased when citrate or 1-phosphoglycerol was added, both can serve as substrate for cellular respiration in this system. **(d)** *Experiment I:* Citrate is causing much more O_2 consumption than would be expected from its complete oxidation. Each molecule of citrate seems to be acting as though it were more than one molecule. The only possible explanation is that each molecule of citrate functions more than once in the reaction—which is how a catalyst operates. *Experiment II:* The key is to calculate the excess O_2 consumed by each sample compared with the control (sample 1).

Sample	Substrate(s) added	μL O_2 absorbed	Excess μL O_2 consumed
1	No extra	342	0
2	0.3 mL 0.2 M 1-phosphoglycerol	757	415
3	0.15 mL 0.02 M citrate	431	89
4	0.3 mL 0.2 M 1-phosphoglycerol + 0.15 mL 0.02 M citrate	1,385	1,043

If both citrate and 1-phosphoglycerol were simply substrates for the reaction, you would expect the excess O_2 consumption by sample 4 to be the sum of the individual excess consumptions by samples 2 and 3 (415 μL + 89 μL = 504 μL). However, the excess consumption when both substrates are present is roughly twice this amount (1,043 μL). Thus citrate increases the ability of the tissue to metabolize 1-phosphoglycerol. This behavior is typical of a catalyst. Both experiments (I and II) are required to make this case convincing. Based on experiment I only, citrate is somehow accelerating the reaction, but it is not clear whether it acts by helping substrate metabolism or by some other mechanism. Based on experiment II only, it is not clear which molecule is the catalyst, citrate or 1-phosphoglycerol. Together, the experiments show that citrate is acting as a "catalyst" for the oxidation of 1-phosphoglycerol.

(e) Given that the pathway can consume citrate (see sample 3), if citrate is to act as a catalyst it must be regenerated. If the set of reactions first consumes then regenerates citrate, it must be a circular rather than a linear pathway. **(f)** When the pathway is blocked at α-ketoglutarate dehydrogenase, citrate is converted to α-ketoglutarate but the pathway goes no further. Oxygen is consumed by reoxidation of the NADH produced by isocitrate dehydrogenase.

(g)



This differs from Fig. 16-7 in that it does not include *cis*-aconitate and isocitrate (between citrate and α-ketoglutarate), or succinyl-CoA, or acetyl-CoA. **(h)** Establishing a quantitative conversion was essential to rule out a branched or other, more complex pathway.

Chapter 17

1. The fatty acid portion; the carbons in fatty acids are more reduced than those in glycerol.

2. Response to glucagon or epinephrine would be prolonged, mobilizing more fatty acids in adipocytes.

3. Fatty acyl groups condensed with CoA in the cytosol are first transferred to carnitine, releasing CoA, then transported into the mitochondrion, where they are again condensed with CoA. The cytosolic and mitochondrial pools of CoA are thus kept separate, and no radioactive CoA from the cytosolic pool enters the mitochondrion.

4. Malonyl-CoA would no longer inhibit entry of fatty acids into the mitochondrion for β oxidation, so there might be a futile cycle of simultaneous fatty acid synthesis in the cytosol and fatty acid breakdown in mitochondria.

5. (a) The carnitine-mediated entry of fatty acids into mitochondria is the rate-limiting step in fatty acid oxidation. Carnitine deficiency slows fatty acid oxidation; added carnitine increases the rate. **(b)** All increase the metabolic need for fatty acid oxidation. **(c)** Carnitine deficiency might result from a deficiency of a carnitine precursor (such as lysine), or from a defect in one of the enzymes in the biosynthesis of carnitine.

6. (a) 4.0×10^5 kJ (9.6×10^4 kcal) **(b)** 48 days **(c)** 0.48 lb/day

7. The first step in fatty acid oxidation is analogous to the conversion of succinate to fumarate; the second step, to the conversion of fumarate to malate; the third step, to the conversion of malate to oxaloacetate.

8. 8 cycles; the last releases 2 acetyl-CoA.

9. (a) R—COO$^-$ + ATP $\rightarrow$ acyl-AMP + PP$_i$
Acyl-AMP + CoA $\rightarrow$ acyl-CoA + AMP

(b) Irreversible hydrolysis of PP$_i$ to 2P$_i$ by cellular inorganic pyrophosphatase

10. *cis*-Δ^3-Dodecanoyl-CoA; it is converted to *cis*-Δ^2-dodecanoyl-CoA, then β-hydroxydodecanoyl-CoA.

11. 4 acetyl-CoA and 1 propionyl-CoA

12. Yes. Some of the tritium is removed from palmitate during the dehydrogenation reactions of β oxidation. The removed tritium appears as tritiated water.

13. (a) In the pigeon, β oxidation predominates; in the pheasant, anaerobic glycolysis of glycogen predominates. **(b)** Pigeon muscle would consume more O_2. **(c)** Fat contains more energy per gram than glycogen does. In addition, the anaerobic breakdown of glycogen is limited by the tissue's tolerance to lactate buildup. Thus the pigeon, using the oxidative catabolism of fats, is the long-distance flyer. **(d)** The enzymes listed in the table are the regulatory enzymes of their respective pathways and thus limit ATP production rate; however, triose phosphate isomerase and malate dehydrogenase are not regulatory enzymes of their respective pathways.

14. Oxidation of fats releases metabolic water; 1.5 L of water per kg of tripalmitoylglycerol (ignores the small contribution of glycerol to the mass).

15. (a) M_r 136; phenylacetic acid **(b)** Even; removal of two carbons at a time from odd-number chains would leave phenylpropionate.

16. Because the mitochondrial pool of CoA is small, CoA must be recycled from acetyl-CoA via the formation of ketone bodies. This allows the operation of the β-oxidation pathway, necessary for energy production.

17. (a) Glucose yields pyruvate via glycolysis, and pyruvate is the main source of oxaloacetate. Without glucose in the diet, [oxaloacetate] drops and the citric acid cycle slows. **(b)** Odd-number; propionate conversion to succinyl-CoA provides intermediates for the citric acid cycle and four-carbon precursors for gluconeogenesis.

18. For the odd-number heptanoic acid, β oxidation produces propionyl-CoA, which can be converted in several steps to oxaloacetate, a starting material for gluconeogenesis. The even-number fatty acid cannot support gluconeogenesis, because it is entirely oxidized to acetyl-CoA.

19. β Oxidation of ω-fluorooleate forms fluoroacetyl-CoA, which enters the citric acid cycle and produces fluorocitrate, a powerful inhibitor of aconitase. Inhibition of aconitase shuts down the citric acid cycle. Without reducing equivalents from the citric acid cycle, oxidative phosphorylation (ATP synthesis) is fatally slowed.

20. Ser to Ala: the Ala side chain in ACC cannot be phosphorylated (and thereby inactivated). Malonyl-CoA continues to be made, which inhibits carnitine acyltransferase 1. β oxidation in mitochondria is blocked. Ser to Asp: ACC has a negative charge where Ser would normally be phosphorylated, so it remains inactive. Fatty acid synthesis is blocked and β oxidation is stimulated (derepressed).

21. Enz-FAD, having a more positive standard reduction potential, is a better electron acceptor than NAD^+, and the reaction is driven in the direction of fatty acyl–CoA oxidation. This more favorable equilibrium is obtained at the cost of 1 ATP; only 1.5 ATP are produced per $FADH_2$ oxidized in the respiratory chain (vs. 2.5 per NADH).

22. 9 turns; arachidic acid, a 20-carbon saturated fatty acid, yields 10 molecules of acetyl-CoA, the last two formed in the ninth turn.

23. See Fig. 17-12. [3-^{14}C]Succinyl-CoA is formed, which gives rise to oxaloacetate labeled at C-2 and C-3.

24. Phytanic acid → pristanic acid → propionyl-CoA →→→ succinyl-CoA → succinate → fumarate → malate. All malate carbons would be labeled, but C-1 and C-4 would have only half as much label as C-2 and C-3.

25. ATP hydrolysis in the energy-requiring reactions of a cell takes up water in the reaction $ATP + H_2O \rightarrow ADP + P_i$; thus, in the steady state, there is no *net* production of H_2O.

26. Digestion of propionate requires methylmalonyl-CoA mutase, which, in turn, requires the cobalt-containing cofactor formed from vitamin B_{12}.

27. Mass lost per day is about 0.66 kg, or about 140 kg in seven months. Ketosis could be avoided by degradation of nonessential body proteins to supply amino acid skeletons for gluconeogenesis.

28. (a) Fatty acids are converted to their CoA derivatives by enzymes in the cytoplasm; the acyl-CoAs are then imported into mitochondria for oxidation. Given that the researchers were using isolated mitochondria, they had to use CoA derivatives. **(b)** Stearoyl-CoA was rapidly converted to 9 acetyl-CoA by the β-oxidation pathway. All intermediates reacted rapidly, and none were detectable at significant levels. **(c)** Two rounds. Each round removes two carbon atoms, thus two rounds convert an 18-carbon to a 14-carbon fatty acid and 2 acetyl-CoA. **(d)** The K_m is higher for the trans isomer than for the cis, so a higher concentration of trans isomer is required for the same rate of breakdown. Roughly speaking, the trans isomer binds less well than the cis, probably because differences in shape, even though not at the target site for the enzyme, affect substrate binding to the enzyme. **(e)** The substrate for LCAD/VLCAD builds up differently, depending on the particular substrate; this is expected for the rate-limiting step in a pathway. **(f)** The kinetic parameters show that the trans isomer is a poorer substrate than the cis for LCAD, but there is little difference for VLCAD. Because it is a poorer substrate, the trans isomer accumulates to higher levels than the cis. **(g)** One possible pathway is shown below (indicating "inside" and "outside" mitochondria).

$$\underset{\text{(outside)}}{\text{Elaidoyl-CoA}} \xrightarrow{\text{carnitine acyltransferase 1}} \underset{\text{(outside)}}{\text{elaidoyl-carnitine}} \xrightarrow{\text{transport}}$$

$$\underset{\text{(inside)}}{\text{elaidoyl-carnitine}} \xrightarrow{\text{carnitine acyltransferase 2}} \underset{\text{(inside)}}{\text{elaidoyl-CoA}} \xrightarrow{\text{2 rounds of } \beta \text{ oxidation}}$$

$$\underset{\text{(inside)}}{\text{5-}\textit{trans}\text{-tetradecenoyl-CoA}} \xrightarrow{\text{thioesterase}} \underset{\text{(inside)}}{\text{5-}\textit{trans}\text{-tetradecanoic acid}} \xrightarrow{\text{diffusion}}$$

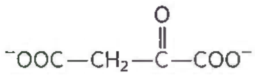
$$\underset{\text{(outside)}}{\text{5-}\textit{trans}\text{-tetradecanoic acid}}$$

(h) It is correct insofar as trans fats are broken down less efficiently than cis fats, and thus trans fats may "leak" out of mitochondria. It is incorrect to say that trans fats are not broken down by cells; they are broken down, but at a slower rate than cis fats.

Chapter 18

1.

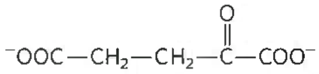
(a)



$$^{-}OOC{-}CH_2{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}COO^{-}$$

Oxaloacetate

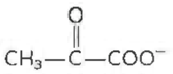
(b)



$$^{-}OOC{-}CH_2{-}CH_2{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}COO^{-}$$

α-Ketoglutarate

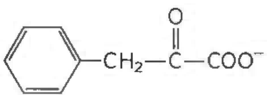
(c)



$$CH_3{-}\overset{\overset{\displaystyle O}{\|}}{C}{-}COO^{-}$$

Pyruvate

(d)



Phenylpyruvate

2. This is a coupled-reaction assay. The product of the slow transamination (pyruvate) is rapidly consumed in the subsequent "indicator reaction" catalyzed by lactate dehydrogenase, which consumes NADH. Thus the rate of disappearance of NADH is a measure of the rate of the aminotransferase reaction. The indicator reaction is monitored by observing the decrease in absorption of NADH at 340 nm with a spectrophotometer.

3. Alanine and glutamine play special roles in the transport of amino groups from muscle and from other nonhepatic tissues, respectively, to the liver.

4. GTP is a product of the citric acid cycle, generated in the second step after the formation of α-ketoglutarate. Elimination of the

GTP inhibition of glutamate dehydrogenase leads to uncon
production of α-ketoglutarate, which is oxidized to produc
levels of ATP. This in turn leads to insulin secretion.

5. No. The nitrogen in alanine can be transferred to oxaloa
transamination, to form aspartate.

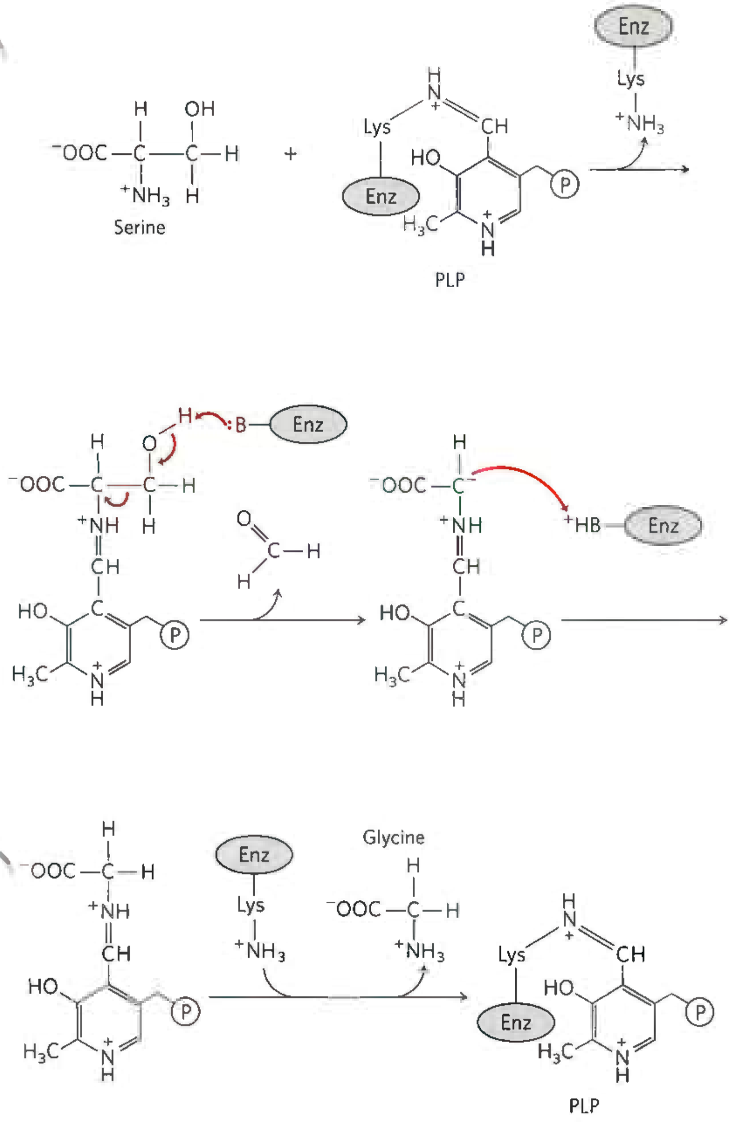
6. 15 mol of ATP per mol of lactate; 13 mol of ATP per mol
when nitrogen removal is included

7. **(a)** Fasting resulted in low blood glucose; subsequent
administration of the experimental diet led to rapid catab
of glucogenic amino acids. **(b)** Oxidative deamination ca
rise in NH_3 levels; the absence of arginine (an intermedia
urea cycle) prevented conversion of NH_3 to urea; arginin
synthesized in sufficient quantities in the cat to meet the
imposed by the stress of the experiment. This suggests t
is an essential amino acid in the cat's diet. **(c)** Ornithine
to arginine by the urea cycle.

8. H_2O + glutamate + NAD^+ → α-ketoglutarate + NH_4^+ + N

[redacted]

19. A likely mechanism is



The formaldehyde (HCHO) produced in the second step reacts rapidly with tetrahydrofolate at the enzyme active site to produce N^5,N^{10}-methylenetetrahydrofolate (see Fig. 18-17).

20. (a) Transamination; no analogies; PLP **(b)** Oxidative decarboxylation; analogous to oxidative decarboxylation of pyruvate to acetyl-CoA prior to entry into the citric acid cycle, and of α-ketoglutarate to succinyl-CoA in the citric acid cycle; NAD^+, FAD, lipoate, and TPP **(c)** Dehydrogenation (oxidation); analogous to dehydrogenation of succinate to fumarate in the citric acid cycle, and of fatty acyl–CoA to enoyl-CoA in β oxidation; FAD **(d)** Carboxylation; no analogies in citric acid cycle or β oxidation; ATP and biotin **(e)** Hydration; analogous to hydration of fumarate to malate in the citric acid cycle, and of enoyl-CoA to 3-hydroxyacyl-CoA in β oxidation; no cofactors **(f)** Reverse aldol reaction; analogous to reverse of citrate synthase reaction in the citric acid cycle; no cofactors

21. Most amino acid catabolism occurs in the liver, including the key steps that are blocked in maple syrup urine disease. A liver transplant from a suitable donor with a normally functioning branched-chain α-keto acid dehydrogenase complex could alleviate disease symptoms.

22. (a) Leucine; valine; isoleucine **(b)** Cysteine (derived from cystine). If cysteine were decarboxylated as shown in Fig. 18-6, it would yield $H_3N—CH_2—CH_2—SH$, which could be oxidized to taurine. **(c)** The January 1957 blood shows significantly elevated levels of isoleucine, leucine, methionine, and valine; the January 1957 urine, significantly elevated isoleucine, leucine, taurine, and valine. **(d)** All patients had high levels of isoleucine, leucine, and valine in both blood and urine, suggesting a defect in the breakdown of these amino acids. Given that the urine also contained high levels of the keto forms of these three amino acids, the block in the pathway must occur after deamination but before dehydrogenation (as shown in Fig. 18-28). **(e)** The model does not explain the high levels of methionine in blood and taurine in urine. The high taurine levels may be due to the death of brain cells during the end stage of the disease. However, the reasons for high levels of methionine in blood are unclear; the pathway of methionine degradation is not linked with the degradation of branched-chain amino acids. Increased methionine could be a secondary effect of buildup of the other amino acids. It is important to keep in mind that the January 1957 samples were from an individual who was dying, so comparing blood and urine results with those of a healthy individual may not be appropriate. **(f)** The following information is needed (and was eventually obtained by other workers): (1) The dehydrogenase activity is significantly reduced or missing in individuals with maple syrup urine disease. (2) The disease is inherited as a single-gene defect. (3) The defect occurs in a gene encoding all or part of the dehydrogenase. (4) The genetic defect leads to production of inactive enzyme.

Chapter 19

1. *Reaction 1:* (a), (d) NADH; (b), (e) E-FMN; (c) $NAD^+/NADH$ and E-FMN/$FMNH_2$

Reaction 2: (a), (d) E-$FMNH_2$; (b), (e) Fe^{3+}; (c) E-FMN/$FMNH_2$ and Fe^{3+}/Fe^{2+}

Reaction 3: (a), (d) Fe^{2+}; (b), (e) Q; (c) Fe^{3+}/Fe^{2+} and Q/QH_2

2. The side chain makes ubiquinone soluble in lipids and allows it to diffuse in the semifluid membrane.

3. From the difference in standard reduction potential ($\Delta E'^\circ$) for each pair of half-reactions, we can calculate $\Delta G'^\circ$. The oxidation of succinate by FAD is favored by the negative standard free-energy change ($\Delta G'^\circ = -3.7$ kJ/mol). Oxidation by NAD^+ would require a large, positive, standard free-energy change ($\Delta G'^\circ = 68$ kJ/mol).

4. (a) All carriers reduced; CN^- blocks the reduction of O_2 catalyzed by cytochrome oxidase. **(b)** All carriers reduced; in the absence of O_2, the reduced carriers are not reoxidized. **(c)** All carriers oxidized **(d)** Early carriers more reduced; later carriers more oxidized

5. (a) Inhibition of NADH dehydrogenase by rotenone decreases the rate of electron flow through the respiratory chain, which in turn decreases the rate of ATP production. If this reduced rate is unable to meet the organism's ATP requirements, the organism dies. **(b)** Antimycin A strongly inhibits the oxidation of Q in the respiratory chain, reducing the rate of electron transfer and leading to the consequences described in (a). **(c)** Because antimycin A blocks *all* electron flow to oxygen, it is a more potent poison than rotenone, which blocks electron flow from NADH but not from $FADH_2$.

6. (a) The rate of electron transfer necessary to meet the ATP demand increases, and thus the P/O ratio decreases. **(b)** High concentrations of uncoupler produce P/O ratios near zero. The P/O ratio decreases and more fuel must be oxidized to generate the same amount of ATP. The extra heat released by this oxidation raises the body temperature. **(c)** Increased activity of the respiratory chain in the presence of an uncoupler requires the degradation of additional fuel. By oxidizing more fuel (including fat reserves) to produce the same amount of ATP, the body loses weight. When the P/O ratio approaches zero, the lack of ATP results in death.

7. Valinomycin acts as an uncoupler. It combines with K^+ to form a complex that passes through the inner mitochondrial membrane, dissipating the membrane potential. ATP synthesis decreases, which causes the rate of electron transfer to increase. This results in an increase in the H^+ gradient, O_2 consumption, and amount of heat released.

8. The steady-state concentration of P_i in the cell is much higher than that of ADP. The P_i released by ATP hydrolysis changes total $[P_i]$ very little.

9. Superoxide dismutase catalyzes the reduction of superoxide to hydrogen peroxide. The hydrogen peroxide can then be eliminated by glutathione peroxidase. This is a major pathway for eliminating the superoxide generated during respiration, helping to ameliorate the damaging effects of reactive oxygen species.

10. **(a)** External medium: 4.0×10^{-8} M; matrix: 2.0×10^{-8} M **(b)** $[H^+]$ gradient contributes 1.7 kJ/mol toward ATP synthesis. **(c)** 21 **(d)** No **(e)** From the overall transmembrane potential

11. **(a)** 0.91 μmol/s • g **(b)** 5.5 s; to provide a constant level of ATP, regulation of ATP production must be tight and rapid.

12. 53 μmol/s • g. With a steady state [ATP] of 7.0 μmol/g, this is equivalent to 10 turnovers of the ATP pool per second; the reservoir would last about 0.13 s.

13. The citric acid cycle is stalled for lack of an acceptor of electrons from NADH. Pyruvate produced by glycolysis cannot enter the cycle as acetyl-CoA; accumulated pyruvate is transaminated to alanine and exported to the liver.

14. Cytosolic malate dehydrogenase plays a key role in the transport of reducing equivalents across the inner mitochondrial membrane via the malate-aspartate shuttle.

15. The inner mitochondrial membrane is impermeable to NADH, but the reducing equivalents of NADH are transferred (shuttled) through the membrane indirectly: they are transferred to oxaloacetate in the cytosol, the resulting malate is transported into the matrix, and mitochondrial NAD^+ is reduced to NADH.

16. Pyruvate dehydrogenase is located in mitochondria; glyceraldehyde 3-phosphate dehydrogenase, in the cytosol. The NAD pools are separated by the inner mitochondrial membrane.

17. **(a)** Glycolysis becomes anaerobic. **(b)** Oxygen consumption ceases. **(c)** Lactate formation increases. **(d)** ATP synthesis decreases to 2 ATP/glucose.

18. The response to **(a)**, increased [ADP], is faster because the response to **(b)**, reduced pO_2, requires protein synthesis.

19. **(a)** NADH is reoxidized via electron transfer instead of lactic acid fermentation. **(b)** Oxidative phosphorylation is more efficient. **(c)** The high mass-action ratio of the ATP system inhibits phosphofructokinase-1.

20. The Pasteur effect is not observed, because the citric acid cycle and respiratory chain are inactive. Fermentation to ethanol could be accomplished in the presence of O_2, which is an advantage because strict anaerobic conditions are difficult to maintain.

21. Reactive oxygen species react with macromolecules, including DNA. If a mitochondrial defect leads to increased production of ROS, proto-oncogenes in the nuclear chromosomes can be damaged, producing oncogenes and leading to unregulated cell division and cancer (see Section 12.9).

22. Different extents of heteroplasmy for the defective gene produce different degrees of defective mitochondrial function.

23. Complete lack of glucokinase (two defective alleles) makes it impossible to carry out glycolysis at a sufficient rate to raise [ATP] to the threshold required for insulin secretion.

24. Defects in Complex II result in increased production of ROS, damage to DNA, and mutations that lead to unregulated cell division (cancer; see Section 12.9). It is not clear why the cancer tends to occur in the midgut.

25. **(a)** Unsaturated fatty acids increase membrane fluidity. **(b)** The cells cannot survive if their membranes contain less than 15% unsaturated fatty acids. **(c)** Oxygen is required for respiration. If cell growth is affected by membrane fluidity only in the presence of oxygen, then logically respiration rates may be affected by membrane fluidity. **(d)** The first observation indicates that something that migrates in the membrane is limiting the rate of respiration. The second observation suggests that respiration is inhibited when unsaturated fatty acid content is low. This might occur if membrane viscosity somehow affected the function of enzymes embedded in the membranes, the passive permeation of oxygen, or the rate of diffusion of a key factor in the membrane itself. **(e)** The overall conclusion is that ubiquinone diffusion through the membrane limits the rate of respiration.

Chapter 20

1. For the maximum photosynthetic rate, PSI (which absorbs light of 700 nm) and PSII (which absorbs light of 680 nm) must be operating simultaneously.

2. From water consumed in the overall reaction

3. H_2S is the hydrogen donor in photosynthesis. No O_2 is evolved, because H_2O is not split; the single photosystem lacks the water-splitting cofactor.

4. **(a)** Stops **(b)** Slows; some electron flow continues by the cyclic pathway.

5. During illumination, a proton gradient is established. When ADP and P_i are added, ATP synthesis is driven by the gradient, which becomes exhausted in the absence of light.

6. DCMU blocks electron transfer between PSII and the first site of ATP production.

7. Venturicidin blocks proton movement through the CF_oCF_1 complex; electron flow (O_2 evolution) continues only until the free-energy cost of pumping protons against the rising proton gradient equals the free energy available in a photon. DNP, by dissipating the proton gradient, restores electron flow and O_2 evolution.

8. From the difference in reduction potentials, you can calculate $\Delta G'^{\circ} = 15$ kJ/mol for the redox reaction. Fig. 20-4 shows that the energy of photons in any region of the visible spectrum is more than sufficient to drive this endergonic reaction. Even photons in the infrared spectrum can provide sufficient energy.

9. 1.35×10^{-77}; the reaction is highly unfavorable! In chloroplasts, the input of light energy overcomes this barrier.

10. –968 kJ/mol

11. No. The electrons from H_2O flow to the artificial electron acceptor Fe^{3+}, not to $NADP^+$.

12. About once every 0.1 s; 1 in 10^8 is excited

13. Light of 700 nm excites PSI, but not PSII; electrons flow from P700 to $NADP^+$, but no electrons flow from P680 to replace them. When light of 680 nm excites PSII, electrons tend to flow to PSI, but the electron carriers between the two photosystems quickly become completely reduced.

14. No. The excited electron from P700 returns to refill the electron "hole" created by illumination. PSII is not needed to supply electrons, and no O_2 is evolved from H_2O. No NADPH is formed, because the excited electron returns to P700. Cyclic photophosphorylation produces ATP rather than NADPH.

15. ATP and NADPH are generated in the light and are essential for CO_2 fixation; conversion stops as the supply of ATP and NADPH becomes exhausted. Some enzymes are switched off in the dark.

16. X is 3-phosphoglycerate; Y is ribulose 1,5-bisphosphate.

17. Ribulose 5-phosphate kinase, fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, and glyceraldehyde 3-phosphate dehydrogenase; all are activated by reduction of a critical disulfide bond to a pair of sulfhydryls, which iodoacetate then blocks irreversibly.

18. The reductive pentose phosphate pathway regenerates ribulose 1,5-bisphosphate from triose phosphates produced during photosynthesis. The oxidative pentose phosphate pathway provides NADPH for reductive biosynthesis and ribose 5-phosphate for nucleotide synthesis.

19. Both types of "respiration" occur in plants, consume O_2, and produce CO_2. (Mitochondrial respiration also occurs in animals.) Mitochondrial respiration takes place continuously, though primarily at night or on cloudy days; electrons derived from various fuels are passed through a chain of carriers in the inner mitochondrial membrane to O_2. Photorespiration takes place in chloroplasts, peroxisomes, and mitochondria, during the daytime, when photosynthetic carbon fixation is occurring. Electron transfer in photorespiration is shown in Fig. 20-43; that for mitochondrial respiration, in Fig. 19-19.

20. In maize, the C_4 pathway fixes CO_2. Phosphoenolpyruvate (PEP) carboxylase carboxylates PEP to form oxaloacetate. Some of the oxaloacetate undergoes transamination to aspartate, but most undergoes reduction to malate in the mesophyll cells. Only after subsequent decarboxylation does the CO_2 enter the Calvin cycle.

21. Measure the amount of $^{14}CO_2$ fixed in leaves during an hour of darkness and an hour of bright illumination. The CAM plant will take up much more CO_2 at night. Alternatively, measure the concentration of organic acids in the vacuoles by titrating an extract of leaves. In darkness, the C_4 plant will have a lower level of titratable acidity.

22. Isocitrate dehydrogenase reaction

23. Rates of photorespiration, which occurs when rubisco uses O_2 rather than CO_2 as a substrate, are increased at higher light intensities and higher leaf temperatures. C_4 plants evolved mechanisms to minimize photorespiration, resulting in an increased ability to perform photosynthesis under these conditions. Because PEP carboxylase has a higher affinity for CO_2 than rubisco, C_4 plants take up more CO_2 under conditions of low $[CO_2]$. Thus, species 1 is a C_4 plant and species 2 is a C_3 plant.

24. $[PP_i]$ is high in the cytosol because the cytosol lacks inorganic pyrophosphatase.

25. (a) Low $[P_i]$ in the cytosol and high [triose phosphate] in the chloroplast **(b)** High $[P_i]$ and [triose phosphate] in the cytosol

26. 3-Phosphoglycerate is the primary product of photosynthesis; $[P_i]$ rises when light-driven synthesis of ATP from ADP and P_i slows.

27. (a) Sucrose + $(\text{glucose})_n \rightarrow (\text{glucose})_{n+1}$ + fructose **(b)** Fructose generated in the synthesis of dextran is readily imported and metabolized by the bacteria.

28. The first enzyme in each path is under reciprocal allosteric regulation. Inhibition of one path shunts isocitrate into the other path.

29. (a) (1) The presence of Mg^{2+} supports the hypothesis that chlorophyll is directly involved in catalysis of the phosphorylation reaction, ADP + $P_i \rightarrow$ ATP. (2) Many enzymes (or other proteins) that contain Mg^{2+} are not phosphorylating enzymes, so the presence of Mg^{2+} in chlorophyll does not prove its role in phosphorylation reactions. (3) The presence of Mg^{2+} is essential to chlorophyll's photochemical properties: light absorption and electron transfer. **(b)** (1) Enzymes catalyze reversible reactions, so an isolated enzyme that can, under certain lab conditions, catalyze removal of a phosphoryl group could probably, under different conditions (such as in cells), catalyze addition of a phosphoryl group. So, chlorophyll could be involved in the phosphorylation of ADP. (2) There are two possible explanations: the chlorophyll protein is a phosphatase only and does not catalyze ADP phosphorylation under cellular conditions, or the crude preparation contains a contaminating phosphatase activity that is unconnected to the photosynthetic reactions. (3) The preparation was probably contaminated with a nonphotosynthetic phosphatase activity. **(c)** (1) This light inhibition would be expected if the chlorophyll protein catalyzed the reaction ADP + P_i + light $\rightarrow$ ATP. Without light, the reverse reaction, a dephosphorylation, would be favored. In the presence of light, energy is provided and the equilibrium would shift to the right, reducing the phosphatase activity. (2) This inhibition must be an artifact of the isolation or assay methods. (3) The crude preparation methods in use at the time were unlikely to preserve intact chloroplast membranes, so the inhibition must be an artifact. **(d)** In the presence of light, (1) ATP is synthesized and other phosphorylated intermediates are consumed; (2) glucose is produced and is metabolized by cellular respiration to produce ATP, with changes in the levels of phosphorylated intermediates; (3) ATP is produced and other phosphorylated intermediates are consumed. **(e)** Light energy is used to produce ATP (as in the Emerson model) *and* is used to produce reducing power (as in the Rabinowitch model). **(f)** The approximate stoichiometry for photophosphorylation is that 8 photons yield 2 NADPH and about 3 ATP. To reduce 1 CO_2 requires 2 NADPH and 3 ATP. Thus, at a minimum, 8 photons are required per CO_2 molecule reduced, in good agreement with Rabinowitch's value. **(g)** Because the energy of light is used to produce *both* ATP and NADPH, each photon absorbed contributes more than just 1 ATP for photosynthesis. The process of energy extraction from light is more efficient than Rabinowitch supposed, and plenty of energy is available for this process—even with red light.

Chapter 21

1. (a) The 16 carbons of palmitate are derived from 8 acetyl groups of 8 acetyl-CoA molecules. The ^{14}C-labeled acetyl-CoA gives rise to malonyl-CoA labeled at C-1 and C-2. **(b)** The metabolic pool of malonyl-CoA, the source of all palmitate carbons except C-16 and C-15, does not become labeled with small amounts of ^{14}C-labeled acetyl-CoA. Hence, only $[15,16\text{-}^{14}C]$ palmitate is formed.

2. Both glucose and fructose are degraded to pyruvate in glycolysis. Pyruvate is converted to acetyl-CoA by the pyruvate dehydrogenase complex. Some of this acetyl-CoA enters the citric acid cycle, which produces reducing equivalents, NADH and NADPH. Mitochondrial electron transfer to O_2 yields ATP.

3. $$8\ \text{Acetyl-CoA} + 15\text{ATP} + 14\text{NADPH} + 9H_2O \rightarrow \text{palmitate} + 8\text{CoA} + 15\text{ADP} + 15P_i + 14\text{NADP}^+ + 2H^+$$

4. (a) 3 deuteriums per palmitate; all located on C-16; all other two-carbon units are derived from unlabeled malonyl-CoA **(b)** 7 deuteriums per palmitate; located on all *even*-numbered carbons except C-16

5. By using the three-carbon unit malonyl-CoA, the activated form of acetyl-CoA (recall that malonyl-CoA synthesis requires ATP), metabolism is driven in the direction of fatty acid synthesis by the exergonic release of CO_2.

6. (a) The rate-limiting step in fatty acid synthesis is carboxylation of acetyl-CoA, catalyzed by acetyl-CoA carboxylase. High [citrate] and [isocitrate] indicate that conditions are favorable for fatty acid synthesis: an active citric acid cycle is providing a plentiful supply of ATP, reduced pyridine nucleotides, and acetyl-CoA. Citrate stimulates (increases the V_{max} of) acetyl-CoA carboxylase **(b)**. Because citrate binds more tightly to the filamentous (active) form of the enzyme, high [citrate] drives the protomer $\rightleftharpoons$ filament equilibrium in the direction of the active form. In contrast, palmitoyl-CoA (the end product of fatty acid synthesis) drives the equilibrium in the direction of the inactive (protomer) form. Hence, when the end product of fatty acid synthesis accumulates, the biosynthetic path slows.

7. (a) $\text{Acetyl-CoA}_{(mit)} + \text{ATP} + \text{CoA}_{(cyt)} \rightarrow$

$$\text{acetyl-CoA}_{(cyt)} + \text{ADP} + \text{P}_i + \text{CoA}_{(mit)}$$

(b) 1 ATP per acetyl group **(c)** Yes

8. No. The double bond in palmitoleate is introduced by an oxidation catalyzed by fatty acyl–CoA desaturase, a mixed-function oxidase that requires O_2 as a cosubstrate.

9. $3\ \text{Palmitate} + \text{glycerol} + 7\text{ATP} + 4H_2O \rightarrow$

$$\text{tripalmitin} + 7\text{ADP} + 7\text{P}_i + 7\text{H}^+$$

10. In adult rats, stored triacylglycerols are maintained at a steady-state level through a balance of rates of degradation and biosynthesis. Hence, triacylglycerols of adipose (fat) tissue are constantly turned over, which explains the incorporation of ^{14}C label from dietary glucose.

11. Net reaction:

$\text{Dihydroxyacetone phosphate} + \text{NADH} + \text{palmitate} + \text{oleate} + 3\text{ATP} + \text{CTP} + \text{choline} + 4H_2O \rightarrow$

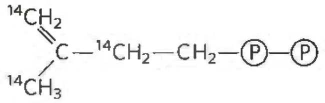
$$\text{phosphatidylcholine} + \text{NAD}^+ + 2\text{AMP} + \text{ADP} + \text{H}^+ + \text{CMP} + 5\text{P}_i$$

7ATP per molecule of phosphatidylcholine

12. Methionine deficiency reduces the level of adoMet, which is required for de novo synthesis of phosphatidylcholine. The salvage pathway does not employ adoMet, but uses available choline. Thus phosphatidylcholine can be synthesized even when the diet is deficient in methionine, as long as choline is available.

13. During cholesterol biosynthesis, the two Claisen condensations involving acetyl-CoA and leading to HMG-CoA are both thermodynamically unfavorable. However, the product HMG-CoA is rapidly siphoned off by more thermodynamically favorable subsequent reactions. In fatty acid synthesis, all of the condensation reactions involving each new malonyl-CoA are identical. If acetyl-CoA were utilized instead of malonyl-CoA, all would be thermodynamically unfavorable. As subsequent reduction steps that expend NADPH in each four-step cycle are also thermodynamically unfavorable, there are no downstream processes capable of balancing the thermodynamics and pulling the sequence toward synthesis. The synthesis of long fatty acids would not be chemically feasible without the use of malonyl-CoA and the thermodynamic boost provided by decarboxylation.

14. ^{14}C label appears in three places in the activated isoprene:



15. (a) ATP **(b)** UDP-glucose **(c)** CDP-ethanolamine **(d)** UDP-galactose **(e)** Fatty acyl–CoA **(f)** *S*-Adenosylmethionine **(g)** Malonyl-CoA **(h)** Δ^3-Isopentenyl pyrophosphate

16. Linoleate is required in the synthesis of prostaglandins. Animals cannot transform oleate to linoleate, so linoleate is an essential fatty acid. Plants can convert oleate to linoleate, and they provide animals with the required linoleate (see Fig. 21-12).

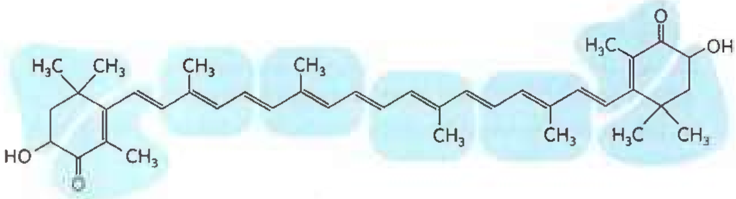
17. The rate-determining step in the biosynthesis of cholesterol is the synthesis of mevalonate, catalyzed by HMG-CoA reductase. This enzyme is allosterically regulated by mevalonate and cholesterol derivatives. High intracellular [cholesterol] also reduces transcription of the gene encoding HMG-CoA reductase.

18. When cholesterol levels decline because of treatment with a statin, cells attempt to compensate by increasing expression of the gene encoding HMG-CoA reductase; however, statins are good competitive inhibitors of HMG-CoA reductase activity and reduce overall production of cholesterol.

19. Note: In the absence of detailed knowledge of the literature on this enzyme, students might propose several plausible alternatives. *Thiolase reaction:* Begins with nucleophilic attack of an active-site Cys residue on the first acetyl-CoA substrate, displacing —S-CoA and forming a covalent thioester link between Cys and the acetyl group. A base on the enzyme then extracts a proton from the methyl group of the second acetyl-CoA, leaving a carbanion that attacks the carbonyl carbon of the thioester formed in the first step. The sulfhydryl of the Cys residue is displaced, creating the product acetoacetyl-CoA. *HMG-CoA synthase reaction:* Begins in the same way, with a covalent thioester link formed between the enzyme's Cys residue and the acetyl group of acetyl-CoA, with displacement of —S-CoA. The —S-CoA dissociates as CoA-SH, and acetoacetyl-CoA binds to the enzyme. A proton is abstracted from the methyl group of the enzyme-linked acetyl, forming a carbanion that attacks the ketone carbonyl of the acetoacetyl-CoA substrate. The carbonyl is converted to a hydroxyl ion in this reaction, which is protonated to create —OH. The thioester link with the enzyme is then cleaved hydrolytically to generate the HMG-CoA product. *HMG-CoA reductase reaction:* Two successive hydride ions derived from NADPH first displace the —S-CoA, and then reduce the aldehyde to a hydroxyl group.

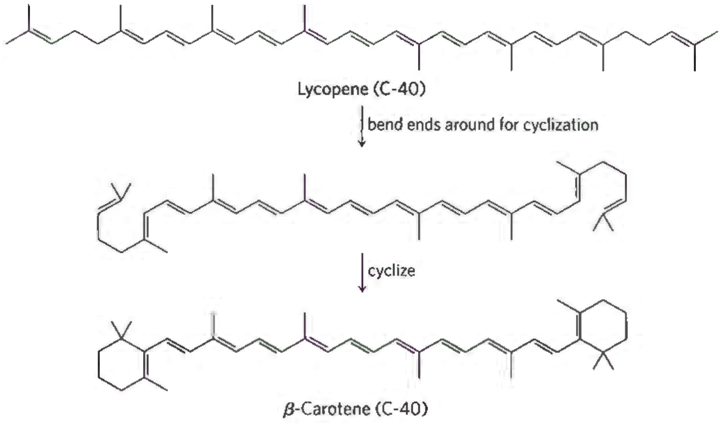
20. Statins inhibit HMG-CoA reductase, an enzyme in the pathway to the synthesis of activated isoprenes, which are precursors of cholesterol and a wide range of isoprenoids, including coenzyme Q (ubiquinone). Hence, statins might reduce the levels of coenzyme Q available for mitochondrial respiration. Ubiquinone is obtained in the diet as well as by direct biosynthesis, but it is not yet clear how much is required and how well dietary sources can substitute for reduced synthesis. Reductions in the levels of particular isoprenoids may account for some side effects of statins.

21. (a)



Astaxanthin

(b) Head-to-head. There are two ways to look at this. First, the "tail" of geranylgeranyl pyrophosphate has a branched dimethyl structure, as do both ends of phytoene. Second, no free —OH is formed by the release of PP_i, indicating that the two —O—Ⓟ—Ⓟ "heads" are linked to form phytoene. **(c)** Four rounds of dehydrogenation convert four single bonds to double bonds. **(d)** No. A count of single and double bonds in the reaction below shows that one double bond is replaced by two single bonds—so, there is no net oxidation or reduction:



(e) Steps ❶ through ❸. The enzyme can convert IPP and DMAP to geranylgeranyl pyrophosphate, but it catalyzes no further reactions in the pathway, as confirmed by results with the other substrates. **(f)** Strains 1 through 4 lack *crtE* and have much lower astaxanthin production than strains 5 through 8, all of which overexpress *crtE*. Thus, overexpression of *crtE* leads to a substantial increase in astaxanthin production. Wild-type *E. coli* has some step ❸ activity, but this conversion of farnesyl pyrophosphate to geranylgeranyl pyrophosphate is strongly rate-limiting. **(g)** IPP isomerase. Comparing strains 5 and 6 shows that adding *ispA*, which catalyzes steps ❶ and ❷, has little effect on astaxanthin production, so these steps are not rate-limiting. However, comparing strains 5 and 7 shows that adding *idi* substantially increases astaxanthin production, so IPP isomerase must be the rate-limiting step when *crtE* is overexpressed.

Chapter 22

1. In their symbiotic relationship with the plant, bacteria supply ammonium ion by reducing atmospheric nitrogen, a process that requires large quantities of ATP because of the very high activation energy of ammonia production from N_2.

2. Fixed nitrogen is limiting in most environments, including marine ecosystems. Large increases in fixed nitrogen help to feed algae blooms, with an accompanying increase in aerobic respiration, which depletes oxygen in the affected waters.

3. A link is formed between enzyme-bound PLP and the phosphohomoserine substrate, with rearrangement to generate the ketimine at the α carbon of the substrate. This activates the β carbon for proton abstraction, leading to displacement of the phosphate and formation of a double bond between the β and γ carbons. A rearrangement (beginning with proton abstraction at the pyridoxal carbon adjacent to the substrate amino nitrogen) moves the α–β double bond and converts the ketimine to the aldimine form of PLP. Attack of water at the β carbon is then facilitated by the linked pyridoxal, followed by hydrolysis of the imine link between PLP and the product, to generate threonine.

4. In the mammalian route, toxic ammonium ions are transformed to glutamine, reducing toxic effects on the brain.

5. Glucose + $2CO_2$ + $2NH_3$ → 2 aspartate + $2H^+$ + $2H_2O$

6. The amino-terminal glutaminase domain is similar in *all* glutamine amidotransferases. A drug that targeted this active site would probably inhibit many enzymes and produce many more side effects than a more specific inhibitor that targets the unique carboxyl-terminal synthetase active site.

7. If phenylalanine hydroxylase is defective, the biosynthetic route to tyrosine is blocked and tyrosine must be obtained from the diet.

8. The biosynthetic pathway requires reduction of the γ-carboxyl group of glutamate to a carbonyl. Prior acetylation of the α-amino group prevents a spontaneous cyclization reaction that leads not to arginine, but to proline.

9. In adoMet synthesis, triphosphate is released from ATP. Hydrolysis of the triphosphate renders the reaction thermodynamically more favorable.

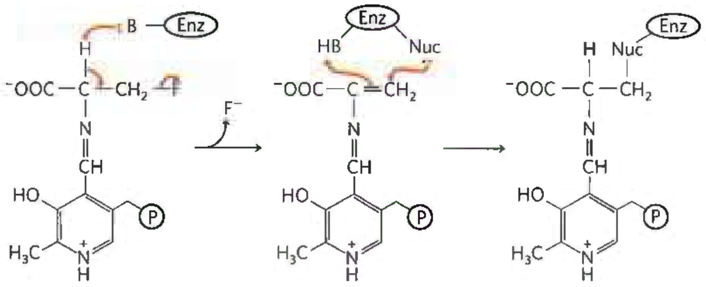
10. If the inhibition of glutamine synthase were not concerted, saturating concentrations of histidine would shut down the enzyme and cut off production of glutamine, which the bacterium needs to synthesize other products.

11. Folic acid is a precursor of tetrahydrofolate (see Fig. 18-16), required in the biosynthesis of glycine (see Fig. 22-14), a precursor of porphyrins. A folic acid deficiency therefore impairs hemoglobin synthesis.

12. This is a PLP-catalyzed decarboxylation.

13. *Glycine auxotrophs:* adenine and guanine; *glutamine auxotrophs:* adenine, guanine, and cytosine; *aspartate auxotrophs:* adenine, guanine, cytosine, and uridine

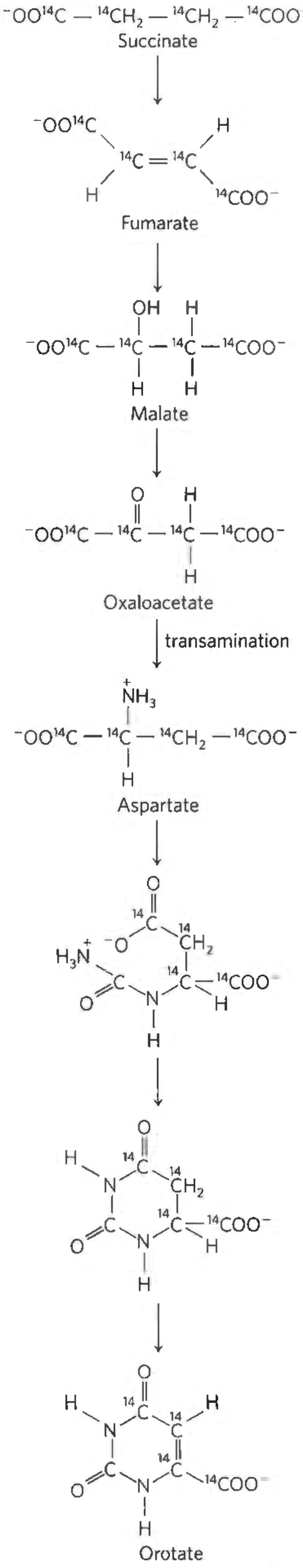
14. See Fig. 18-6, step ❷, for the reaction mechanism of amino acid racemization. The F atom of fluoroalanine is an excellent leaving group. Fluoroalanine causes irreversible (covalent) inhibition of alanine racemase. One plausible mechanism (where Nuc denotes any nucleophilic amino acid side chain in the enzyme active site) is



15. (a) As shown in Fig. 18-16, *p*-aminobenzoate is a component of tetrahydrofolate (H_4 folate), the cofactor involved in the transfer of one-carbon units. **(b)** In the presence of sulfanilamide, a structural analog of *p*-aminobenzoate, bacteria are unable to synthesize tetrahydrofolate, a cofactor necessary for converting AICAR to FAICAR; thus, AICAR accumulates. **(c)** The competitive inhibition by sulfanilamide of the enzyme involved in tetrahydrofolate biosynthesis is overcome by the addition of excess substrate (*p*-aminobenzoate).

16. (b) and **(d)**

17. The ^{14}C-labeled orotate arises from the following pathway (the first three steps are part of the citric acid cycle):

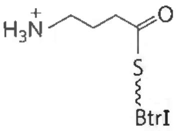


18. Organisms do not store nucleotides to be used as fuel, and they do not completely degrade them, but rather hydrolyze them to release the bases, which can be recovered in salvage pathways. The low C:N ratio of nucleotides makes them poor sources of energy.

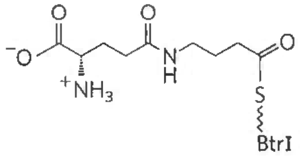
19. Treatment with allopurinol has two consequences. (1) It inhibits conversion of hypoxanthine to uric acid, causing accumulation of hypoxanthine, which is more soluble and more readily excreted; this alleviates the clinical problems associated with AMP degradation. (2) It inhibits conversion of guanine to uric acid, causing accumulation of xanthine, which is less soluble than uric acid; this is the source of xanthine stones. Because the amount of GMP degradation is low relative to AMP degradation, the kidney damage caused by xanthine stones is less than the damage caused by untreated gout.

20. Tetrahydrofolate is utilized in thymidylate synthesis, and also in the synthesis of glycine from serine.

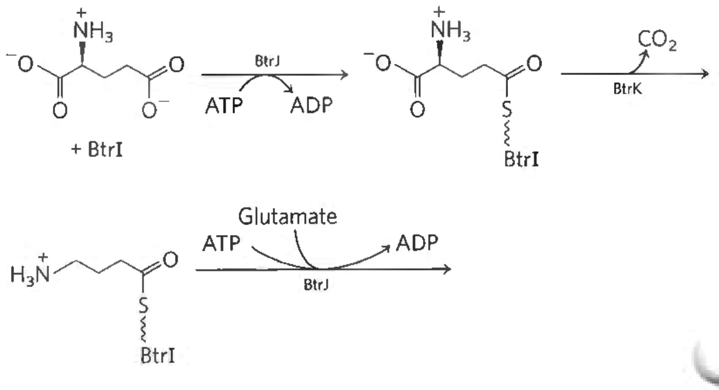
21. (a) The α-carboxyl group is removed and an —OH is added to the γ carbon. **(b)** BtrI has sequence homology with acyl carrier proteins. The molecular weight of BtrI increases when incubated under conditions in which CoA could be added to the protein. Adding CoA to a Ser residue would replace an —OH, formula weight (FW) 17, with a 4′-phosphopantetheine group (see Fig. 21-5; formula $C_{11}H_{21}N_2O_7PS$), FW 356. Thus, $11{,}182 - 17 + 356 = 12{,}151$, which is very close to the observed M_r of 12,153. **(c)** The thioester could form with the α-carboxyl group. **(d)** In the most common reaction for removing the α-carboxyl group of an amino acid (see Fig. 18-6, reaction Ⓒ), the carboxyl group must be free. Furthermore, it is difficult to imagine a decarboxylation reaction starting with a carboxyl group in its thioester form. **(e)** $12{,}240 - 12{,}281 = 41$, close to the M_r of CO_2 (44). Given that BtrK is probably a decarboxylase, the most likely structure is the decarboxylated form:

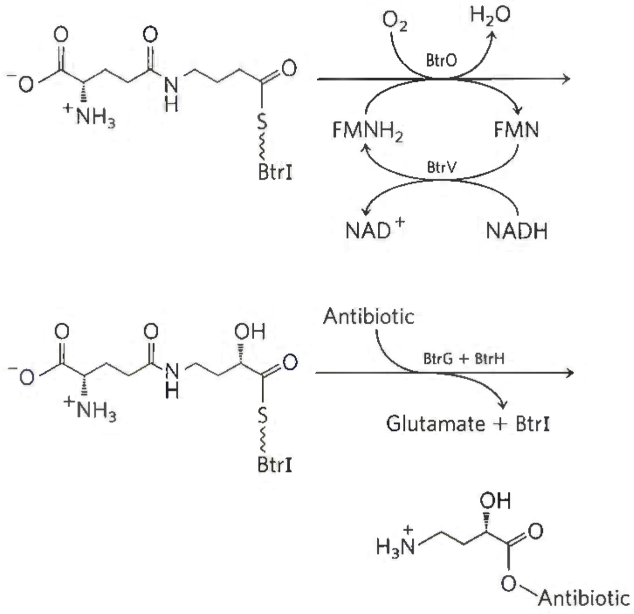


(f) $12{,}370 - 12{,}240 = 130$. Glutamic acid ($C_5H_9NO_4$; M_r 147), minus the —OH (FW 17) removed in the glutamylation reaction, leaves a glutamyl group of FW 130; thus, γ-glutamylating the molecule shown above would add 130 to its M_r. BtrJ is capable of γ-glutamylating other substrates, so it may γ-glutamylate this molecule. The most likely site for this is the free amino group, giving the following structure:



(g)





Chapter 23

1. They are recognized by two different receptors, typically found in different cell types, and are coupled to different downstream effectors.

2. Ammonia is highly toxic to nervous tissue, especially the brain. In healthy individuals, excess NH_3 is removed by transformation of glutamate to glutamine, which travels to the liver and is subsequently transformed to urea. The additional glutamine arises from conversion of glucose to α-ketoglutarate, transamination of α-ketoglutarate to glutamate, and conversion of glutamate to glutamine.

3. Glucogenic amino acids are used to make glucose for the brain; others are deaminated, then oxidized in mitochondria via the citric acid cycle.

4. From glucose, by the following route: Glucose → dihydroxyacetone phosphate (in glycolysis); dihydroxyacetone phosphate + NADH + H^+ → glycerol 3-phosphate + NAD^+ (glycerol 3-phosphate dehydrogenase reaction)

5. **(a)** Increased muscular activity increases the demand for ATP, which is met by increased O_2 consumption. **(b)** After the sprint, lactate produced by anaerobic glycolysis is converted to glucose and glycogen, which requires ATP and therefore O_2.

6. Glucose is the primary fuel for the brain. TPP-dependent oxidative decarboxylation of pyruvate to acetyl-CoA is essential to complete glucose metabolism. TPP synthesis requires the vitamin thiamine.

7. 5.5×10^7 L. For comparison, an Olympic pool, 50 m long, 25 m wide, and 2 m deep, holds 2.5×10^6 L.

8. **(a)** Inactivation provides a rapid means to change the concentration of active hormone and thus end its effects. **(b)** Changes in the rate of release from storage, the rate of conversion from prohormone to active hormone, and the rate of inactivation can rapidly change the level of a circulating peptide hormone.

9. Water-soluble hormones bind to receptors on the outer surface of the cell, triggering formation of a second messenger (e.g., cAMP) inside the cell. Lipid-soluble hormones can pass through the plasma membrane to act on target molecules or receptors directly.

10. **(a)** Skeletal muscle does not express glucose 6-phosphatase. Any glucose 6-phosphate produced enters the glycolytic pathway and, under O_2-deficient conditions, is converted to lactate via pyruvate. **(b)** In a "fight-or-flight" situation, the concentration of glycolytic precursors must be high in preparation for muscular activity. Phosphorylated intermediates cannot escape from myocytes because the membrane is not permeable to charged species, and glucose 6-phosphate is not exported by the glucose transporter. In the liver, glucose is formed from glucose 6-phosphate and enters the bloodstream to maintain the blood glucose level in the homeostatic range.

11. **(a)** Excessive uptake and use of blood glucose by the liver, leading to hypoglycemia; shutdown of amino acid and fatty acid catabolism **(b)** Little circulating fuel is available for ATP requirements. Brain damage results because glucose is the main source of fuel for the brain.

12. As an uncoupler of oxidative phosphorylation, thyroxine would decrease the efficiency of the process (lower the P/O ratio), forcing the tissue to increase respiration to meet the normal demand for ATP. This less-efficient respiration would dissipate as heat a greater proportion of the energy potentially available for making ATP. Thermogenesis could also be due to the increased rate of ATP use by the thyroid-stimulated tissue. In this case, the efficiency of oxidative phosphorylation (the P/O ratio) would be unchanged. But because some energy is always dissipated as heat in the process, the increased production of ATP demanded by the stimulated tissue would produce more heat overall.

13. Because prohormones are inactive, they can be stored in quantity in secretory granules. Rapid activation is achieved by enzymatic cleavage in response to an appropriate signal.

14. In animals, glucose can be synthesized from many precursors (see Fig. 14-15). In humans, the principal precursors are glycerol from TAGs, glucogenic amino acids from protein degradation, and oxaloacetate formed by pyruvate carboxylase.

15. The *ob/ob* mouse, which is initially obese, will lose weight. The *OB/OB* mouse will retain its normal body weight.

16. BMI = 39.3. For a BMI of 25, weight must be 75 kg; he must lose 43 kg, or 95 lb.

17. Reduced insulin secretion. Valinomycin has the same effect as opening the K^+ channel, allowing K^+ exit and consequent hyperpolarization.

18. The liver does not receive the insulin message and therefore continues to have high levels of glucose 6-phosphatase and gluconeogenesis, increasing blood glucose both during a fast and after a glucose-containing meal. The elevated blood glucose triggers insulin release from pancreatic β cells, hence the high level of insulin in the blood.

19. Some things to consider: What do the data show about the frequency of heart attack attributable to the drug among people taking the drug? How does this frequency compare with the data on individuals spared the long-term consequences of type 2 diabetes? Are other, equally effective treatment options with fewer adverse effects available?

20. Without intestinal glucosidase activity, absorption of glucose from dietary glycogen and starch is reduced, blunting the usual rise in blood glucose after a meal. The undigested oligosaccharides are fermented by intestinal bacteria, and the gases released cause intestinal discomfort.

21. **(a)** Increased; closing the ATP-gated K^+ channel would depolarize the membrane, increasing insulin release. **(b)** Type 2 diabetes results from decreased sensitivity to insulin, not a deficit of insulin production; increasing circulating insulin levels will reduce the symptoms associated with this disease. **(c)** Individuals with type 1 diabetes have deficient pancreatic β cells, so glyburide will have no beneficial effect. **(d)** Iodine, like chlorine (the atom it replaces in the labeled glyburide), is a halogen, but it is a larger atom and has slightly different chemical properties. The iodinated glyburide might not bind to SUR. If it bound to another molecule instead, the experiment would result in cloning of the gene for this other, incorrect protein. **(e)** Although a protein has been "purified," the "purified" preparation might be a mixture of several proteins that co-purify under those experimental conditions. In this case, the amino acid sequence could be that of a protein that co-purifies with SUR. Using antibody binding to show that the peptide sequences are present in SUR excludes this possibility. **(f)** Although the cloned gene does encode the 25 amino acid sequence found in SUR, it could be a gene that, coincidentally, encodes the same sequence in another protein. In this case, this other gene would most likely be expressed in different cells than the *SUR* gene. The mRNA hybridization results are consistent with the putative *SUR* cDNA actually encoding SUR. **(g)** The excess unlabeled glyburide competes with labeled glyburide for the binding site on SUR. As a result, there is significantly less binding of labeled glyburide, so little or no radioactivity is detected in the 140 kDa protein. **(h)** In the absence of excess unlabeled glyburide, labeled 140 kDa protein is found only in the presence of the putative *SUR* cDNA. Excess unlabeled glyburide competes with the labeled glyburide, and no ^{125}I-labeled 140 kDa protein is detected. This shows that the cDNA produces a glyburide-binding protein of the same molecular weight as SUR—strong evidence that the cloned gene encodes the SUR protein. **(i)** Several additional steps are possible, such as the following: (1) Express the putative *SUR* cDNA in CHO (Chinese hamster ovary) cells and show that the transformed cells have ATP-gated K^+ channel activity. (2) Show that HIT cells with mutations in the putative *SUR* gene lack ATP-gated K^+ channel activity. (3) Show that humans or experimental animals with mutations in the putative *SUR* gene are unable to secrete insulin.

Chapter 24

1. 6.1×10^4 nm; 290 times longer than the T2 phage head

2. The number of A residues does not equal the number of T residues, nor does the number of G equal the number of C, so the DNA is not a base-paired double helix; the M13 DNA is single-stranded.

3. $M_r = 3.8 \times 10^8$; length = 200 μm; $Lk_0 = 55{,}200$; $Lk = 51{,}900$

4. The exons contain 3 bp/amino acid × 192 amino acids = 576 bp. The remaining 864 bp are in introns, possibly in a leader or signal sequence, and/or in other noncoding DNA.

5. 5,000 bp. **(a)** Doesn't change; *Lk* cannot change without breaking and re-forming the covalent backbone of the DNA. **(b)** Becomes undefined; a circular DNA with a break in one strand has, by definition, no *Lk*. **(c)** Decreases; in the presence of ATP, gyrase underwinds DNA. **(d)** Doesn't change; this assumes that neither of the DNA strands is broken in the heating process.

6. For *Lk* to remain unchanged, the topoisomerase must introduce the same number of positive and negative supercoils.

7. $\sigma = -0.067$; >70% probability

8. (a) Undefined; the strands of a nicked DNA could be separated and thus have no *Lk*. **(b)** 476 **(c)** The DNA is already relaxed, so the topoisomerase does not cause a net change; *Lk* = 476. **(d)** 460; gyrase plus ATP reduces the *Lk* in increments of 2. **(e)** 464; eukaryotic type I topoisomerases increase the *Lk* of underwound or negatively supercoiled DNA in increments of 1. **(f)** 460; nucleosome binding does not break any DNA strands and thus cannot change *Lk*.

9. A fundamental structural unit in chromatin repeats about every 200 bp; the DNA is accessible to the nuclease only at 200 bp intervals. The brief treatment was insufficient to cleave the DNA at every accessible point, so a ladder of DNA bands is created in which the DNA fragments are multiples of 200 bp. The thickness of the DNA bands suggests that the distance between cleavage sites varies somewhat. For instance, not all the fragments in the lowest band are exactly 200 bp long.

10. A right-handed helix has a positive *Lk*; a left-handed helix (such as Z-DNA) has a negative *Lk*. Decreasing the *Lk* of a closed circular B-DNA by underwinding facilitates formation of regions of Z-DNA within certain sequences. (See Chapter 8, p. 273, for a description of sequences that permit the formation of Z-DNA.)

11. (a) Both strands must be covalently closed, and the molecule must be either circular or constrained at both ends. **(b)** Formation of cruciforms, left-handed Z-DNA, plectonemic or solenoidal supercoils, and unwinding of the DNA are favored. **(c)** *E. coli* DNA topoisomerase II or DNA gyrase **(d)** It binds the DNA at a point where it crosses on itself, cleaves both strands of one of the crossing segments, passes the other segment through the break, then reseals the break. The result is a change in *Lk* of –2.

12. Centromere, telomeres, and an autonomous replicating sequence or replication origin

13. The bacterial nucleoid is organized into domains approximately 10,000 bp long. Cleavage by a restriction enzyme relaxes the DNA within a domain, but not outside the domain. Any gene in the cleaved domain for which expression is affected by DNA topology will be affected by the cleavage; genes outside the domain will not.

14. (a) The lower, faster-migrating band is negatively supercoiled plasmid DNA. The upper band is nicked, relaxed DNA. **(b)** DNA topoisomerase I would relax the supercoiled DNA. The lower band would disappear, and all of the DNA would converge on the upper band. **(c)** DNA ligase would produce little change in the pattern. Some minor additional bands might appear near the upper band, due to the trapping of topoisomers not quite perfectly relaxed by the ligation reaction. **(d)** The upper band would disappear, and all of the DNA would be in the lower band. The supercoiled DNA in the lower band might become even more supercoiled and migrate somewhat faster.

15. (a) When DNA ends are sealed to create a relaxed, closed circle, some DNA species are completely relaxed but others are trapped in slightly underwound or overwound states. This gives rise to a distribution of topoisomers centered on the most relaxed species. **(b)** Positively supercoiled **(c)** The DNA that is relaxed despite the addition of dye is DNA with one or both strands broken. DNA isolation procedures inevitably introduce small numbers of strand breaks in some of the closed-circular molecules. **(d)** –0.05. This is determined by simply comparing native DNA with samples of known σ. In both gels, the native DNA migrates most closely with the sample of $\sigma = -0.049$.

16. 62 million (the genome refers to the haploid genetic content of the cell; the cell is actually diploid, so the number of nucleosomes is doubled). The number is obtained by dividing 3.1 billion bp by 200 bp/nucleosome (giving 15.5 million nucleosomes), multiplying by 2 copies of H2A per nucleosome, and again multiplying by 2 to account for the diploid state of the cell. The 62 million would double upon replication.

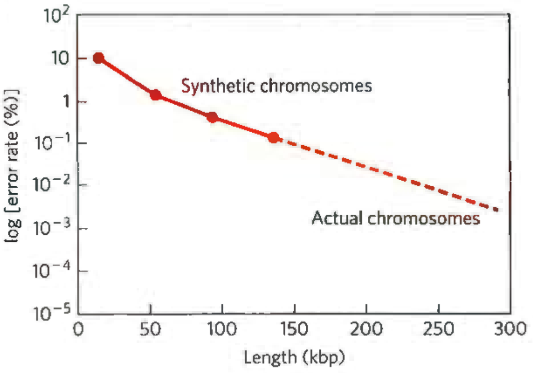
17. DNA topoisomerase IV is needed to decatenate the two circular chromosome products of DNA replication prior to cell division.

18. A TAD, or topologically associating domain, is a DNA loop that is bound and constrained at its base. Supercoiling within the TAD is maintained in part by the restriction to free DNA rotation imposed by the protein binding at the base of the loop.

19. (a) In nondisjunction, one daughter cell and all of its descendants get two copies of the synthetic chromosome and are white; the other daughter cell and all of its descendants get no copies of the synthetic chromosome and are red. This gives rise to a half-white, half-red colony. **(b)** In chromosome loss, one daughter cell and all of its descendants get one copy of the synthetic chromosome and are pink; the other daughter cell and all of its descendants get no copies of the synthetic chromosome and are red. This gives rise to a half-pink, half-red colony. **(c)** The minimum functional centromere must be smaller than 0.63 kbp, because all fragments of this size or larger confer relative mitotic stability. **(d)** Telomeres are required to fully replicate only linear DNA; a circular molecule can replicate without them.

(e) The larger the chromosome, the more faithfully it is segregated. The data show neither a minimum size below which the synthetic chromosome is completely unstable nor a maximum size above which stability no longer changes.

(f)

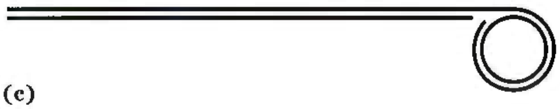


As shown in the graph, even if the synthetic chromosomes were as long as the normal yeast chromosomes, they would not be as stable. This suggests that other, as yet undiscovered, elements are required for stability.

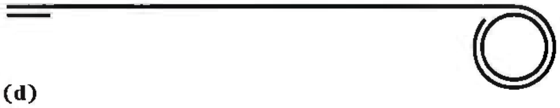
Chapter 25

1. (a) Structure **1**

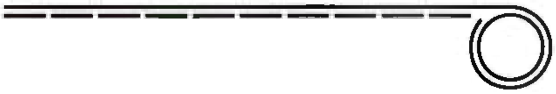
(b)



(c)



(d)



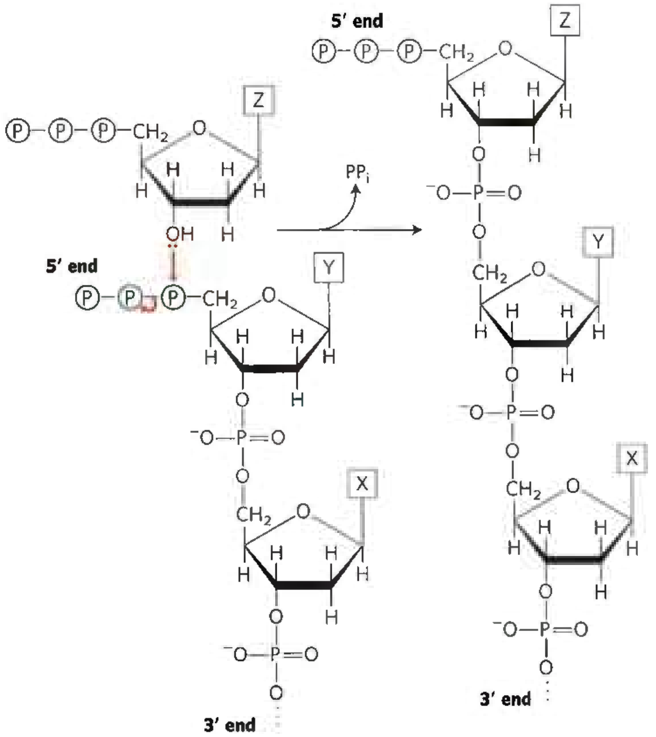
2. This is an extension of the classic Meselson-Stahl experiment. After three generations the molar ratio of ^{15}N–^{14}N DNA to ^{14}N–^{14}N DNA is 2/6 = 0.33.

3. (a) 4.42×10^5 turns **(b)** 40 min. In cells dividing every 20 min, a replicative cycle is initiated every 20 min, each cycle beginning before the prior one is complete. **(c)** 2,000 to 5,000 Okazaki fragments. The fragments are 1,000 to 2,000 nucleotides long. The ligation of Okazaki fragments does not occur randomly. Each fragment is stably base-paired with the lagging strand template prior to ligation with its neighbor, ensuring proper ordering.

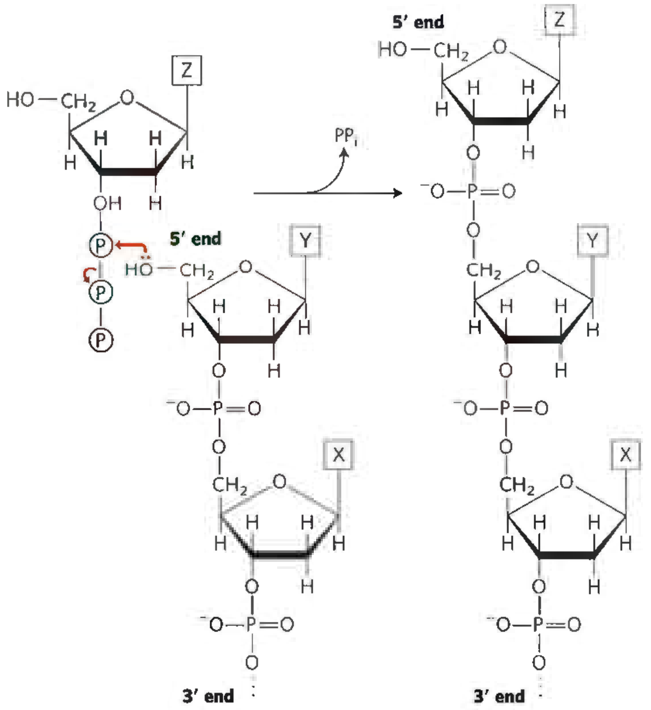
4. A, 28.7%; G, 21.3%; C, 21.3%; T, 28.7%. The DNA strand made from the template strand: A, 32.7%; G, 18.5%; C, 24.1%; T, 24.7%; the DNA strand made from the complementary template strand: A, 24.7%; G, 24.1%; C, 18.5%; T, 32.7%. This assumes that the two template strands are replicated completely.

5. (a) No. Incorporation of ^{32}P into DNA results from the synthesis of new DNA, which requires the presence of *all four* nucleotide precursors. **(b)** Yes. Although all four nucleotide precursors must be present for DNA synthesis, only one of them has to be radioactive for radioactivity to appear in the new DNA. **(c)** No. Radioactivity is incorporated only if the ^{32}P label is in the α phosphate; DNA polymerase cleaves off pyrophosphate—that is, the β- and γ-phosphate groups.

6. *Mechanism 1:* 3′-OH group of an incoming dNTP attacks the α phosphate of the triphosphate at the 5′ end of the growing DNA strand, displacing pyrophosphate. This mechanism uses normal dNTPs, and the growing end of the DNA always has a triphosphate on the 5′ end.



Mechanism 2: This uses a new type of precursor, nucleotide 3′-triphosphates. The growing end of the DNA strand has a 5′-OH group, which attacks the α phosphate of an incoming deoxynucleoside 3′-triphosphate, displacing pyrophosphate. Note that this mechanism would require the evolution of new metabolic pathways to supply the needed deoxynucleoside 3′-triphosphates.



7. The DNA polymerase contains a 3′→5′ exonuclease activity that degrades DNA to produce [^{32}P]dNMPs. The activity is not a 5′→3′

exonuclease, because the addition of unlabeled dNTPs inhibits the production of [^{32}P]dNMPs (polymerization activity would suppress a proofreading exonuclease but not an exonuclease operating downstream of the polymerase). Addition of pyrophosphate would generate [^{32}P]dNTPs through reversal of the polymerase reaction.

8. *Leading strand:* Precursors: dATP, dGTP, dCTP, dTTP (also needs a template DNA strand and DNA primer); enzymes and other proteins: DNA gyrase, helicase, single-stranded DNA–binding protein, DNA polymerase III, topoisomerases, and pyrophosphatase. *Lagging strand:* Precursors: ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, dTTP (also needs an RNA primer); enzymes and other proteins: DNA gyrase, helicase, single-stranded DNA–binding protein, primase, DNA polymerase III, DNA polymerase I, DNA ligase, topoisomerases, and pyrophosphatase. NAD$^+$ is also required as a cofactor for DNA ligase.

9. Mutants with defective DNA ligase produce a DNA duplex in which one of the strands remains in pieces (as Okazaki fragments). When this duplex is denatured, sedimentation results in one fraction containing the intact single strand (the high molecular weight band) and one fraction containing the unspliced fragments (the low molecular weight band).

10. Watson-Crick base pairing between template and leading strand; proofreading and removal of wrongly inserted nucleotides by the 3′-exonuclease activity of DNA polymerase III. Yes—perhaps. Because the factors ensuring fidelity of replication are operative in both the leading and the lagging strands, the lagging strand would probably be made with the same fidelity. However, the greater number of distinct chemical operations involved in making the lagging strand might provide a greater opportunity for errors to arise.

11. ~1,200 bp (600 in each direction)

12. A small fraction (13 of 10^9 cells) of the histidine-requiring mutants spontaneously undergo back-mutation and regain their capacity to synthesize histidine. 2-Aminoanthracene increases the rate of back-mutations about 1,800-fold and is therefore mutagenic. Since most carcinogens are mutagenic, 2-aminoanthracene is probably carcinogenic.

13. Spontaneous deamination of 5-methylcytosine (see Fig. 8-29a) produces thymine, and thus a G–T mismatched pair. These are among the most common mismatches in the DNA of eukaryotes. The specialized repair system restores the G≡C pair.

14. ~1,950 (650 in the DNA degraded between the mismatch and GATC, plus 650 in DNA synthesis to fill the resulting gap, plus 650 in degradation of the pyrophosphate products to inorganic phosphate). ATP is hydrolyzed by the MutSL complex and by the UvrD helicase.

15. (a) UV irradiation produces pyrimidine dimers; in normal fibroblasts, these are excised by cleavage of the damaged strand by a special excinuclease. Thus the denatured single-stranded DNA contains the many fragments created by the cleavage, and the average molecular weight is lowered. These fragments of single-stranded DNA are absent from the XPG samples, as indicated by the unchanged average molecular weight. **(b)** The absence of fragments in the single-stranded DNA from the XPG cells after irradiation suggests the special excinuclease is defective or missing.

16. Most cancerous tumors consist of cells that are deficient in some aspect of DNA repair, relative to the normal surrounding tissue. They thus can be more sensitive to the DNA damaging agent. Tumor cells also tend to be actively dividing, a state in which cells are more sensitive to DNA damage that might be encountered by replication forks.

17. Using G* to represent O^6-meG:

(a) (5′)AACG*TGCAC
TTG T ACGTG

(5′)AACGTGCAC
TTGCACGTG

(b) (5′)AACGTGCAC
TTGCACGTG

(5′)AACGTGCAC
TTGCACGTG

(c) (5′)AACG*TGCAC
TTG T ACGTG

(5′)AACATGCAC
TTGTACGTG

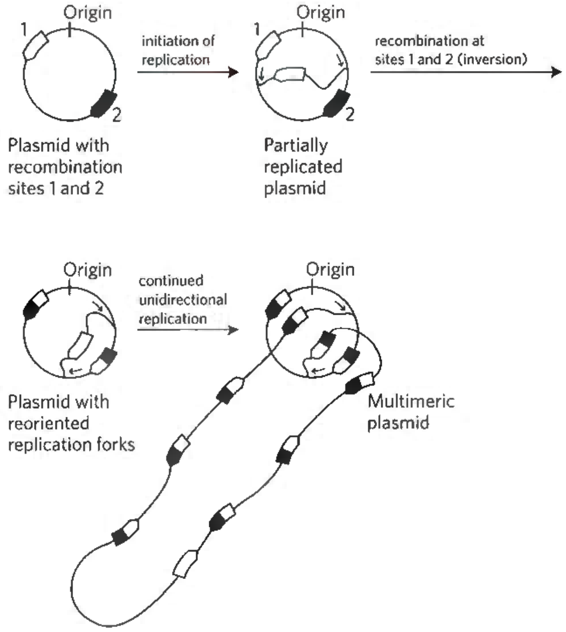
2 × (5′)AACGTGCAC
TTGCACGTG

18. Once paired with a complement after strand invasion, the 3′ end, unlike a 5′ end, can be extended by a DNA polymerase.

19. During homologous genetic recombination, a Holliday intermediate may be formed almost anywhere within the two paired, homologous chromosomes; the branch point of the intermediate can move extensively by branch migration. In site-specific recombination, the Holliday intermediate is formed between two specific sites, and branch migration is generally restricted by heterologous sequences on either side of the recombination sites.

20. (a) Points Y **(b)** Points X

21. Once replication has proceeded from the origin to a point where one recombination site has been replicated but the other has not, site-specific recombination not only inverts the DNA between the recombination sites but also changes the direction of one replication fork relative to the other. The forks will chase each other around the DNA circle, generating many tandem copies of the plasmid. The multimeric circle can be resolved to monomers by additional site-specific recombination events.


Origin
1
2
Plasmid with recombination sites 1 and 2
initiation of replication
Origin
1
2
Partially replicated plasmid
recombination at sites 1 and 2 (inversion)
Origin
Plasmid with reoriented replication forks
continued unidirectional replication
Origin
Multimeric plasmid

22. (a) Even in the absence of an added mutagen, background mutations occur due to radiation, cellular chemical reactions, and so forth. **(b)** If the DNA is sufficiently damaged, a substantial fraction of gene products is nonfunctional and the cell is nonviable. **(c)** Cells with reduced DNA repair capability are more sensitive to mutagens. Because they less readily repair lesions caused by R7000, Uvr$^-$ bacteria have an increased mutation rate and increased chance of lethal effects. **(d)** In the Uvr$^+$ strain, the excision-repair system removes DNA bases with attached [^{3}H]R7000, decreasing the amount of ^{3}H in these cells over time. In the Uvr$^-$ strain, the DNA is not repaired and the ^{3}H level increases as [^{3}H]R7000 continues to react with the DNA.

(e) All mutations listed in the table except A=T to G≡C show significant increases over background. Each type of mutation results from a different type of interaction between R7000 and DNA. Because different types of interactions are not equally likely (due to differences in reactivity, steric constraints, etc.), the resulting mutations occur with different frequencies. **(f)** No. Only those that start with a G≡C base pair are explained by this model. Thus A=T to C≡G and A=T to T=A must be due to R7000 attaching to an A or a T. **(g)** R7000—G pairs with A. First, R7000 adds to G≡C to give R7000—G≡C. (Compare this with what happens with the CH_3—G in Fig. 25-27b.) If this is not repaired, one strand is replicated as R7000—G=A, which is repaired to T=A. The other strand is wild-type. If the replication produces R7000—G=T, a similar pathway leads to an A=T base pair. **(h)** No. Compare data in the two tables, and keep in mind that different mutations occur at different frequencies.

A=T to C≡G: moderate in both strains; but better repair in Uvr$^+$

G≡C to A=T: moderate in both; no real difference

G≡C to C≡G: higher in Uvr$^+$; certainly less repair!

G≡C to T=A: high in both; no real difference

A=T to T=A: high in both; no real difference

A=T to G≡C: low in both; no real difference

Certain adducts may be more readily recognized by the repair apparatus than others, and these are repaired more rapidly and result in fewer mutations.

Chapter 26

1. **(a)** 60 to 100 s **(b)** 500 to 900 nucleotides **(c)** 6 to 11 h

2. A single base error in DNA replication, if not corrected, would cause one of the two daughter cells, and all its progeny, to have a mutated chromosome. A single base error in RNA transcription would not affect the chromosome; it would lead to formation of some defective copies of one protein, but because mRNAs turn over rapidly, most copies of the protein would not be defective. The progeny of this cell would be normal.

3. Normal posttranscriptional processing at the 3′ end (cleavage and polyadenylation) would be inhibited or blocked.

4. Because the template-strand RNA does not encode the enzymes needed to initiate viral infection, it would probably be inert or simply degraded by cellular ribonucleases. Replication of the template-strand RNA and propagation of the virus could occur only if intact RNA replicase (RNA-dependent RNA polymerase) were introduced into the cell along with the template strand.

5. AUGUCCAAAAUCGUA

6. (1) Use of a template strand of nucleic acid; (2) synthesis in the 5′→3′ direction; (3) use of nucleoside triphosphate substrates, with formation of a phosphodiester bond and displacement of PP_i. Polynucleotide phosphorylase forms phosphodiester bonds but differs in all other listed properties.

7. Most of the RNA transcribed in the nucleus was intronic and removed from the mRNAs.

8. No, at this time it is not possible to determine a cell's transcriptome based solely on the genome. Different cells have different transcriptomes based on which promoters are being used and how the transcripts are processed by factors present in the cell.

9. Generally two: one to cleave the phosphodiester bond at one intron-exon junction, the other to link the resulting free exon end to the exon at the other end of the intron. If the nucleophile in the first step were water, this step would be a hydrolysis, and only one transesterification step would be required to complete the splicing process.

10. Many snoRNAs, required for rRNA modification reactions, are encoded in introns. If splicing does not occur, snoRNAs are not produced.

11. **(a)** Water attacks the C-6 position of adenine, forming a tetrahedral intermediate, which then eliminates ammonia to form inosine. **(b)** Inosine can no longer pair correctly with U residues found on the opposite RNA strand prior to ADAR activity. This results in disruption of the RNA duplex. **(c)** Inosine does not have the same pairing properties as adenine and could potentially recode that particular codon so that a different amino acid is incorporated into the protein.

12. Physical separation of the processes prevents translation of primary or precursor transcripts that have not yet been processed by the cell. It also prevents the RNA processing and translational machineries from competing with one another for mRNAs.

13. **(a)** This could change how proteins or other RNAs recognize a particular RNA sequence and interfere with splicing, poly(A) formation, RNA modification, or other steps in which sequence-specific RNA recognition is important. **(b)** *S*-adenosylmethionine serves as the methyl donor in the synthesis of N^6-methyladenosine.

14. These enzymes lack a 3′→5′ proofreading exonuclease and have a high error rate; the likelihood of a replication error that would inactivate the virus is much lower in a small genome than in a large one.

15. **(a)** $4^{36} = 4.7 \times 10^{21}$ **(b)** 0.00002% **(c)** For the "unnatural selection" step, use a chromatographic resin with a bound molecule that is a transition-state analog of the ester hydrolysis reaction.

16. Though RNA synthesis is quickly halted by α-amanitin toxin, it takes several days for the critical mRNAs and proteins in the liver to degrade, causing liver dysfunction and death.

17. **(a)** After lysis of the cells and partial purification of the contents, an antibody-based assay could detect the β subunit. The β subunit could then be subjected to tandem mass spectrometry, which could detect the difference in amino acid residues between the normal β subunit and the mutated form. **(b)** Direct DNA sequencing (by the Sanger method)

18. **(a)** 384 **(b)** 1,620 nucleotide pairs **(c)** Most of the nucleotides are untranslated regions at the 3′ and 5′ ends of the mRNA. Also, most mRNAs code for a signal sequence (Chapter 27) in their protein products, which is eventually removed to produce the mature, functional protein.

19. **(a)** Injection of the antisense oligo cleaves the c-*mos* RNA and removes its poly(A) tail. This correlates with a loss in oocyte maturation. The noncoding poly(A) tail of the c-*mos* mRNA must be important for its function in maturation. **(b)** The sense oligo control shows that these results depend on complementarity to the c-*mos* mRNA. **(c)** The prosthetic RNA likely base-paired with the amputated c-*mos* mRNA and restored its function. Therefore, poly(A) tails can function "in trans"—they do not need to be covalently connected to the mRNAs they regulate. **(d)** The poly(A) tail stimulates protein expression in the reporter. This is likely also occurring with c-*mos*, and this protein expression is important for oocyte maturation. **(e)** These results suggest that the expression of genes can be artificially controlled either by cutting off their poly(A) tails or by attaching synthetic ones derived from other genes. This could be useful for fine-tuning gene expression in bioengineered cells or turning expression on or off.

Chapter 27

1. **(a)** Gly–Gln–Ser–Leu–Leu–Ile **(b)** Leu–Asp–Ala–Pro **(c)** His–Asp–Ala–Cys–Cys–Tyr **(d)** Met–Asp–Glu in eukaryotes; fMet–Asp–Glu in bacteria

2. UUAAUGUAU, UUGAUGUAU, CUUAUGUAU, CUCAUGUAU, CUAAUGUAU, CUGAUGUAU, UUAAUGUAC, UUGAUGUAC, CUUAUGUAC, CUCAUGUAC, CUAAUGUAC, CUGAUGUAC

3. No. Because nearly all the amino acids have more than one codon (e.g., Leu has six), any given polypeptide can be encoded by several different base sequences. However, some amino acids are encoded by only one codon, and those with multiple codons often share the same nucleotide at two of the three positions, so *certain parts* of the mRNA sequence encoding a protein of known amino acid sequence can be predicted with high certainty.

4. (a) (5′)CGACGGCGCGAAGUCAGGGGUGUUAAG(3′) **(b)** Arg–Arg–Glu–Val–Arg–Gly–Val–Lys **(c)** No. The complementary antiparallel strands in double-helical DNA do not have the same base sequence in the 5′→3′ direction. RNA is transcribed from only one specific strand of duplex DNA. The RNA polymerase must therefore recognize and bind to the correct strand.

5. (a), (b), and (c) are correct. **(a)** This outcome would restore the original gene and allow production of the native protein. **(b)** Altering the gene to insert a different amino acid at this position would allow for the generation of a full-length protein. Some activity may be present, especially if the new amino acid represented a conservative alteration (such as Ile substituting for Val). **(c)** This outcome would be similar to (c), inserting an amino acid (probably a different one) at the affected position and allowing synthesis of a full length and perhaps active protein. This outcome is called nonsense suppression. It works because most cells have multiple copies of particular tRNAs, some of which are expressed at low levels. If a minor one is altered, the genetic code is not disrupted because the other copies of the tRNA provide normal function. **(d)** This outcome would rarely work, since it would tend to introduce too many amino acid changes into the protein.

6. The labeled amino acids were found at the carboxyl end. Dintzis only isolated complete α subunits. With short incubation times, labeled amino acids would only appear in the part of the polypeptide that was synthesized last. Labeled amino acids introduced at the amino terminus would not be seen, because those polypeptides would not have been completed prior to protein isolation.

7. There are two tRNAs for methionine: tRNA$^{\text{fMet}}$, which is the initiating tRNA, and tRNA$^{\text{Met}}$, which can insert a Met residue in interior positions in a polypeptide. Only fMet-tRNA$^{\text{fMet}}$ is recognized by the initiation factor IF2 and is aligned with the initiating AUG positioned at the ribosomal P site in the initiation complex. AUG codons in the interior of the mRNA can bind and incorporate only Met-tRNA$^{\text{Met}}$.

8. (5′)AUG-GGU-CGU-GAG-UCA-UCG-UUA-AUU-GUA-GCU-GGA-GGG-GAG-GAA-UGA(3′) is translated as Met–Gly–Arg–Glu–Ser–Ser–Leu–Ile–Val–Ala–Gly–Gly–Glu–Glu. The peptide is 14 amino acids long instead of 15, because the last codon is a stop codon. Ten tRNAs are needed, one for each type of amino acid.

9. Allow polynucleotide phosphorylase to act on a mixture of UDP and CDP in which UDP has, say, five times the concentration of CDP. The result would be a synthetic RNA polymer with many UUU triplets (coding for Phe), a smaller number of UUC (Phe), UCU (Ser), and CUU (Leu), a much smaller number of UCC (Ser), CUC (Leu), and CCU (Pro), and an even smaller number of CCC (Pro).

10. A minimum of 583 ATP equivalents (based on 4 per amino acid residue added, except that there are only 145 translocation steps). Correction of each error requires 2 ATP equivalents. For glycogen synthesis, 292 ATP equivalents are required. The extra energy cost for β-globin synthesis reflects the cost of the information content of the protein. At least 20 activating enzymes, 70 ribosomal proteins, 4 rRNAs, 32 or more tRNAs, an mRNA, and 10 or more auxiliary enzymes must be made by the eukaryotic cell in order to synthesize a protein from amino acids. The synthesis of an (α1→4) chain of glycogen from glucose requires only 4 or 5 enzymes (Chapter 15).

11.

Glycine codons	Anticodons
(5′)GGU	(5′)ACC, GCC, ICC
(5′)GGC	(5′)GCC, ICC
(5′)GGA	(5′)UCC, ICC
(5′)GGG	(5′)CCC, UCC

(a) The 3′ and middle position **(b)** Pairings with anticodons (5′)GCC, ICC, and UCC **(c)** Pairings with anticodons (5′)ACC and CCC

12. (a), (c), (e), and (g) only; (b), (d), and (f) cannot be the result of single-base mutations, because (b) and (f) would require substitutions of two bases, and (d) would require substitutions of all three bases.

```
13. (5′)AUGAUAUUGCUAUCUUGGACU
    Changes: CC AU U C      C
             U     C A      A
                   G G      G
```

Of 63 possible one-base changes, 14 would result in no coding change.

14. The two DNA codons for Glu are GAA and GAG, and the four DNA codons for Val are GTT, GTC, GTA, and GTG. A single base change in GAA to form GTA or in GAG to form GTG could account for the Glu → Val replacement in sickle-cell hemoglobin. Much less likely are two-base changes, from GAA to GTG, GTT, or GTC; and from GAG to GTA, GTT, or GTC.

15. Isoleucine is similar in structure to several other amino acids, particularly valine. Distinguishing between valine and isoleucine in the aminoacylation process requires a second filter—a proofreading function. Histidine has a structure unlike that of any other amino acid, providing opportunities for binding specificity adequate to ensure accurate aminoacylation of the cognate tRNA.

16. (a) The Ala-tRNA synthetase recognizes the G^3–U^{70} base pair in the amino acid arm of tRNA$^{\text{Ala}}$. **(b)** The mutant tRNA$^{\text{Ala}}$ would insert Ala residues at codons encoding Pro. **(c)** A mutation that might have similar effects is an alteration in tRNA$^{\text{Pro}}$ that allowed it to be recognized and aminoacylated by Ala-tRNA synthetase. **(d)** Most of the proteins in the cell would be inactivated, so these would be lethal mutations and hence never observed. This represents a powerful selective pressure for maintaining the genetic code.

17. The 15,000 ribosomes in an *E. coli* cell can synthesize more than 23,000 proteins in 20 minutes.

18. *IF2:* The 70S ribosome would form, but initiation factors would not be released and elongation could not start. *EF-Tu:* The second aminoacyl-tRNA would bind to the ribosomal A site, but no peptide bond would form. *EF-G:* The first peptide bond would form, but the ribosome would not move along the mRNA to vacate the A site for binding of a new EF-Tu–tRNA.

19. The amino acid most recently added to a growing polypeptide chain is the only one covalently attached to a tRNA and thus is the only link between the polypeptide and the mRNA encoding it. A proofreading activity would sever this link, halting synthesis of the polypeptide and releasing it from the mRNA.

20. SecA; Drugs that inhibit the ability of SecA to bind bacterial proteins or hydrolyze ATP could significantly disrupt the export of bacterial proteins. SecB is nonessential as bacteria have numerous other chaperone proteins. Antibiotics that target the SecYEG complex, which is homologous to its eukaryotic counterpart (Sec61), could cause significant adverse effects in humans.

21. The protein would be directed into the ER, and from there the targeting would depend on additional signals. SRP binds the amino-terminal signal early in protein synthesis and directs the nascent polypeptide and ribosome to receptors in the ER. Because the protein is translocated into the lumen of the ER as it is synthesized, the NLS is never accessible to the proteins involved in nuclear targeting.

22. Trigger factor is a molecular chaperone that stabilizes an unfolded and translocation-competent conformation of ProOmpA.

23. DNA with a minimum of 5,784 bp; some of the coding sequences must be nested or overlapping.

24. (a) The helices associate through the hydrophobic effect and van der Waals interactions. **(b)** R groups 3, 6, 7, and 10 extend to the left; 1, 2, 4, 5, 8, and 9 extend to the right. **(c)** One possible sequence is

1 2 3 4 5 6 7 8 9 10
N–Phe–Ile–Glu–Val–Met–Asn–Ser–Ala–Phe–Gln–*C*

(d) One possible DNA sequence for the amino acid sequence in (c) is

Nontemplate strand
(5′)TTTATTGAAGTAATGAATAGTGCATTCCAG(3′)
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
(3′)AAATAACTTCATTACTTATCACGTAAGGTC(5′)
Template strand

(e) Phe, Leu, Ile, Met, and Val. All are hydrophobic, but the set does not include *all* the hydrophobic amino acids; Trp, Pro, and Ala are missing. **(f)** Tyr, His, Gln, Asn, Lys, Asp, and Glu. All of these are hydrophilic, although Tyr is less hydrophilic than the others. The set does not include *all* the hydrophilic amino acids; Ser, Thr, and Arg are missing. **(g)** Omitting T from the mixture excludes codons starting or ending with T—thus excluding Tyr, which is not very hydrophilic, and, more importantly, excluding the two possible stop codons (TAA and TAG). No other amino acids in the NAN set are excluded by omitting T. **(h)** Misfolded proteins are often degraded in the cell. Therefore, if a synthetic gene has produced a protein that forms a band on the SDS gel, it is likely that this protein is folded properly. **(i)** Protein folding depends on more than the hydrophobic effect and van der Waals interactions. There are many reasons why a synthesized random-sequence protein might not fold into the four-helix structure. For example, hydrogen bonds between hydrophilic side chains could disrupt the structure. Also, not all sequences have an equal propensity to form an α helix.

Chapter 28

1. (a) Tryptophan synthase levels remain high despite the presence of tryptophan. **(b)** Levels again remain high. **(c)** Levels rapidly decrease, preventing wasteful synthesis of tryptophan.

2. The *E. coli* cells will produce β-galactosidase when they are subjected to high levels of a DNA-damaging agent such as UV light. Under such conditions, RecA binds to single-stranded chromosomal DNA and facilitates autocatalytic cleavage of the LexA repressor, releasing LexA from its binding site and allowing transcription of downstream genes.

3. (a) Constitutive, low-level expression of the operon; most mutations in the operator would make the repressor less likely to bind. **(b)** Constitutive expression; mutation prevents negative regulation of the operon. **(c)** Increased expression; under conditions in which the operon is induced, mutation increases recruitment of RNA polymerase. **(d)** Constant repression; mutation allows repressor to readily bind to operator. **(e)** Decreased expression; under conditions in which the operon is induced, mutation decreases recruitment of RNA polymerase.

4. 7,000 copies

5. 8×10^{-9} M, about 10^5 times greater than the dissociation constant. With 10 copies of active repressor in the cell, the operator site is always bound by the repressor molecule.

6. (a) through **(e)**. Each condition decreases expression of *lac* operon genes.

7. (a) Less attenuation. The ribosome completing the translation of sequence 1 would no longer overlap and block sequence 2; sequence 2 would always be available to pair with sequence 3, preventing formation of the attenuator structure. **(b)** More attenuation. Sequence 2 would pair less efficiently with sequence 3; the attenuator structure would be formed more often, even when sequence 2 was not blocked by a ribosome. **(c)** No attenuation. The only regulation would be that afforded by the Trp repressor. **(d)** Attenuation loses its sensitivity to Trp tRNA. It might become sensitive to His tRNA. **(e)** Attenuation would rarely, if ever, occur. Sequences 2 and 3 always block formation of the attenuator. **(f)** Constant attenuation. Attenuator always forms, regardless of the availability of tryptophan.

8. Induction of the SOS response could not occur, making the cells more sensitive to high levels of DNA damage.

9. Each *Salmonella* cell would have flagella made up of both types of flagellar protein, and the cell would be vulnerable to antibodies generated in response to either protein.

10. A dissociable factor necessary for activity (e.g., a specificity factor similar to the σ subunit of the *E. coli* enzyme) may have been lost during purification of the polymerase.

11.

Gal4 protein

Gal4p DNA-binding domain	Gal4p activator domain

Engineered protein

Lac repressor DNA-binding domain	Gal4p activator domain

The engineered protein cannot bind to the Gal4p-binding site in the *GAL* gene (UAS_G) because it lacks the Gal4p DNA-binding domain. Modify the Gal4p-binding site in the DNA to give it the nucleotide sequence to which the Lac repressor normally binds (using methods described in Chapter 9).

12. Methylamine. The reaction proceeds with attack of water on the guanidinium carbon of the modified arginine.

13. Synthesis of a protein first requires the synthesis of an mRNA long enough to encode the protein and to include any necessary regulatory sequences, with one ribonucleoside triphosphate used up for every nucleotide residue included in the mRNA. Then, the mRNA must be translated—one of the most energy-intensive processes in the cell (Chapter 27). To maintain repression, the repressor protein would need to be synthesized repeatedly. With the use of RNA as repressor, the RNA can be shorter than a protein repressor–encoding mRNA, and no translation step is required.

14. The *bcd* mRNA needed for development is contributed to the egg by the mother. The fertilized egg develops normally even if its genotype is *bcd*$^-$/*bcd*$^-$, as long as the mother has one normal *bcd* gene and the *bcd*$^-$ allele is recessive. However, the adult *bcd*$^-$/*bcd*$^-$ female will be sterile because she has no normal *bcd* mRNA to contribute to her eggs.

15. (a) For 10% expression (90% repression), 10% of the repressor has bound inducer and 90% is free and available to bind the operator. The calculation uses Eqn 5-8 (p. 151), with $Y = 0.1$ and $K_\text{d} = 10^{-4}$ M:

$$Y = \frac{[\text{IPTG}]}{[\text{IPTG}] + K_\text{d}} = \frac{[\text{IPTG}]}{[\text{IPTG}] + 10^{-4}\ \text{M}}$$

$$0.1 = \frac{[\text{IPTG}]}{[\text{IPTG}] + 10^{-4}\ \text{M}}$$

$$0.9[\text{IPTG}] = 10^{-5} \qquad \text{or} \qquad [\text{IPTG}] = 1.1 \times 10^{-5}\ \text{M}$$

For 90% expression, 90% of the repressor has bound inducer, so $Y = 0.9$. Entering the values for Y and K_d in Eqn 5-8 gives $[\text{IPTG}] = 9 \times 10^{-4}$ M. Thus, gene expression varies 10-fold over a roughly 10-fold [IPTG] range. **(b)** You would expect the protein levels to be low before induction, to rise during induction, and then to decay as synthesis stops and the proteins are degraded. **(c)** As shown in (a), the *lac* operon has more levels of expression than just on or off; thus it does not have characteristic A. As shown in (b), expression of the *lac* operon subsides once the inducer is removed; thus it lacks characteristic B. **(d)** *GFP-on:* rep^{ts} (designating the protein product of the *rep*^{ts} gene) and GFP are expressed at high levels; rep^{ts} represses OP$_\lambda$, so no LacI is produced. *GFP-off:* LacI is expressed at a high level; LacI represses OP$_{lac}$, so rep^{ts} and GFP are not produced. **(e)** IPTG treatment switches the system from GFP-off to GFP-on. IPTG has an effect only when LacI is present, so it affects only the GFP-off state. Adding IPTG relieves the repression of OP$_{lac}$, allowing high-level expression of rep^{ts}, which turns off expression of LacI, and high-level expression of GFP. **(f)** Heat treatment switches the system from GFP-on to GFP-off. Heat has an effect only when rep^{ts} is present, so affects only the GFP-on state. Heat inactivates rep^{ts} and relieves the repression of OP$_\lambda$, allowing high-level expression of LacI. LacI then acts at OP$_{lac}$ to repress synthesis of rep^{ts} and GFP. **(g)** *Characteristic A:* The system is not stable in the intermediate state. At some point, one repressor will act more strongly than the other due to chance fluctuations in expression; this shuts off expression of the other repressor and locks the system in one state. *Characteristic B:* Once one repressor is expressed, it prevents the synthesis of the other; thus the system remains in one state even after the switching stimulus has been removed. **(h)** At no time does any cell express an intermediate level of GFP—this is a confirmation of characteristic A. At the intermediate concentration (X) of inducer, some cells have switched to GFP-on while others have not yet made the switch and remain in the GFP-off state; none are in between. The bimodal distribution of expression levels at [IPTG] = X is caused by the mixed population of GFP-on and GFP-off cells.

Glossary

a

ABC transporters: Plasma membrane proteins with sequences that make up *A*TP-*b*inding *c*assettes; serve to transport a large variety of substrates, including inorganic ions, lipids, and nonpolar drugs, out of the cell, using ATP as the energy source.

absolute configuration: The configuration of four different substituent groups around an asymmetric carbon atom, in relation to D- and L-glyceraldehyde.

absorption: Transport of the products of digestion from the intestinal tract into the blood.

acceptor control: Regulation of the rate of respiration by the availability of ADP as phosphate group acceptor.

accessory pigments: Visible light–absorbing pigments (carotenoids, xanthophylls, and phycobilins) in plants and photosynthetic bacteria that complement chlorophylls in trapping energy from sunlight.

acid dissociation constant: The dissociation constant (K_a) of an acid, describing its dissociation into its conjugate base and a proton.

acidosis: A metabolic condition in which the capacity of the body to buffer H^+ is diminished; usually accompanied by decreased blood pH. *Compare* alkalosis.

actin: A protein that makes up the thin filaments of muscle; also an important component of the cytoskeleton of many eukaryotic cells.

action spectrum: A plot of the efficiency of light at promoting a light-dependent process such as photosynthesis as a function of wavelength.

activation energy ($\Delta G^\ddagger$): The amount of energy (in joules) required to convert all the molecules in 1 mol of a reacting substance from the ground state to the transition state.

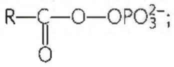
activator: (1) A DNA-binding protein that positively regulates the expression of one or more genes; that is, transcription rates increase when an activator is bound to the DNA. (2) A positive modulator of an allosteric enzyme.

active site: The region of an enzyme surface that binds the substrate molecule and catalytically transforms it; also known as the catalytic site.

active transporter: Membrane protein that moves a solute across a membrane against an electrochemical gradient in an energy-requiring reaction.

activity: The true thermodynamic activity or potential of a substance, as distinct from its molar concentration.

acyl phosphate: Any molecule with the general chemical form



$R{-}\underset{\underset{O}{\|}}{C}{-}O{-}OPO_3^{2-}$;

an acid anhydride between a carboxylic acid and phosphoric acid.

adaptor proteins: Signaling proteins, generally lacking enzymatic activities, that have binding sites for two or more cellular components and serve to bring those components together.

adenosine 3′,5′-cyclic monophosphate: *See* cyclic AMP.

adenosine diphosphate: *See* ADP.

adenosine triphosphate: *See* ATP.

***S*-adenosylmethionine (adoMet):** An enzymatic cofactor involved in methyl group transfers.

adenylate kinase: The enzyme that catalyzes the reversible reaction ATP + AMP → 2 ADP. When anabolic activities have depleted the supply of ATP, this enzyme produces more ATP from ADP. Also called myokinase. *Compare* nucleoside monophosphate kinase.

adipocyte: An animal cell specialized for the storage of fats (triacylglycerols).

adipose tissue: Connective tissue specialized for the storage of large amounts of triacylglycerols. *See also* beige adipose tissue; brown adipose tissue; white adipose tissue.

ADP (adenosine diphosphate): A ribonucleoside 5′-diphosphate serving as phosphate group acceptor in the cell energy cycle.

adoMet: *See S*-adenosylmethionine.

aerobe: An organism that lives in air and uses oxygen as the terminal electron acceptor in respiration.

aerobic: Requiring or occurring in the presence of oxygen.

aerobic glycolysis: Cellular energy generation by glycolysis alone (without subsequent oxidation of pyruvate) even though oxygen is available. *See* glycolysis.

agonist: A compound, typically a hormone or neurotransmitter, that elicits a physiological response when it binds to its specific receptor.

AKAPs (A kinase anchoring proteins): A family of proteins that share a domain that binds the R subunit of protein kinase A (PKA). Each also has a domain with affinity for one of a number of diverse proteins, so that each AKAP anchors PKA in a specific location or to a specific protein.

alcohol fermentation: *See* ethanol fermentation.

aldose: A simple sugar in which the carbonyl carbon atom is an aldehyde; that is, the carbonyl carbon is at one end of the carbon chain.

alkalosis: A metabolic condition in which the capacity of the body to buffer OH^- is diminished; usually accompanied by an increase in blood pH. *Compare* acidosis.

allosteric enzyme: A regulatory enzyme with catalytic activity modulated by the noncovalent binding of a specific metabolite at a site other than the active site.

allosteric protein: A protein (generally with multiple subunits) with multiple ligand-binding sites, such that ligand binding at one site affects ligand binding at another.

allosteric site: The specific site on the surface of an allosteric protein molecule to which the modulator or effector molecule binds.

α helix: A helical conformation of a polypeptide chain, usually right-handed, with maximal intrachain hydrogen bonding; one of the most common secondary structures in proteins.

α oxidation: An alternative path for the oxidation of β-methyl fatty acids in peroxisomes, as distinct from β oxidation and ω oxidation.

alternative splicing: A process in which exons are selectively spliced (linked) in alternative ways to generate different mature mRNAs.

Ames test: A simple bacterial test for carcinogenicity, based on the assumption that carcinogens are mutagens.

amino acid activation: ATP-dependent enzymatic esterification of the carboxyl group of an amino acid to the 3′-hydroxyl group of its corresponding tRNA.

amino acids: α-Amino–substituted carboxylic acids, the building blocks of proteins.

aminoacyl-tRNA: An aminoacyl ester of a tRNA.

aminoacyl-tRNA synthetases: Enzymes that catalyze synthesis of an aminoacyl-tRNA at the expense of ATP energy.

amino-terminal residue: The only amino acid residue in a polypeptide chain that has a free α-amino group; defines the amino terminus of the polypeptide.

aminotransferases: Enzymes that catalyze the transfer of amino groups from α-amino to α-keto acids; also called transaminases.

ammonotelic: Excreting excess nitrogen in the form of ammonia.

AMP-activated protein kinase (AMPK): A protein kinase allosterically activated by 5′-adenosine monophosphate (AMP) and inhibited by ATP. AMPK action generally shifts metabolism away from biosynthesis toward energy production.

amphibolic pathway: A metabolic pathway used in both catabolism and anabolism.

amphipathic: Containing both polar and nonpolar domains.

amphitropic proteins: Proteins that associate reversibly with the membrane and thus can be found in the cytosol, in the membrane, or in both places.

ampholyte: A substance that can act as either a base or an acid.

amphoteric: Capable of donating and accepting protons, thus able to serve as an acid or a base.

AMPK: *See* AMP-activated protein kinase.

amyloidoses: A variety of progressive diseases characterized by abnormal deposits of misfolded proteins in one or more organs or tissues.

anabolism: The phase of intermediary metabolism concerned with the energy-requiring biosynthesis of cell components from smaller precursors, typically a reductive process.

anaerobe: An organism that lives without oxygen. Obligate anaerobes die when exposed to oxygen.

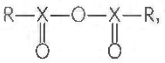
anaerobic: Occurring in the absence of air or oxygen.

analyte: A molecule to be analyzed by mass spectrometry.

anammox: Anaerobic oxidation of ammonia to N_2, using nitrite as electron acceptor; carried out by specialized chemolithotrophic bacteria.

anaplerotic reaction: An enzyme-catalyzed reaction that can replenish the supply of intermediates in the citric acid cycle.

angstrom (Å): A unit of length (10^{-8} cm) used to indicate molecular dimensions. 10 Å = 1 nm.

anhydride: The product of the condensation of two carboxyl or phosphate groups in which the elements of water are eliminated to form a compound with the general structure  $R-\underset{\underset{O}{\|}}{X}-O-\underset{\underset{O}{\|}}{X}-R$, where X is either carbon or phosphorus.

anion-exchange resin: A polymeric resin with fixed cationic groups, used in the chromatographic separation of anions.

anomeric carbon: The carbon atom in a sugar at the new chiral center formed when a sugar cyclizes to form a hemiacetal. This is the carbonyl carbon of aldehydes and ketones.

anomers: Two stereoisomers of a given sugar that differ only in the configuration about the carbonyl (anomeric) carbon atom.

anorexigenic: Tending to decrease appetite and food consumption. *Compare* orexigenic.

antagonist: A compound that interferes with the physiological action of another substance (the agonist), usually at a hormone or neurotransmitter receptor.

antibiotic: One of many different organic compounds that are formed and secreted by various species of microorganisms and plants, are toxic to other microbial species, and presumably have a defensive function.

antibody: A defense protein synthesized by the immune system of vertebrates and circulated in the blood. *See also* immunoglobulin.

anticodon: A specific sequence of three nucleotides in a tRNA, complementary to a codon for an amino acid in an mRNA.

antigen: A molecule capable of eliciting the synthesis of a specific antibody in vertebrates.

antiparallel: Describes two linear polymers that are opposite in polarity or orientation.

antiport: Cotransport of two solutes across a membrane in opposite directions.

apoenzyme: The protein portion of an enzyme, exclusive of any organic or inorganic cofactors or prosthetic groups that might be required for catalytic activity.

apolipoprotein: The protein component of a lipoprotein.

apoprotein: The protein portion of a protein, exclusive of any organic or inorganic cofactors or prosthetic groups that might be required for activity.

apoptosis: Process in which a cell in an organism brings about its own death and lysis, in response to a signal from outside or programmed in its genes, by systematically degrading its own macromolecules for salvage by the organism.

aptamer: An oligonucleotide that binds specifically to one molecular target, usually selected by an iterative cycle of affinity-based enrichment (SELEX).

aquaporins (AQPs): A family of integral membrane proteins that mediate the flow of water across membranes.

archaea: Members of Archaea, one of the three domains of living organisms; include many species that thrive in extreme environments of high ionic strength, high temperature, or low pH.

arcuate nucleus: A group of neurons in the hypothalamus that function in regulation of hunger and feeding behavior.

asymmetric carbon atom: A carbon atom that is covalently bonded to four different groups and thus may exist in two different tetrahedral configurations.

ATP (adenosine triphosphate): A ribonucleoside 5′-triphosphate functioning as a phosphate group donor in the cellular energy cycle; carries chemical energy between metabolic pathways by serving as a shared intermediate coupling endergonic and exergonic reactions.

ATPase: An enzyme that hydrolyzes ATP to yield ADP and phosphate, usually coupled to a process requiring energy.

ATP synthase: An enzyme complex that forms ATP from ADP and phosphate during oxidative phosphorylation in the inner mitochondrial membrane or the bacterial plasma membrane, and during photophosphorylation in chloroplasts.

attenuator: An RNA sequence involved in regulating the expression of certain genes; functions as a transcription terminator.

autophagy: Catabolic lysosomal degradation of cellular proteins and other components.

autophosphorylation: Strictly, the phosphorylation of an amino acid residue in a protein that is catalyzed by the same protein molecule; often extended to include phosphorylation of one subunit of a homodimer by the other subunit.

autotroph: An organism that can synthesize its own complex molecules from very simple carbon and nitrogen sources, such as carbon dioxide and ammonia.

auxin: A plant growth hormone.

auxotrophic mutant (auxotroph): A mutant organism defective in the synthesis of a particular biomolecule, which must therefore be supplied for the organism's growth.

Avogadro's number (*N*): The number of molecules in a gram molecular weight (a mole) of any compound (6.02×10^{23}).

b

bacteria: Members of Bacteria, one of the three domains of living organisms; they have a plasma membrane but no internal organelles or nucleus.

bacteriophage: A virus capable of replicating in a bacterial cell; also called a phage.

baculovirus: Any of a group of double-stranded DNA viruses that infect invertebrates, particularly insects; widely used for protein expression in biotechnology.

Barr body: A condensed, inactive form of the X chromosome found in the cells of female mammals.

basal metabolic rate: An animal's rate of oxygen consumption when at complete rest, long after a meal.

base pair: Two nucleotides in nucleic acid chains that are paired by hydrogen bonding of their bases; for example, A with T or U, and G with C.

BAT: *See* brown adipose tissue.

B cell: *See* B lymphocyte.

beige adipose tissue: Thermogenic adipose tissue activated by cooling of the individual; expresses the uncoupling protein UCP1 (thermogenin) at a high level. *Compare* brown adipose tissue; white adipose tissue.

β conformation: An extended, zigzag arrangement of a polypeptide chain; a common secondary structure in proteins.

β oxidation: Oxidative degradation of fatty acids into acetyl-CoA by successive oxidations at the β-carbon atom; as distinct from α oxidation and ω oxidation.

β sheet: The side-by-side, hydrogen-bonded arrangement of polypeptide chains in the extended β conformation.

β turn: A type of protein secondary structure consisting of four amino acid residues arranged in a tight turn so that the polypeptide turns back on itself.

bilayer: A double layer of oriented amphipathic lipid molecules, forming the basic structure of biological membranes. The hydrocarbon tails face inward to form a continuous nonpolar phase.

bile acids: Polar derivatives of cholesterol, secreted by the liver into the intestine, that serve to emulsify dietary fats, facilitating lipase action.

bile salts: Amphipathic steroid derivatives with detergent properties, participating in digestion and absorption of lipids.

binding energy (ΔG_B): The energy derived from noncovalent interactions between enzyme and substrate or receptor and ligand.

binding site: The crevice or pocket on a protein in which a ligand binds.

bioassay: A method for measuring the amount of a biologically active substance (such as a hormone) in a sample by quantifying the biological response to aliquots of that sample.

biochemical standard free-energy change ($\Delta G'^{\circ}$): The free-energy change for a reaction occurring under a set of standard conditions: temperature, 298 K; pressure, 1 atm (101.3 kPa); all solutes at 1 M concentration; at pH 7.0 in 55.5 M water. Also called standard transformed free-energy change. *Compare* standard free-energy change (ΔG°).

biochemistry: A molecular description of the structures, mechanisms, and chemical processes of living things in all their diverse forms.

bioinformatics: The computerized analysis of biological data, using methods derived from statistics, linguistics, mathematics, chemistry, biochemistry, and physics. The data are often nucleic acid or protein sequences or structural data.

biosphere: All the places on or in the earth, the seas, and the atmosphere occupied by living matter.

biotin: A vitamin; an enzymatic cofactor in carboxylation reactions.

B lymphocyte (B cell): One of a class of blood cells (lymphocytes), responsible for the production of circulating antibodies.

body mass index (BMI): A measure of obesity, calculated as weight in kilograms divided by (height in meters)^2. A BMI of more than 27.5 is defined as overweight; more than 30, as obese.

bond energy: The energy required to break a bond.

branch migration: Movement of the branch point in a branched DNA formed from two DNA molecules with identical sequences. *See also* Holliday intermediate.

brown adipose tissue (BAT): Thermogenic adipose tissue rich in mitochondria that contain the uncoupling protein UCP1 (thermogenin), which uncouples electron transfer through the respiratory chain from ATP synthesis. *Compare* beige adipose tissue; white adipose tissue.

buffer: A system capable of resisting changes in pH, consisting of a conjugate acid-base pair in which the ratio of proton acceptor to proton donor is near unity.

C

C_3 plants: Plants in which the first product of CO_2 fixation is the three-carbon compound 3-phosphoglycerate. *Compare* C_4 plants.

C_4 pathway: The metabolic pathway in which CO_2 is first added to phosphoenolpyruvate by the enzyme PEP carboxylase to produce the four-carbon compound within mesophyll cells that is later transported to the bundle-sheath cells, where the CO_2 is released for use in the Calvin cycle.

C_4 plants: Plants (generally tropical) in which CO_2 is first fixed into a four-carbon compound, oxaloacetate or malate, before entering the Calvin cycle via the rubisco reaction. *Compare* C_3 plants.

calorie: The amount of heat required to raise the temperature of 1.0 g of water from 14.5 to 15.5 °C. One calorie (cal) equals 4.18 joules (J). The nutritional calorie, Cal, is equal to 1,000 calories, or 1 kcal.

Calvin cycle: The cyclic pathway in plants that fixes carbon dioxide and produces triose phosphates.

CAM plants: Succulent plants of hot, dry climates, in which CO_2 is fixed into oxaloacetate in the dark, then fixed by rubisco in the light when stomata close to exclude O_2.

cAMP: *See* cyclic AMP.

cAMP receptor protein (CRP): In bacteria, a specific regulatory protein that controls initiation of transcription of the genes that produce the enzymes required to use some other nutrient when glucose is lacking; also called catabolite gene activator protein (CAP).

cAMP-dependent protein kinase (protein kinase A; PKA): A protein kinase that phosphorylates Ser or Thr residues when bound by its allosteric activator, cAMP.

CAP: *See* cAMP receptor protein.

capsid: The protein coat of a virion, or virus particle.

carbanion: A negatively charged carbon atom.

carbocation: A positively charged carbon atom; also called a carbonium ion.

carbohydrate: A polyhydroxy aldehyde or ketone, or substance that yields such a compound on hydrolysis. Many carbohydrates have the empirical formula $(CH_2O)_n$; some also contain nitrogen, phosphorus, or sulfur.

carbon-assimilation reactions: Formerly referred to as dark reactions. *See* CO_2 assimilation.

carbon fixation: *See* CO_2 fixation.

carbonium ion: *See* carbocation.

carboxyl-terminal domain (CTD): The carboxyl-terminal domain of eukaryotic RNA polymerase II, containing many repeats of the heptapeptide YSPTSPS. Phosphorylation of the CTD changes during transcription. Many cellular factors interact with the CTD to regulate gene expression.

carboxyl-terminal residue: The only amino acid residue in a polypeptide chain that has a free α-carboxyl group; defines the carboxyl terminus of the polypeptide.

cardiolipin: A membrane phospholipid in which two phosphatidic acid moieties share a single glycerol head group.

carnitine shuttle: A mechanism for moving fatty acids from the cytosol to the mitochondrial matrix as fatty esters of carnitine.

carotenoids: Lipid-soluble photosynthetic pigments made up of isoprene units.

cascade: *See* enzyme cascade; regulatory cascade.

catabasis: Resolution of inflammation.

catabolism: The phase of intermediary metabolism concerned with the energy-yielding degradation of nutrient molecules, typically an oxidative process.

catabolite gene activator protein (CAP): *See* cAMP receptor protein.

catalytic site: *See* active site.

catecholamines: Hormones, such as epinephrine, that are amino derivatives of catechol.

catenane: Two or more circular polymeric molecules interlinked by one or more noncovalent topological links, resembling the links of a chain.

cation-exchange resin: An insoluble polymer with fixed negative charges, used in the chromatographic separation of cationic substances.

CD spectroscopy: *See* circular dichroism spectroscopy.

cDNA: *See* complementary DNA.

cDNA library: A collection of cloned DNA fragments derived entirely from the complement of mRNA being expressed in a particular organism or cell type under a defined set of conditions.

cellular differentiation: The process in which a precursor cell becomes specialized to carry out a particular function, by acquiring a new complement of proteins and RNA.

central dogma: An organizing principle of molecular biology that is now accepted as fact rather than dogma: genetic information flows between nucleic acids and from nucleic acid to protein, but not from protein to nucleic acid or from protein to protein.

centromere: A specialized site in a chromosome, serving as the attachment point for the mitotic or meiotic spindle.

cerebroside: A sphingolipid containing one sugar residue as a head group.

channeling: *See* substrate channeling.

chaperone: Any of several classes of proteins or protein complexes that catalyze the accurate folding of proteins in all cells.

chaperonin: One of two major classes of chaperones in virtually all organisms; a complex of proteins that functions in protein folding: GroES/GroEL in bacteria; Hsp60 in eukaryotes.

chemiosmotic coupling: Coupling of ATP synthesis to electron transfer by a transmembrane difference in charge and pH.

chemiosmotic theory: The theory that energy derived from electron transfer reactions is temporarily stored as a transmembrane difference in charge and pH, which subsequently drives formation of ATP in oxidative phosphorylation and photophosphorylation.

chemotaxis: A cell's sensing of and movement toward or away from a specific chemical agent.

chemotroph: An organism that obtains energy by metabolizing organic compounds derived from other organisms.

chiral center: An atom with substituents arranged so that the molecule is not superposable on its mirror image.

chiral compound: A compound that contains an asymmetric center (chiral atom or chiral center) and thus can occur in two nonsuperposable mirror-image forms (enantiomers).

chlorophylls: A family of green pigments that function as receptors of light energy in photosynthesis; magnesium-porphyrin complexes.

chloroplast: A chlorophyll-containing photosynthetic organelle in some eukaryotic cells.

chondroitin sulfate: One of a family of sulfated glycosaminoglycans, a major component of the extracellular matrix.

chromatin: A filamentous complex of DNA, histones, and other proteins, constituting the eukaryotic chromosome.

chromatography: A process in which complex mixtures of molecules are separated by many repeated partitionings between a flowing (mobile) phase and a stationary phase.

chromatophore: A compound or moiety (natural or synthetic) that absorbs visible or ultraviolet light.

chromosome: A single large DNA molecule and its associated proteins and often associated regulatory or structural RNA; containing many genes; stores and transmits genetic information.

chromosome territory: A region of the nucleus preferentially occupied by a particular chromosome.

chylomicron: A plasma lipoprotein consisting of a large droplet of triacylglycerols stabilized by a coat of protein and phospholipid; carries lipids from the intestine to tissues.

circular dichroism (CD) spectroscopy: A method used to characterize the degree of folding in a protein, based on differences in the absorption of right-handed versus left-handed circularly polarized light.

cis-trans isomers: *See* geometric isomers.

cistron: A unit of DNA or RNA corresponding to one gene.

citric acid cycle: A cyclic pathway for the oxidation of acetyl residues to carbon dioxide, in which formation of citrate is the first step; also known as the Krebs cycle or tricarboxylic acid cycle.

Cleland nomenclature: A shorthand notation developed by W. W. Cleland for describing the progress of enzymatic reactions with multiple substrates and products.

clones: The descendants of a single cell.

cloning: The production of large numbers of identical DNA molecules, cells, or organisms from a single, ancestral DNA molecule, cell, or organism.

closed system: A system that exchanges neither matter nor energy with the surroundings. *See also* system.

CO_2 assimilation: Reaction sequence in which atmospheric CO_2 is converted into organic compounds.

cobalamin: *See* coenzyme B_{12}.

coding strand: In DNA transcription, the DNA strand identical in base sequence to the RNA transcribed from it, with U in the RNA in place of T in the DNA; as distinct from the template strand. Also called the nontemplate strand.

codon: A sequence of three adjacent nucleotides in a nucleic acid that codes for a specific amino acid.

coenzyme: An organic cofactor required for the action of certain enzymes; often is synthesized from a vitamin.

coenzyme A: A coenzyme with two sulfhydryl groups that serve both as acyl group carriers and redox cofactors.

coenzyme B_{12}: An enzymatic cofactor derived from the vitamin cobalamin, involved in certain types of carbon skeletal rearrangements.

cofactor: An inorganic ion or a coenzyme required for enzyme activity.

CO_2 fixation: The reaction, catalyzed by rubisco during photosynthesis or by other carboxylases, in which atmospheric CO_2 is initially incorporated (fixed) into an organic compound.

cognate: Describes two biomolecules that normally interact; for example, an enzyme and its usual substrate, or a receptor and its usual ligand.

cohesive ends: *See* sticky ends.

cointegrate: An intermediate in the migration of certain DNA transposons in which the donor DNA and target DNA are covalently attached.

colligative properties: The properties of a solution that depend on the number of solute particles per unit volume; for example, freezing-point depression.

combinatorial control: The use of combinations of a limited repertoire of regulatory proteins to provide gene-specific regulation of many individual genes.

comparative genomics: A discipline in which genomic information is compared within one organism or between two species or many. Comparisons may focus on, but are not limited to, gene sequences, gene order on a chromosome, regulatory sequences, gene modifications, and gene evolution.

competitive inhibition: A type of enzyme inhibition reversed by increasing the substrate concentration; a competitive inhibitor generally competes with the normal substrate or ligand for a protein's binding site.

complementary: Having a molecular surface with chemical groups arranged to interact specifically with chemical groups on another molecule.

complementary DNA (cDNA): A DNA complementary to a specific RNA, usually made through the use of reverse transcriptase.

Complex I: Respiratory chain complex that catalyzes the transfer to ubiquinone of a hydride ion from NADH and a proton from the mitochondrial matrix, coupled to the transfer of four protons from the matrix to the intermembrane space. Complex I is composed of 45 different polypeptide chains, including an FMN-containing flavoprotein and at least 8 iron-sulfur centers. Also called NADH:ubiquinone oxidoreductase and NADH dehydrogenase.

Complex II: Membrane-bound respiratory chain complex and citric acid cycle component that couples the oxidation of succinate with the reduction of ubiquinone. Complex II has four different protein subunits, a heme group, three 2Fe-2S centers, bound FAD, and binding sites for succinate and ubiquinone. Also called succinate dehydrogenase.

Complex III: Respiratory chain complex that couples the transfer of electrons from ubiquinol to cytochrome *c* with the vectorial transport of protons from the matrix to the intermembrane space (from the cytosol to the periplasmic space in bacteria). Complex III functions as a homodimer; each monomer consists of three proteins: cytochrome *b*, cytochrome c_1, and the Rieske iron-sulfur protein. Also called cytochrome bc_1 complex and ubiquinone:cytochrome *c* oxidoreductase.

Complex IV: Respiratory chain complex that carries electrons from cytochrome *c* to molecular oxygen, reducing it to H_2O. Also called cytochrome oxidase.

condensation: A reaction type in which two compounds are joined with the elimination of water.

configuration: The spatial arrangement of an organic molecule conferred by the presence of (1) double bonds, about which there is no freedom of rotation, or (2) chiral centers, around which substituent groups are arranged in a specific sequence. Configurational isomers cannot be interconverted without breaking one or more covalent bonds.

conformation: A spatial arrangement of substituent groups that are free to assume different positions in space, without breaking any bonds, because of the freedom of bond rotation.

conjugate acid-base pair: A proton donor and its corresponding deprotonated species; for example, acetic acid (donor) and acetate (acceptor).

conjugated protein: A protein containing one or more prosthetic groups.

conjugate redox pair: An electron donor and its corresponding electron acceptor; for example, Cu^+ (donor) and Cu^{2+} (acceptor), or NADH (donor) and NAD^+ (acceptor).

consensus sequence: A DNA or amino acid sequence consisting of the residues that most commonly occur at each position in a set of similar sequences.

conservative substitution: Replacement of an amino acid residue in a polypeptide by another residue with similar properties; for example, substitution of Glu by Asp.

constitutive enzymes: Enzymes required at all times by a cell and present at some constant level; for example, many enzymes of the central metabolic pathways. Sometimes called housekeeping enzymes.

contig: A series of overlapping clones or a continuous sequence defining an uninterrupted section of a chromosome.

contour length: The length of a nucleic acid molecule as measured along its helical axis.

cooperativity: The characteristic of an enzyme or other protein in which binding of the first molecule of a ligand changes the affinity for the second molecule. In positive cooperativity, the affinity for the second ligand molecule increases; in negative cooperativity, it decreases.

cotransport: The simultaneous transport, by a single transporter, of two solutes across a membrane. *See also* antiport; symport; uniport.

coupled reactions: Two chemical reactions that have a common intermediate and thus a means of energy transfer from one to the other.

covalent bond: A chemical bond that involves sharing of electron pairs.

CRISPR/Cas: Bacterial systems that evolved to provide a defense against bacteriophage infection. CRISPR stands for *c*lustered, *r*egularly *i*nterspaced *s*hort *p*alindromic *r*epeats. Cas stands for *C*RISPR-*as*sociated. Engineered CRISPR/Cas systems are used for efficient, targeted genome editing in a wide range of organisms.

cristae: Infoldings of the inner mitochondrial membrane.

CRP: *See* cAMP receptor protein.

cruciform: A secondary structure in double-stranded RNA or DNA in which the double helix is denatured at palindromic repeat sequences in each strand, and each separated strand is paired internally to form opposing hairpin structures. *See also* hairpin.

cryo-electron microscopy (cryo-EM): A technique for determining the structure of proteins or protein complexes; molecules are quick-frozen on a grid in random orientations and visualized by EM. Images of individual molecules are computationally aligned and combined, yielding a three-dimensional map into which a structure can be modeled.

cyclic AMP (cAMP; adenosine 3′,5′-cyclic monophosphate): A second messenger; its formation from ATP in a cell by adenylyl cyclase is stimulated by certain hormones or other molecular signals.

cyclic electron transfer: In chloroplasts, the light-induced transfer of electrons originating from and returning to photosystem I.

cyclic photophosphorylation: ATP synthesis driven by cyclic electron transfer through cytochrome b_6f and photosystem I.

cyclin: One of a family of proteins that activate cyclin-dependent protein kinases and thereby regulate the cell cycle.

cytochrome P-450: A family of heme-containing enzymes, with a characteristic absorption band at 450 nm, that participate in biological hydroxylations with O_2.

cytochromes: Heme proteins serving as electron carriers in respiration, photosynthesis, and other oxidation-reduction reactions.

cytokine: One of a family of small secreted proteins (such as interleukins and interferons) that activate cell division or differentiation by binding to plasma membrane receptors in target cells.

cytokinesis: The final separation of daughter cells following mitosis.

cytoplasm: The portion of a cell's contents outside the nucleus but within the plasma membrane; includes organelles such as mitochondria.

cytoskeleton: The filamentous network that provides structure and organization to the cytoplasm; includes actin filaments, microtubules, and intermediate filaments.

cytosol: The continuous aqueous phase of the cytoplasm, with its dissolved solutes; excludes the organelles such as mitochondria.

d

dalton: Unit of atomic or molecular weight; 1 dalton (Da) is the weight of a hydrogen atom (1.66×10^{-24} g).

dark reactions: *See* carbon-assimilation reactions.

deamination: The enzymatic removal of amino groups from biomolecules such as amino acids or nucleotides.

degenerate code: A code in which a single element in one language is specified by more than one element in a second language.

dehydrogenases: Enzymes that catalyze the removal of pairs of hydrogen atoms from substrates, often with NAD as a coenzyme.

deletion mutation: A mutation resulting from deletion of one or more nucleotides from a gene or chromosome.

ΔG: *See* free-energy change.

$\Delta G^\ddagger$: *See* activation energy.

ΔG°: *See* standard free-energy change.

$\Delta G'^\circ$: *See* biochemical standard free-energy change.

ΔG_B: *See* binding energy.

ΔG_p: *See* phosphorylation potential.

denaturation: Partial or complete unfolding of the specific native conformation of a polypeptide chain, protein, or nucleic acid such that the function of the molecule is lost.

denatured protein: A protein that has lost enough of its native conformation by exposure to a destabilizing agent such as heat or detergent that its function is lost.

de novo pathway: A pathway for the synthesis of a biomolecule, such as a nucleotide, from simple precursors; as distinct from a salvage pathway.

deoxyribonucleic acid: *See* DNA.

deoxyribonucleotides: Nucleotides containing 2-deoxy-D-ribose as the pentose component.

desaturases: Enzymes that catalyze the introduction of double bonds into the hydrocarbon portion of fatty acids.

desensitization: A universal process by which sensory mechanisms cease to respond after prolonged exposure to the specific stimulus they detect.

desolvation: In aqueous solution, the release of bound water surrounding a solute.

diabetes mellitus: A group of metabolic diseases with symptoms that result from a deficiency in insulin production or responsiveness; characterized by an inability to transport glucose from the blood into cells at normal glucose concentrations.

dialysis: Removal of small molecules from a solution of a macromolecule by their diffusion through a semipermeable membrane into a suitably buffered solution.

dideoxy sequencing: *See* Sanger sequencing.

differential centrifugation: Separation of cell organelles or other particles of different size by their different rates of sedimentation in a centrifugal field.

differentiation: Specialization of cell structure and function during growth and development.

diffusion: Net movement of molecules in the direction of lower concentration.

digestion: Enzymatic hydrolysis of major nutrients in the gastrointestinal system to yield their simpler components.

diploid: Having two sets of genetic information; describes a cell with two chromosomes of each type. *Compare* haploid.

disaccharide: A carbohydrate consisting of two covalently joined monosaccharide units.

dissociation constant (K_d): An equilibrium constant for the dissociation of a complex of two or more biomolecules into its components; for example, dissociation of a substrate from an enzyme.

disulfide bond: A covalent bond resulting from the oxidative linkage of two Cys residues, from the same or different polypeptide chains, forming a cystine residue.

DNA (deoxyribonucleic acid): A polynucleotide with a specific sequence of deoxyribonucleotide units covalently joined through 3′,5′-phosphodiester bonds; serves as the carrier of genetic information.

DNA chimera: DNA containing genetic information derived from two different species.

DNA chip: An informal term for a DNA microarray, referring to the small size of typical microarrays.

DNA cloning: *See* cloning.

DNA library: A collection of cloned DNA fragments.

DNA ligases: Enzymes that create a phosphodiester bond between the 3′ end of one DNA segment and the 5′ end of another.

DNA looping: The interaction of proteins bound at distant sites on a DNA molecule so that the intervening DNA forms a loop.

DNA microarray: A collection of DNA sequences immobilized on a solid surface, with individual sequences laid out in patterned arrays that can be probed by hybridization.

DNA polymerases: Enzymes that catalyze template-dependent synthesis of DNA from its deoxyribonucleoside 5′-triphosphate precursors. The bacterium *Escherichia coli* has five DNA polymerases, numbered I through V; eukaryotes have a larger number.

DNA supercoiling: The coiling of DNA upon itself, generally as a result of bending, underwinding, or overwinding of the DNA helix.

DNA transposition: *See* transposition.

domain: A distinct structural unit of a polypeptide; domains may have separate functions and may fold as independent, compact units.

double helix: The natural coiled conformation of two complementary, antiparallel DNA chains.

double-reciprocal plot: A plot of $1/V_0$ versus $1/[S]$, which allows a more accurate determination of V_{max} and K_m than a plot of V_0 versus [S]; also called the Lineweaver-Burk plot.

e

***E*°:** *See* standard reduction potential.

***E*′°:** Standard transformed reduction potential. *See* standard reduction potential.

ECM: *See* extracellular matrix.

eicosanoid: Any of several classes of hydrophobic signaling molecules derived from the lipid arachidonate, including prostaglandins, thromboxanes, and leukotrienes, that regulate physiological responses in humans such as inflammation, blood pressure, and fever.

electrochemical gradient: The resultant of the gradients of concentration and of electric charge of an ion across a membrane; the driving force for oxidative phosphorylation and photophosphorylation.

electrochemical potential: The energy required to maintain a separation of charge and of concentration across a membrane.

electrogenic: Contributing to an electrical potential across a membrane.

electron acceptor: A substance that receives electrons in an oxidation-reduction reaction.

electron carrier: A protein, such as a flavoprotein or a cytochrome, that can reversibly gain and lose electrons; functions in the transfer of electrons from organic nutrients to oxygen or some other terminal acceptor.

electron donor: A substance that donates electrons in an oxidation-reduction reaction.

electron transfer: Movement of electrons from electron donor to electron acceptor; especially, from substrates to oxygen via the carriers of the respiratory (electron-transfer) chain.

electron-transfer flavoprotein (ETF): Flavoprotein that carries electrons from fatty acid β-oxidation to the mitochondrial electron transfer chain.

electrophile: An electron-deficient group with a strong tendency to accept electrons from an electron-rich group (nucleophile).

electrophoresis: Movement of charged solutes in response to an electrical field; often used to separate mixtures of ions, proteins, or nucleic acids.

ELISA: *See* enzyme-linked immunosorbent assay.

elongation factors: (1) Proteins that function in the elongation phase of eukaryotic transcription. (2) Specific proteins required in the elongation of polypeptide chains by ribosomes.

eluate: The effluent from a chromatographic column.

enantiomers: Stereoisomers that are nonsuperposable mirror images of each other.

endergonic reaction: A chemical reaction that consumes energy (i.e., for which ΔG is positive).

endocannabinoids: Endogenous substances capable of binding to and functionally activating cannabinoid receptors.

endocrine: Pertaining to cellular secretions that enter the bloodstream and have their effects on distant tissues.

endocytosis: The uptake of extracellular material by its inclusion in a vesicle (endosome) formed by invagination of the plasma membrane.

endonucleases: Enzymes that hydrolyze the interior phosphodiester bonds of a nucleic acid—that is, act at bonds other than the terminal bonds.

endoplasmic reticulum: An extensive system of double membranes in the cytoplasm of eukaryotic cells; it encloses secretory channels and is often studded with ribosomes (rough endoplasmic reticulum).

endothermic reaction: A chemical reaction that takes up heat (i.e., for which ΔH is positive).

end-product inhibition: *See* feedback inhibition.

energy transduction: The conversion of energy from one form to another.

enhancers: DNA sequences that facilitate the expression of a given gene; may be located a few hundred, or even thousand, base pairs away from the gene.

enthalpy (H): The heat content of a system.

enthalpy change (ΔH): For a reaction, approximately equal to the difference between the energy used to break bonds and the energy gained by formation of new bonds.

entropy (S): The extent of randomness or disorder in a system.

enzyme: A biomolecule, either protein or RNA, that catalyzes a specific chemical reaction. It does not affect the equilibrium of the catalyzed reaction; it enhances the rate of the reaction by providing a reaction path with a lower activation energy.

enzyme cascade: A series of reactions, often involved in regulatory events, in which one enzyme activates another (often by phosphorylation), which activates a third, and so on. The effect of a catalyst activating a catalyst is a large amplification of the signal that initiated the cascade. *See also* regulatory cascade.

enzyme-linked immunosorbent assay (ELISA): A sensitive immunoassay that uses an enzyme linked to an antibody or antigen to detect a specific protein.

epigenetic: Describes any inherited characteristic of a living organism that is acquired by means that do not involve the nucleotide sequence of the parental chromosomes; for example, covalent modifications of histones.

epimerases: Enzymes that catalyze the reversible interconversion of two epimers.

epimers: Two stereoisomers differing in configuration at one asymmetric center in a compound having two or more asymmetric centers.

epithelial cell: Any cell that forms part of the outer covering of an organism or organ.

epitope: An antigenic determinant; the particular chemical group or groups in a macromolecule (antigen) to which a given antibody binds.

epitope tag: A protein sequence or domain bound by a well-characterized antibody.

equilibrium: The state of a system in which no further net change is occurring; the free energy is at a minimum.

equilibrium constant (K_{eq}): A constant, characteristic for each chemical reaction, that relates the specific concentrations of all reactants and products at equilibrium at a given temperature and pressure.

erythrocyte: A cell containing large amounts of hemoglobin and specialized for oxygen transport, but without a nucleus or mitochondria; a red blood cell.

essential amino acids: Amino acids that cannot be synthesized by humans and must be obtained from the diet.

essential fatty acids: The group of polyunsaturated fatty acids produced by plants, but not by humans; required in the human diet.

ETF: *See* electron-transferring flavoprotein.

ethanol fermentation: The anaerobic conversion of glucose to ethanol via glycolysis; also called alcohol fermentation. *See also* fermentation.

euchromatin: The regions of interphase chromosomes that are more open (less condensed), where genes are being actively expressed. *Compare* heterochromatin.

eukaryotes: Members of Eukarya, one of the three domains of living organisms; unicellular or multicellular organisms with cells having a membrane-bounded nucleus, multiple chromosomes, and internal organelles.

exchange factors: Enzymes that catalyze the exchange of histone variants within eukaryotic nucleosomes.

excited state: An energy-rich state of an atom or molecule, produced by absorption of light energy; as distinct from ground state.

exergonic reaction: A chemical reaction that proceeds with the release of free energy (i.e., for which ΔG is negative).

exocytosis: The fusion of an intracellular vesicle with the plasma membrane, releasing the vesicle contents to the extracellular space.

exon: The segment of a eukaryotic gene that encodes a portion of the final product of the gene; a segment of RNA that remains after posttranscriptional processing and is transcribed into a protein or incorporated into the structure of an RNA. *See also* intron.

exonucleases: Enzymes that hydrolyze only those phosphodiester bonds that are in the terminal positions of a nucleic acid.

exosome: In eukaryotes, a large protein complex involved in RNA degradation that is composed of a barrel-like assembly of proteins through which RNA is fed into a nuclease.

exothermic reaction: A chemical reaction that releases heat (i.e., for which ΔH is negative).

expression vector: A vector incorporating sequences that allow the transcription and translation of a cloned gene. *See* vector.

extracellular matrix (ECM): An interwoven combination of glycosaminoglycans, proteoglycans, and proteins, just outside the plasma membrane, that provides cell anchorage, positional recognition, and traction during cell migration.

extrahepatic: Describes all tissues outside the liver; implies the centrality of the liver in metabolism.

f

FAD (flavin adenine dinucleotide): The coenzyme of some oxidation-reduction enzymes; contains riboflavin.

F_1 ATPase: The multiprotein subunit of ATP synthase that has the ATP-synthesizing catalytic sites. It interacts with the F_o subunit of ATP synthase, coupling proton movement to ATP synthesis.

fatty acid: A long-chain aliphatic carboxylic acid in natural fats and oils; also a component of membrane phospholipids and glycolipids.

feedback inhibition: Inhibition of an allosteric enzyme at the beginning of a metabolic sequence by the end product of the sequence; also known as end-product inhibition.

fermentation: Energy-yielding anaerobic breakdown of a nutrient molecule, such as glucose, without net oxidation; yields lactate, ethanol, or some other simple product.

ferredoxin: One of a family of small, soluble Fe-S proteins that serve as one-electron carriers between PSI and NADH in photosynthetic organisms.

fibrin: A protein factor that forms the cross-linked fibers in blood clots.

fibrinogen: The inactive precursor protein of fibrin.

fibroblast: A cell of the connective tissue that secretes connective tissue proteins such as collagen.

fibrous proteins: Insoluble proteins that serve a protective or structural role; contain polypeptide chains that generally share a common secondary structure.

first law of thermodynamics: The law stating that, in all processes, the total energy of the universe remains constant.

Fischer projection formulas: A method for representing molecules that shows the configuration of groups around chiral centers; also known as projection formulas.

5′ end: The end of a nucleic acid that lacks a nucleotide bound at the 5′ position of the terminal residue.

flagellum: A cell appendage used in propulsion. Bacterial flagella have a much simpler structure than eukaryotic flagella, which are similar to cilia.

flavin adenine dinucleotide: *See* FAD.

flavin-linked dehydrogenases: Dehydrogenases requiring one of the riboflavin coenzymes, FMN or FAD.

flavin mononucleotide: *See* FMN.

flavin nucleotides: Nucleotide coenzymes (FMN and FAD) containing riboflavin.

flavoprotein: An enzyme containing a flavin nucleotide as a tightly bound prosthetic group.

flippases: Membrane proteins in the ABC transporter family that catalyze movement of phospholipids from the extracellular leaflet (monolayer) to the cytosolic leaflet of a membrane bilayer.

floppases: Membrane proteins in the ABC transporter family that catalyze movement of phospholipids from the cytosolic leaflet (monolayer) to the extracellular leaflet of a membrane bilayer.

fluid mosaic model: The model describing biological membranes as a fluid lipid bilayer with embedded proteins; the bilayer exhibits both structural and functional asymmetry.

fluorescence: Emission of light by excited molecules as they revert to the ground state.

fluorescence recovery after photobleaching: *See* FRAP.

fluorescence resonance energy transfer: *See* FRET.

FMN (flavin mononucleotide): Riboflavin phosphate, a coenzyme of certain oxidation-reduction enzymes.

fold: *See* motif.

footprinting: A technique for identifying the nucleic acid sequence bound by a DNA- or RNA-binding protein.

fraction: A portion of a biological sample that has been subjected to a procedure designed to separate macromolecules based on a property such as solubility, net charge, molecular weight, or function.

fractionation: The process of separating the proteins or other components of a complex molecular mixture into fractions based on differences in properties such as solubility, net charge, molecular weight, or function.

frame shift: A mutation caused by insertion or deletion of one or more paired nucleotides, changing the reading frame of codons during protein synthesis; the polypeptide product has a garbled amino acid sequence beginning at the mutated codon.

FRAP (fluorescence recovery after photobleaching): A technique used to quantify the diffusion of membrane components (lipids or proteins) in the plane of the bilayer.

free energy (*G*): The component of the total energy of a system that can do work at constant temperature and pressure.

free energy of activation ($\Delta G^{\ddagger}$): *See* activation energy.

free-energy change (ΔG): The amount of free energy released (negative ΔG) or absorbed (positive ΔG) in a reaction at constant temperature and pressure.

free radical: *See* radical.

FRET (fluorescence resonance energy transfer): A technique for estimating the distance between two proteins or two domains of a protein by measuring the nonradiative transfer of energy between reporter chromophores when one protein or domain is excited and the fluorescence emitted from the other is quantified.

functional group: The specific atom or group of atoms that confers a particular chemical property on a biomolecule.

furanose: A simple sugar containing the five-membered furan ring.

fusion protein: (1) One of a family of proteins that facilitate membrane fusion. (2) The protein product of a gene created by the fusion of two distinct genes or portions of genes.

futile cycle: *See* substrate cycle.

g

G_i: *See* inhibitory G protein.

G_s: *See* stimulatory G protein.

gametes: Reproductive cells with a haploid gene content; sperm or egg cells.

ganglioside: A sphingolipid containing a complex oligosaccharide as a head group; especially common in nervous tissue.

GAPs: *See* GTPase activator proteins.

GEFs: *See* guanosine nucleotide–exchange factors.

gel filtration: *See* size-exclusion chromatography.

gene: A chromosomal segment that codes for a single functional polypeptide chain or RNA molecule.

gene expression: Transcription, and, in the case of proteins, translation, to yield the product of a gene; a gene is expressed when its biological product is present and active.

gene fusion: The enzymatic attachment of one gene, or part of a gene, to another.

general acid-base catalysis: Catalysis involving proton transfer(s) to or from a molecule other than water.

general transcription factors: Protein factors required for transcription by RNA polymerase II in eukaryotes.

genetic code: The set of triplet code words in DNA (or mRNA) coding for the amino acids of proteins.

genetic engineering: Any process by which genetic material, particularly DNA, is altered by a molecular biologist.

genetic map: A diagram showing the relative sequence and position of specific genes along a chromosome.

genome: All the genetic information encoded in a cell or virus.

genome annotation: The process of assigning actual or likely functions to genes discovered during genomic DNA sequencing projects.

genomic library: A DNA library containing DNA segments that represent all (or most) of the sequences in an organism's genome.

genomics: A science devoted broadly to the understanding of cellular and organism genomes.

genotype: The genetic constitution of an organism, as distinct from its physical characteristics, or phenotype.

geometric isomers: Isomers related by rotation about a double bond; also called cis-trans isomers.

germ-line cell: A type of animal cell that is formed early in embryogenesis and may multiply by mitosis or produce, by meiosis, cells that develop into gametes (egg or sperm cells).

GFP: *See* green fluorescent protein.

globular proteins: Soluble proteins with a globular (somewhat rounded) shape.

glucogenic: Capable of being converted into glucose or glycogen by the process of gluconeogenesis.

gluconeogenesis: The biosynthesis of a carbohydrate from simpler, noncarbohydrate precursors such as oxaloacetate or pyruvate.

GLUT: Designation for a family of membrane proteins that transport glucose.

glycan: A polymer of monosaccharide units joined by glycosidic bonds; polysaccharide.

glyceroneogenesis: The synthesis in adipocytes of glycerol 3-phosphate from pyruvate for use in triacylglycerol synthesis.

glycerophospholipid: An amphipathic lipid with a glycerol backbone; fatty acids are ester-linked to C-1 and C-2 of the glycerol, and a polar alcohol is attached through a phosphodiester linkage to C-3.

glycoconjugate: A compound containing a carbohydrate component bound covalently to a protein or lipid, forming a glycoprotein or glycolipid.

glycogenesis: The process of converting glucose to glycogen.

glycogenin: The protein that both primes the synthesis of new glycogen chains and catalyzes the polymerization of the first few sugar residues of each chain before glycogen synthase continues the extension.

glycogenolysis: The enzymatic breakdown of stored (not dietary) glycogen.

glycolate pathway: The metabolic pathway in photosynthetic organisms that converts glycolate produced during photorespiration into 3-phosphoglycerate.

glycolipid: A lipid containing a carbohydrate group.

glycolysis: The catabolic pathway by which a molecule of glucose is broken down into two molecules of pyruvate. *Compare* aerobic glycolysis.

glycome: The full complement of carbohydrates and carbohydrate-containing molecules of a cell or tissue under a particular set of conditions.

glycomics: The systematic characterization of the glycome.

glycoprotein: A protein containing a carbohydrate group, typically a complex oligosaccharide.

glycosaminoglycan: A heteropolysaccharide of two alternating units: one is either *N*-acetylglucosamine or *N*-acetylgalactosamine; the other is a uronic acid (usually glucuronic acid). Formerly called a mucopolysaccharide.

glycosidic bonds: *See O*-glyosidic bonds.

glycosphingolipid: An amphipathic lipid with a sphingosine backbone to which are attached a long-chain fatty acid and a polar alcohol head group.

glyoxylate cycle: A variant of the citric acid cycle, for the net conversion of acetate into succinate and, eventually, new carbohydrate; present in bacteria and some plant cells.

glyoxysome: A specialized peroxisome containing the enzymes of the glyoxylate cycle; found in cells of germinating seeds.

glypican: A heparan sulfate proteoglycan attached to a membrane through a glycosyl phosphatidylinositol (GPI) anchor.

Golgi complex: A complex membranous organelle of eukaryotic cells; functions in the posttranslational modification of proteins and their secretion from the cell or incorporation into the plasma membrane or organellar membranes.

GPCRs: *See* G protein–coupled receptors.

GPI-anchored protein: A protein held to the outer monolayer of the plasma membrane by its covalent attachment through a short oligosaccharide chain to a phosphatidylinositol molecule in the membrane.

G protein–coupled receptor kinases (GRKs): A family of protein kinases that phosphorylate Ser and Thr residues near the carboxyl terminus of G protein–coupled receptors, initiating their internalization.

G protein–coupled receptors (GPCRs): A large family of membrane receptor proteins with seven transmembrane helical segments, often associating with G proteins to transduce an extracellular signal into a change in cellular metabolism.

G proteins: A large family of GTP-binding proteins that act in intracellular signaling pathways and in membrane trafficking. Active when GTP is bound, they self-inactivate by converting GTP to GDP. Also called guanosine nucleotide–binding proteins.

gram molecular weight: For a compound, the weight in grams that is numerically equal to its molecular weight; the weight of one mole.

grana: Stacks of thylakoids, flattened membranous sacs or disks, in chloroplasts.

green fluorescent protein (GFP): A small protein from a marine organism that produces bright fluorescence in the green region of the visible spectrum. Fusion proteins with GFP are commonly used to determine the subcellular location of the fused protein by fluorescence microscopy.

GRKs: *See* G protein–coupled receptor kinases.

gRNA: *See* guide RNA.

ground state: The normal, stable form of an atom or molecule, as distinct from the excited state.

group transfer potential: A measure of the ability of a compound to donate an activated group (such as a phosphate or acyl group); generally expressed as the standard free energy of hydrolysis.

growth factors: Proteins or other molecules that act from outside a cell to stimulate cell growth and division.

GTPase activator proteins (GAPs): Regulatory proteins that bind activated G proteins and stimulate their intrinsic GTPase activity, speeding their self-inactivation.

guanosine nucleotide–binding proteins: *See* G proteins.

guanosine nucleotide–exchange factors (GEFs): Regulatory proteins that bind to and activate G proteins by stimulating the exchange of bound GDP for GTP.

guide RNA (gRNA): An RNA found in CRISPR systems that has sequences complementary to those in a target DNA such as a phage DNA.

h

hairpin: Secondary structure in single-stranded RNA or DNA, in which complementary parts of a palindromic repeat fold back and pair to form an antiparallel duplex helix closed at one end. *See also* cruciform.

half-life: The time required for the disappearance or decay of one-half of a given component in a system.

haploid: Having a single set of genetic information; describes a cell with one chromosome of each type. *Compare* diploid.

haplotype: A combination of alleles of different genes located sufficiently close together on a chromosome that they tend to be inherited together.

hapten: A small molecule that, when linked to a larger molecule, elicits an immune response.

Haworth perspective formulas: A method for representing cyclic chemical structures so as to define the configuration of each substituent group; commonly used for representing sugars.

helicases: Enzymes that catalyze separation of strands of double-stranded DNA or RNA. Helicases are required for gene replication and expression.

heme: The iron-porphyrin prosthetic group of heme proteins.

heme protein: A protein containing a heme as prosthetic group.

hemoglobin: A heme protein in erythrocytes; functions in oxygen transport.

Henderson-Hasselbalch equation: An equation relating pH, pK_a, and ratio of the concentrations of proton-acceptor (A^-) and proton-donor (HA) species in a solution:

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[\mathrm{A}^-]}{[\mathrm{HA}]}.$$

heparan sulfate: A sulfated polymer of alternating *N*-acetylglucosamine and a uronic acid, either glucuronic or iduronic acid; typically found in the extracellular matrix.

hepatocyte: The major cell type of liver tissue.

heterochromatin: The regions of chromosomes that are condensed, in which gene expression is generally suppressed. *Compare* euchromatin.

heteropolysaccharide: A polysaccharide containing more than one type of sugar.

heterotroph: An organism that requires complex nutrient molecules, such as glucose, as a source of energy and carbon.

heterotropic: Describes an allosteric modulator that is distinct from the normal ligand.

heterotropic enzyme: An allosteric enzyme requiring a modulator other than its substrate.

hexose: A simple sugar with a backbone containing six carbon atoms.

hexose monophosphate pathway: *See* pentose phosphate pathway.

high-performance liquid chromatography (HPLC): Chromatographic procedure, often conducted at relatively high pressures using automated equipment, which permits refined and highly reproducible profiles.

Hill coefficient: A measure of cooperative interaction between protein subunits.

Hill reaction: The evolution of oxygen and photoreduction of an artificial electron acceptor by a chloroplast preparation in the absence of carbon dioxide.

histones: The family of basic proteins that associate tightly with DNA in the chromosomes of all eukaryotic cells.

Holliday intermediate: An intermediate in genetic recombination in which two double-stranded DNA molecules are joined by a reciprocal crossover involving one strand of each molecule.

holoenzyme: A catalytically active enzyme, including all necessary subunits, prosthetic groups, and cofactors.

homeobox: A conserved DNA sequence of 180 base pairs that encodes a protein domain found in many proteins with a regulatory role in development.

homeodomain: The protein domain encoded by the homeobox; a regulatory unit that determines the segmentation of a body plan.

homeostasis: The maintenance of a dynamic steady state by regulatory mechanisms that compensate for changes in external circumstances.

homeotic genes: Genes that regulate development of the pattern of segments in the *Drosophila* body plan; similar genes are found in most vertebrates.

homologous genetic recombination: Recombination between two DNA molecules of similar sequence, taking place in all cells; occurs during meiosis and mitosis in eukaryotes.

homologous proteins: Proteins having similar sequences and functions in different species; for example, the hemoglobins.

homologs: Genes or proteins that possess a clear sequence and functional relationship to each other.

homopolysaccharide: A polysaccharide made up of one type of monosaccharide unit.

homotropic: Describes an allosteric modulator that is identical to the normal ligand.

homotropic enzyme: An allosteric enzyme that uses its substrate as a modulator.

hormone: A chemical substance, synthesized in small amounts by an endocrine tissue, that is carried in the blood to another tissue, or diffuses to a nearby cell, where it acts as a messenger to regulate the function of the target tissue or organ.

hormone receptor: A protein in, or on the surface of, target cells that binds a specific hormone and initiates the cellular response.

hormone response element (HRE): A short (12 to 20 base pairs) DNA sequence that binds receptors for steroid, retinoid, thyroid, and vitamin D hormones, altering expression of the contiguous genes. Each hormone has a consensus sequence preferred by the cognate receptor.

housekeeping genes: Genes that encode products (such as the enzymes of the central energy-yielding pathways) needed by cells at all times; also called constitutive genes because they are expressed under all conditions.

HPLC: *See* high-performance liquid chromatography.

HRE: *See* hormone response element.

hyaluronan: A high molecular weight, acidic polysaccharide typically composed of the alternating disaccharide GlcUA(β1→3)GlcNAc; major component of the extracellular matrix, forming larger complexes (proteoglycans) with proteins and other acidic polysaccharides. Also called hyaluronic acid.

hybridoma: Stable, antibody-producing cell lines that grow well in tissue culture; created by fusing an antibody-producing B lymphocyte with a myeloma cell.

hydrogen bond: A weak electrostatic attraction between one electronegative atom (such as oxygen or nitrogen) and a hydrogen atom covalently linked to a second electronegative atom.

hydrolases: Enzymes (e.g., proteases, lipases, phosphatases, nucleases) that catalyze hydrolysis reactions.

hydrolysis: Cleavage of a bond, such as an anhydride or peptide bond, by addition of the elements of water, yielding two or more products.

hydronium ion: The hydrated hydrogen ion (H_3O^+).

hydropathy index: A scale that expresses the relative hydrophobic and hydrophilic tendencies of a chemical group.

hydrophilic: Polar or charged; describes molecules or groups that associate with (dissolve easily in) water.

hydrophobic: Nonpolar; describes molecules or groups that are insoluble in water.

hydrophobic effect: The aggregation of nonpolar molecules in aqueous solution, excluding water molecules; caused largely by an entropic effect related to the hydrogen-bonding structure of the surrounding water.

hyperchromic effect: The large increase in light absorption at 260 nm as a double-helical DNA unwinds (melts).

hypoxia: The metabolic condition in which the supply of oxygen is severely limited.

i

Ig: *See* immunoglobulin.

immune response: The capacity of a vertebrate to generate antibodies to an antigen, a macromolecule foreign to the organism.

immunoblotting: A technique using antibodies to detect the presence of a protein in a biological sample after the proteins have been separated by gel electrophoresis, transferred to a membrane, and immobilized; also called Western blotting.

immunoglobulin (Ig): An antibody protein generated against, and capable of binding specifically to, an antigen.

induced fit: An enzyme conformation change in response to substrate binding that renders the enzyme catalytically active; also denotes a conformation change in any macromolecule in response to ligand binding, such that the binding site better conforms to the shape of the ligand.

inducer: A signal molecule that, when bound to a regulatory protein, increases the expression of a given gene.

induction: An increase in the expression of a gene in response to a change in the activity of a regulatory protein.

informational macromolecules: Biomolecules containing information in the form of specific sequences of different monomers; for example, many proteins, lipids, polysaccharides, and nucleic acids.

inhibitory G protein (G_i): A trimeric GTP-binding protein that, when activated by an associated plasma membrane receptor, inhibits a neighboring membrane enzyme such as adenylyl cyclase. *Compare* stimulatory G protein (G_s).

initiation codon: AUG (sometimes GUG or, more rarely, UUG in bacteria and archaea); codes for the first amino acid in a polypeptide sequence: *N*-formylmethionine in bacteria; methionine in archaea and eukaryotes.

initiation complex: A complex of a ribosome with an mRNA and the initiating Met-tRNA^{Met} or fMet-tRNA^{fMet}, ready for the elongation steps.

inorganic pyrophosphatase: An enzyme that hydrolyzes a molecule of inorganic pyrophosphate to yield two molecules of (ortho) phosphate; also known as pyrophosphatase.

insertion mutation: A mutation caused by insertion of one or more extra bases, or a mutagen, between successive bases in DNA.

insertion sequence: Specific base sequences at either end of a transposable segment of DNA.

in silico: "In silicon"; that is, by computer simulation.

in situ: "In position"; that is, in its natural position or location.

integral proteins: Proteins firmly bound to a membrane by interactions resulting from the hydrophobic effect; as distinct from peripheral proteins.

integrin: One of a large family of heterodimeric transmembrane proteins that mediate adhesion of cells to other cells or to the extracellular matrix.

intercalation: Insertion between stacked aromatic or planar rings; for example, insertion of a planar molecule between two successive bases in a nucleic acid.

intermediary metabolism: In cells, the enzyme-catalyzed reactions that extract chemical energy from nutrient molecules and use it to synthesize and assemble cell components.

intrinsically disordered proteins: Proteins, or segments of proteins, that lack a definable three-dimensional structure in solution. In some cases folding can be dictated by binding partners.

intron: A sequence of nucleotides in a gene that is transcribed but removed from the RNA transcript before translation; also called intervening sequence. *See also* exon.

in vitro: "In glass"; that is, in the test tube.

in vivo: "In life"; that is, in the living cell or organism.

ion channels: Integral proteins that provide for the regulated transport of a specific ion, or ions, across a membrane.

ion-exchange chromatography: A process for separating complex mixtures of ionic compounds by many repeated partitionings between a flowing (mobile) phase and a stationary phase consisting of a polymeric resin that contains fixed charged groups.

ionizing radiation: A type of radiation, such as x-rays, that causes loss of electrons from some organic molecules, thus making them more reactive.

ionophore: A compound that binds one or more metal ions and is capable of diffusing across a membrane, carrying the bound ion.

ionotropic: Describes a membrane receptor that acts as a ligand-gated ion channel. *Compare* metabotropic.

ion product of water (K_w): The product of the concentrations of H^+ and OH^- in pure water; $K_w = [H^+][OH^-] = 1 \times 10^{-14}$ at 25 °C.

iron-sulfur protein: One of a large family of electron-transfer proteins in which the electron carrier is one or more iron ions associated with two or more sulfur atoms of Cys residues or of inorganic sulfide.

isoelectric focusing: An electrophoretic method for separating macromolecules on the basis of isoelectric pH.

isoelectric pH (isoelectric point, pI): The pH at which a solute has no net electric charge and thus does not move in an electric field.

isoenzymes: *See* isozymes.

isomerases: Enzymes that catalyze the transformation of compounds into their positional isomers.

isomers: Any two molecules with the same molecular formula but a different arrangement of molecular groups.

isoprene: The hydrocarbon 2-methyl-1,3-butadiene, a recurring structural unit of terpenoids.

isoprenoid: Any of a large number of natural products synthesized by enzymatic polymerization of two or more isoprene units; also called terpenoid.

isozymes: Multiple forms of an enzyme that catalyze the same reaction but differ in amino acid sequence, substrate affinity, V_{max}, and/or regulatory properties; also called isoenzymes.

k

K_a: *See* acid dissociation constant.

K_d: *See* dissociation constant.

K_{eq}: *See* equilibrium constant.

K_m: *See* Michaelis constant.

K_t ($K_{transport}$): *See* transport constant.

K_w: *See* ion product of water.

ketoacidosis: A pathological condition sometimes experienced by people with untreated diabetes in which the ketone bodies acetoacetate and D-β-hydroxybutyrate reach extraordinary levels in tissues, urine, and blood (ketosis), which lowers the blood pH (acidosis).

ketogenic: Yielding acetyl-CoA, a precursor for ketone body formation, as a breakdown product.

ketone bodies: Acetoacetate, D-β-hydroxybutyrate, and acetone; water-soluble fuels normally exported by the liver but overproduced during fasting or in untreated diabetes mellitus.

ketose: A simple monosaccharide in which the carbonyl group is a ketone.

ketosis: A condition in which the concentration of ketone bodies in the blood, tissues, and urine is abnormally high.

kinases: Enzymes that catalyze phosphorylation of certain molecules by ATP.

kinetics: The study of reaction rates.

Krebs cycle: *See* citric acid cycle.

l

lagging strand: The DNA strand that, during replication, must be synthesized in the direction opposite to that in which the replication fork moves.

Lands cycle: The process of remodeling the fatty acyl content of phosphatidylcholine.

law of mass action: The law stating that the rate of any given chemical reaction is proportional to the product of the activities (or concentrations) of the reactants.

leader: A short sequence near the amino terminus of a protein or the 5′ end of an RNA that has a specialized targeting or regulatory function.

leading strand: The DNA strand that, during replication, is synthesized in the same direction in which the replication fork moves.

leaky mutant: A mutant gene that gives rise to a product with a detectable level of biological activity.

leaving group: The departing or displaced molecular group in a unimolecular elimination or bimolecular substitution reaction.

lectin: A protein that binds a carbohydrate, commonly an oligosaccharide, with very high affinity and specificity, mediating cell-cell interactions.

lethal mutation: A mutation that inactivates a biological function essential to the life of the cell or organism.

leucine zipper: A protein structural motif involved in protein-protein interactions in many eukaryotic regulatory proteins; consists of two interacting α helices in which Leu residues at every seventh position are a prominent feature of the interacting surfaces.

leukocyte: A white blood cell; involved in the immune response in mammals.

leukotriene (LT): Any of a class of noncyclic eicosanoid signaling lipids with three conjugated double bonds; they mediate inflammatory responses, including smooth muscle activity.

ligand: A small molecule that binds specifically to a larger one; for example, a hormone is the ligand for its specific protein receptor.

ligases: Enzymes that catalyze condensation reactions in which two atoms are joined, using the energy of ATP or another energy-rich compound.

light-dependent reactions: The reactions of photosynthesis that require light and cannot occur in the dark; also known as light reactions.

linear electron transfer: The light-induced transfer of electrons from water to $NADP^+$ in oxygen-evolving photosynthesis; involves photosystems I and II.

Lineweaver-Burk equation: An algebraic transform of the Michaelis-Menten equation, allowing determination of V_{max} and K_m by extrapolation of [S] to infinity:

$$\frac{1}{V_0} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

linking number: The number of times one closed circular DNA strand is wound about another; the number of topological links holding the circles together.

lipases: Enzymes that catalyze the hydrolysis of triacylglycerols.

lipid: A small water-insoluble biomolecule generally containing fatty acids, sterols, or isoprenoid compounds.

lipidome: The full complement of lipid-containing molecules in a cell, organ, or tissue under a particular set of conditions.

lipidomics: The systematic characterization of the lipidome.

lipid transfer protein: A family of proteins that transfer membrane lipids between parts of the endomembrane system at contact points.

lipoate (lipoic acid): A vitamin for some microorganisms; an intermediate carrier of hydrogen atoms and acyl groups in α-keto acid dehydrogenases.

lipoprotein: A lipid-protein aggregate that carries water-insoluble lipids in the blood. The protein component alone is an apolipoprotein.

liposome: A small, spherical vesicle composed of a phospholipid bilayer, forming spontaneously when phospholipids are suspended in an aqueous buffer.

lipoxin (LX): One of a class of hydroxylated linear derivatives of arachidonate that act as potent anti-inflammatory agents.

long noncoding RNA (lncRNA): A functional class of RNA, more than 200 nucleotides long, that does not encode protein, but can have roles in chromosome structure and function.

LT: *See* leukotriene.

LX: *See* lipoxin.

lyases: Enzymes that catalyze removal of a group from a molecule to form a double bond, or addition of a group to a double bond.

lymphocytes: A subclass of leukocytes involved in the immune response. *See also* B lymphocyte; T lymphocyte.

lysis: Destruction of a plasma membrane or (in bacteria) cell wall, releasing the cellular contents and killing the cell.

lysophosphatidylcholine acyltransferases: Enzymes that add polyunsaturated fatty acids of various types to lysophosphatidylcholine at C-2.

lysosome: An organelle of eukaryotic cells; contains many hydrolytic enzymes and serves as a degrading and recycling center for unneeded cellular components.

m

macromolecule: A molecule having a molecular weight in the range of a few thousand to many millions.

MAPKs: *M*itogen-*a*ctivated *p*rotein *k*inases that phosphorylate protein substrates on Ser, Thr, or Tyr residues. They function in protein phosphorylation cascades connecting surface receptors such as the insulin receptor to specific gene expression in the nucleus.

mass-action ratio (*Q*): For the reaction $aA + bB \rightleftharpoons cC + dD$, the ratio $[C]^c[D]^d/[A]^a[B]^b$.

mass spectrometry: A set of methods for the accurate assessment of molecular mass of individual molecules or mixtures of molecules. After samples are introduced to a vacuum and ionized, mass is determined by molecular behavior in successive electrical and magnetic fields, separating ions into a spectrum.

matrix: The space enclosed by the inner membrane of the mitochondrion.

MCS: *See* multiple cloning sites.

mechanistic target of rapamycin complex 1: *See* mTORC1.

Mediator: A highly conserved, multisubunit, eukaryotic coactivator complex required for transcription from many RNA polymerase II promoters.

meiosis: A type of cell division in which diploid cells give rise to haploid cells destined to become gametes or spores.

membrane potential (V_m): The difference in electrical potential across a biological membrane, commonly measured by insertion of a microelectrode; typical values range from −25 mV (by convention, the negative sign indicates that the inside is negative relative to the outside) to more than −100 mV across some plant vacuolar membranes.

membrane transport: Movement of a polar solute across a membrane via a specific membrane protein (a transporter).

messenger ribonucleoprotein complex: A supramolecular complex of messenger RNA and associated proteins. Also called mRNP complex.

messenger RNA (mRNA): A class of RNA molecules that are translated by ribosomes to produce proteins.

metabolic control: The mechanisms by which flux through a metabolic pathway is changed to reflect a cell's altered circumstances.

metabolic regulation: The mechanisms by which a cell resists changes in the concentrations of individual metabolites that would otherwise occur when metabolic control mechanisms alter flux through a pathway.

metabolic syndrome: A combination of medical conditions that together predispose to cardiovascular disease and type 2 diabetes; includes high blood pressure, high concentrations of LDL and triacylglycerol in the blood, slightly elevated fasting blood glucose concentration, and obesity.

metabolism: The entire set of enzyme-catalyzed transformations of organic molecules in living cells; the sum of anabolism and catabolism.

metabolite: A chemical intermediate in the enzyme-catalyzed reactions of metabolism.

metabolome: The complete set of small-molecule metabolites (metabolic intermediates, signals, secondary metabolites) present in a given cell or tissue under specific conditions.

metabolomics: The systematic characterization of the metabolome of a cell or tissue.

metabolon: A supramolecular assembly of sequential metabolic enzymes.

metabotropic: Describes a membrane receptor that acts through a second messenger. *Compare* ionotropic.

metalloprotein: A protein with a metal ion as its prosthetic group.

metamerism: Division of the body into segments, as in insects, for example.

micelle: An aggregate of amphipathic molecules in water, with the nonpolar portions in the interior and the polar portions at the exterior surface, exposed to water.

Michaelis constant (K_m): The substrate concentration at which an enzyme-catalyzed reaction proceeds at one-half its maximum velocity.

Michaelis-Menten equation: The equation describing the hyperbolic dependence of the initial reaction velocity, V_0, on substrate concentration, [S], in many enzyme-catalyzed reactions: $V_0 = \dfrac{V_{max}[S]}{K_m + [S]}$.

Michaelis-Menten kinetics: A kinetic pattern in which the initial rate of an enzyme-catalyzed reaction exhibits a hyperbolic dependence on substrate concentration.

microRNA (miRNA): A class of small RNA molecules (20 to 25 nucleotides after processing is complete) involved in gene silencing by inhibiting translation and/or promoting degradation of particular mRNAs.

microsomes: Membranous vesicles formed by fragmentation of the endoplasmic reticulum of eukaryotic cells; recovered by differential centrifugation.

miRNA: *See* microRNA.

mismatch: A base pair in a nucleic acid that cannot form a normal Watson-Crick pair.

mismatch repair: An enzymatic system for repairing base mismatches in DNA.

mitochondrion: An organelle of eukaryotic cells; contains the enzyme systems required for the citric acid cycle, fatty acid oxidation, respiratory electron transfer, and oxidative phosphorylation.

mitogen-activated protein kinases: *See* MAPKs.

mitosis: In eukaryotic cells, the multistep process that results in chromosome replication and cell division.

mixed-function oxidases: Enzymes that catalyze reactions in which two different substrates are oxidized by molecular oxygen simultaneously, but the oxygen atoms do not appear in the oxidized product.

mixed-function oxygenases: Enzymes (e.g., monooxygenases) that catalyze reactions in which two reductants—one generally NADPH, the other the substrate—are oxidized by molecular oxygen, with one oxygen atom incorporated into the product and the other reduced to H_2O.

mixed inhibition: The reversible inhibition pattern resulting when an inhibitor molecule can bind to either the free enzyme or the enzyme-substrate complex (not necessarily with the same affinity).

modulator: A metabolite that, when bound to the allosteric site of an enzyme, alters its kinetic characteristics.

molar solution: One mole of solute dissolved in water to give a total volume of 1,000 mL.

mole: One gram molecular weight of a compound. *See also* Avogadro's number.

monocistronic mRNA: An mRNA that can be translated into only one protein.

monoclonal antibodies: Antibodies produced by a cloned hybridoma cell, which are therefore identical and directed against the same epitope of the antigen.

monosaccharide: A carbohydrate consisting of a single sugar unit.

moonlighting enzymes: Enzymes that play two distinct roles, at least one of which is catalytic; the other may be catalytic, regulatory, or structural.

motif: Any distinct folding pattern for elements of secondary structure, observed in one or more proteins. A motif can be simple or complex, and can represent all or just a small part of a polypeptide chain. Also called a fold or supersecondary structure.

mRNA: *See* messenger RNA.

mRNP complex: *See* messenger ribonucleoprotein complex.

mTORC1 (mechanistic target of rapamycin complex 1): A multiprotein complex of mTOR (mechanistic target of rapamycin) and several regulatory subunits, which together act as a Ser/Thr protein kinase; stimulated by nutrients and energy-sufficient conditions, it triggers cell growth and proliferation.

mucopolysaccharide: *See* glycosaminoglycan.

multidrug transporters: Plasma membrane transporters in the ABC transporter family that expel several commonly used antitumor drugs, thereby interfering with antitumor therapy.

multienzyme system: A group of related enzymes participating in a given metabolic pathway.

multiple cloning site (MCS): A planned DNA sequence that includes recognition sequences for multiple restriction endonucleases in close proximity.

mutarotation: The change in specific rotation of a pyranose or furanose sugar or glycoside that accompanies equilibration of its α- and β-anomeric forms.

mutases: Enzymes that catalyze the transposition of functional groups.

mutation: An inheritable change in the nucleotide sequence of a chromosome.

myocyte: A muscle cell.

myofibril: A unit of thick and thin filaments of muscle fibers.

myokinase: *See* adenylate kinase.

myosin: A contractile protein; the major component of the thick filaments of muscle and other actin-myosin systems.

n

***N*:** Avogadro's number, the number of molecules in a gram molecular weight (a mole) of any compound (6.02×10^{23}).

NAD, NADP (nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate): Nicotinamide-containing coenzymes that function as carriers of hydrogen atoms and electrons in some oxidation-reduction reactions.

NADH dehydrogenase: *See* Complex I.

Na^+K^+ ATPase: The electrogenic ATP-driven active transporter in the plasma membrane of most animal cells that pumps three Na^+ outward for every two K^+ moved inward.

native conformation: The biologically active conformation of a macromolecule.

ncRNA: *See* noncoding RNA.

negative cooperativity: A property of some multisubunit enzymes or proteins in which binding of a ligand or substrate to one subunit impairs binding to another subunit.

negative feedback: Regulation of a biochemical pathway in which a reaction product inhibits an earlier step in the pathway.

neuron: A cell of nervous tissue specialized for transmission of a nerve impulse.

neurotransmitter: A low molecular weight compound (usually containing nitrogen) secreted from the axon terminal of a neuron and bound by a specific receptor on the next neuron or on a myocyte; transmits a nerve impulse.

NHEJ: *See* nonhomologous end joining.

nitrogenase complex: A system of enzymes capable of reducing atmospheric nitrogen to ammonia in the presence of ATP.

nitrogen fixation: Conversion of atmospheric nitrogen (N_2) into a reduced, biologically available form by nitrogen-fixing organisms.

NMR: *See* nuclear magnetic resonance spectroscopy.

noncoding RNA (ncRNA): Any RNA that does not encode instructions for a protein product.

nonessential amino acids: Amino acids that can be made by humans from simpler precursors and are thus not required in the diet.

nonheme iron proteins: Proteins, usually acting in oxidation-reduction reactions, that contain iron but no porphyrin groups.

nonhomologous end joining (NHEJ): A process in which a double-strand break in a chromosome is repaired by end-processing and ligation, often creating mutations at the ligation site.

nonpolar: Hydrophobic; describes molecules or groups that are poorly soluble in water.

nonsense codon: A codon that does not specify an amino acid, but signals termination of a polypeptide chain.

nonsense mutation: A mutation that results in premature termination of a polypeptide chain.

nonsense suppressor: A mutation, usually in the gene for a tRNA, that causes an amino acid to be inserted into a polypeptide in response to a termination (nonsense) codon.

nontemplate strand: *See* coding strand.

nuclear magnetic resonance (NMR) spectroscopy: A technique that utilizes certain quantum mechanical properties of atomic nuclei to study the structure and dynamics of the molecules of which the nuclei are a part.

nucleases: Enzymes that hydrolyze the internucleotide (phosphodiester) linkages of nucleic acids.

nucleic acids: Biologically occurring polynucleotides in which the nucleotide residues are linked in a specific sequence by phosphodiester bonds; DNA and RNA.

nucleoid: In bacteria, the nuclear zone that contains the chromosome but has no surrounding membrane.

nucleolus: In eukaryotic cells, a densely staining structure in the nucleus; the site of rRNA synthesis and ribosome formation.

nucleophile: An electron-rich group with a strong tendency to donate electrons to an electron-deficient nucleus (electrophile); the entering reactant in a bimolecular substitution reaction.

nucleoplasm: The portion of a eukaryotic cell's contents that is enclosed by the nuclear membrane.

nucleoside: A compound consisting of a purine or pyrimidine base covalently linked to a pentose.

nucleoside diphosphate kinase: The enzyme that catalyzes reversible transfer of the terminal phosphate of a nucleoside 5′-triphosphate to a nucleoside 5′-diphosphate.

nucleoside diphosphate sugar: A coenzyme-like carrier of a sugar molecule, functioning in the enzymatic synthesis of polysaccharides and sugar derivatives.

nucleoside monophosphate kinase: An enzyme that catalyzes transfer of the terminal phosphate of ATP to a nucleoside 5′-monophosphate. *Compare* adenylate kinase.

nucleosome: In eukaryotes, a structural unit for packaging chromatin; consists of a DNA strand wound around a histone core.

nucleotide: A nucleoside phosphorylated at one of its pentose hydroxyl groups.

nucleus: In eukaryotes, an organelle that contains chromosomes.

o

***O*-glycosidic bonds:** Bonds between a sugar and another molecule (typically an alcohol, purine, pyrimidine, or sugar) through an intervening oxygen. Also called simply glycosidic bonds.

oligomer: A short polymer, usually of amino acids, sugars, or nucleotides; the definition of "short" is somewhat arbitrary, but usually fewer than 50 subunits.

oligomeric protein: A multisubunit protein having two or more polypeptide chains.

oligonucleotide: A short polymer of nucleotides (usually fewer than 50).

oligopeptide: A short polymer of amino acids joined by peptide bonds.

oligosaccharide: Several monosaccharide groups joined by glycosidic bonds.

ω oxidation: An alternative mode of fatty acid oxidation in which the initial oxidation is at the carbon most distant from the carboxyl carbon; as distinct from α oxidation and β oxidation.

oncogene: A cancer-causing gene; any of several mutant genes that cause cells to exhibit rapid, uncontrolled proliferation. *See also* proto-oncogene.

open reading frame (ORF): A group of contiguous, nonoverlapping nucleotide codons in a DNA or RNA molecule that does not include a termination codon.

open system: A system that exchanges matter and energy with its surroundings. *See also* system.

operator: A region of DNA that interacts with a repressor protein to control the expression of a gene or group of genes.

operon: A unit of genetic expression consisting of one or more related genes and the operator and promoter sequences that regulate their transcription.

opsin: The protein portion of the visual pigment, which becomes rhodopsin with addition of the chromophore retinal.

optical activity: The capacity of a substance to rotate the plane of plane-polarized light.

optimum pH: The characteristic pH at which an enzyme has maximal catalytic activity.

orexigenic: Tending to increase appetite and food consumption. *Compare* anorexigenic.

ORF: *See* open reading frame.

organelles: Membrane-bounded structures found in eukaryotic cells; contain enzymes and other components required for specialized cell functions.

origin: The nucleotide sequence or site in DNA at which replication is initiated.

orthologs: Genes or proteins from different species that possess a clear sequence and functional relationship to each other.

osmosis: Bulk flow of water through a semipermeable membrane into another aqueous compartment containing solute at a higher concentration.

osmotic pressure: Pressure generated by the osmotic flow of water through a semipermeable membrane into an aqueous compartment containing solute at a higher concentration.

oxidases: Enzymes that catalyze oxidation reactions in which molecular oxygen serves as the electron acceptor, but neither of the oxygen atoms is incorporated into the product. *Compare* oxygenases.

oxidation: The loss of electrons from a compound.

oxidation-reduction reaction: A reaction in which electrons are transferred from a donor to an acceptor molecule; also called a redox reaction.

oxidative phosphorylation: The enzymatic phosphorylation of ADP to ATP coupled to electron transfer from a substrate to molecular oxygen.

oxidizing agent (oxidant): The acceptor of electrons in an oxidation-reduction reaction.

oxygenases: Enzymes that catalyze reactions in which oxygen atoms are directly incorporated into the product, forming a hydroxyl or carboxyl group. In a monooxygenase reaction, only one oxygen atom is incorporated; the other is reduced to H_2O. In a dioxygenase reaction, both oxygens are incorporated into the product. *Compare* oxidases.

oxygen-evolving center: In green plants, the region of photosystem II with the cofactor Mn_4O_4Ca, in which H_2O is oxidized to O_2. Also called the water-splitting center.

oxygenic photosynthesis: Light-driven ATP and NADPH synthesis in organisms that use water as the electron source, producing O_2. *Compare* photosynthesis.

P

palindrome: A segment of duplex DNA in which the base sequences of the two strands exhibit twofold rotational symmetry about an axis.

paradigm: In biochemistry, an experimental model or example.

paralogs: Genes or proteins present in the same species that possess a clear sequence and functional relationship to each other.

partition coefficient: A constant that expresses the ratio in which a given solute will be partitioned or distributed between two given immiscible liquids at equilibrium.

passive transporter: A membrane protein that increases the rate of movement of a solute across the membrane along its electrochemical gradient without the input of energy.

pathogenic: Disease-causing.

PCR: *See* polymerase chain reaction.

PDB (Protein Data Bank): An international data bank (www.rcsb.org) that archives data describing the three-dimensional structure of nearly all macromolecules for which structures have been published.

pentose: A simple sugar with a backbone containing five carbon atoms.

pentose phosphate pathway: A pathway, present in most organisms, that interconverts hexoses and pentoses and is a source of reducing equivalents (NADPH) and pentoses for biosynthetic processes; it begins with glucose 6-phosphate and includes 6-phosphogluconate as an intermediate. Also called the phosphogluconate pathway and hexose monophosphate pathway.

peptidases: Enzymes that hydrolyze peptide bonds.

peptide: Two or more amino acids covalently joined by peptide bonds.

peptide bond: A substituted amide linkage between the α-amino group of one amino acid and the α-carboxyl group of another, with elimination of the elements of water.

peptidoglycan: A major component of bacterial cell walls; generally consists of parallel heteropolysaccharides cross-linked by short peptides.

peptidyl transferase: The enzyme activity that synthesizes the peptide bonds of proteins; a ribozyme, part of the rRNA of the large ribosomal subunit.

peripheral proteins: Proteins loosely bound to a membrane by hydrogen bonds or electrostatic forces; generally water-soluble once released from the membrane. *Compare* integral proteins.

permeases: *See* transporters.

peroxisome: An organelle of eukaryotic cells; contains peroxide-forming and peroxide-destroying enzymes.

peroxisome proliferator-activated receptor: *See* PPAR.

PG: *See* prostaglandin.

pH: The negative logarithm of the hydrogen ion concentration of an aqueous solution.

phage: *See* bacteriophage.

phenotype: The observable characteristics of an organism.

phosphatases: Enzymes that cleave phosphate esters by hydrolysis, the addition of the elements of water.

phosphodiester linkage: A chemical grouping that contains two alcohols esterified to one molecule of phosphoric acid, which thus serves as a bridge between them.

phosphogluconate pathway: *See* pentose phosphate pathway.

phospholipid: A lipid containing one or more phosphate groups.

phosphoprotein phosphatases: *See* protein phosphatases.

phosphorolysis: Cleavage of a compound with phosphate as the attacking group; analogous to hydrolysis.

phosphorylases: Enzymes that catalyze phosphorolysis.

phosphorylation: Formation of a phosphate derivative of a biomolecule, usually by enzymatic transfer of a phosphoryl group from ATP.

phosphorylation potential (ΔG_p): The actual free-energy change of ATP hydrolysis under the nonstandard conditions prevailing in a cell.

photochemical reaction center: The part of a photosynthetic complex where the energy of an absorbed photon causes charge separation, initiating electron transfer.

photon: The ultimate unit (a quantum) of light energy.

photophosphorylation: The enzymatic formation of ATP from ADP coupled to the light-dependent transfer of electrons in photosynthetic cells.

photoreduction: The light-induced reduction of an electron acceptor in photosynthetic cells.

photorespiration: Oxygen consumption occurring in illuminated temperate-zone plants that is largely due to oxidation of phosphoglycolate.

photosynthesis: The use of light energy to produce carbohydrates from carbon dioxide and a reducing agent such as water. *Compare* oxygenic photosynthesis.

photosynthetic phosphorylation: *See* photophosphorylation.

photosystem: In photosynthetic cells, a functional set of light-absorbing pigments and its reaction center, where the energy of an absorbed photon is transduced into a separation of electric charges.

phototroph: An organism that can use the energy of light to synthesize its own fuels from simple molecules such as carbon dioxide, oxygen, and water; as distinct from a chemotroph.

pI: *See* isoelectric pH.

PIC: *See* preinitiation complex.

pK_a: The negative logarithm of an equilibrium constant.

PKA: *See* cAMP-dependent protein kinase.

plasmalogen: A phospholipid with an alkenyl ether substituent on C-1 of glycerol.

plasma membrane: The exterior membrane enclosing the cytoplasm of a cell.

plasma proteins: The proteins present in blood plasma.

plasmid: An extrachromosomal, independently replicating, small circular DNA molecule; commonly employed in genetic engineering.

plastid: In plants, a self-replicating organelle; may differentiate into a chloroplast or amyloplast.

plastocyanin: A small, soluble, copper-containing electron carrier in the thylakoid lumen. It transfers electrons, one at a time, between cytochrome b_6f and the reaction center P700, alternating between its Cu^+ and Cu^{2+} forms.

platelets: Small, enucleated cells that initiate blood clotting; they arise from bone marrow cells called megakaryocytes. Also known as thrombocytes.

plectonemic: Describes a structure in a molecular polymer that has a net twisting of strands about each other in a simple and regular way.

PLP: *See* pyridoxal phosphate.

polar: Hydrophilic, or "water-loving"; describes molecules or groups that are soluble in water.

polarity: (1) In chemistry, the nonuniform distribution of electrons in a molecule; polar molecules are usually soluble in water. (2) In molecular biology, the distinction between the 5′ and 3′ ends of nucleic acids.

poly(A) site choice: The strand cleavage and addition of a 3′-poly(A) tract at alternative locations within an mRNA transcript to generate different mature mRNAs.

poly(A) tail: A length of adenosine residues added to the 3′ end of many mRNAs in eukaryotes (and sometimes in bacteria).

polycistronic mRNA: A contiguous mRNA with more than two genes that can be translated into proteins.

polyclonal antibodies: A heterogeneous pool of antibodies produced in an animal by different B lymphocytes in response to an antigen. Different antibodies in the pool recognize different parts of the antigen.

polymerase chain reaction (PCR): A repetitive laboratory procedure that results in geometric amplification of a specific DNA sequence.

polymorphic: Describes a protein for which amino acid sequence variants are present in a population of organisms, but the variations do not destroy the protein's function.

polynucleotide: A covalently linked sequence of nucleotides in which the 3′ hydroxyl of the pentose of one nucleotide residue is joined by a phosphodiester bond to the 5′ hydroxyl of the pentose of the next residue.

polypeptide: A long chain of amino acids linked by peptide bonds; the molecular weight is generally less than 10,000.

polyribosome: *See* polysome.

polysaccharide: A linear or branched polymer of monosaccharide units linked by glycosidic bonds.

polysome: A complex of an mRNA molecule and two or more ribosomes; also called a polyribosome.

polyunsaturated fatty acid (PUFA): A fatty acid with more than one double bond, generally nonconjugated.

P/O ratio: The number of moles of ATP formed in oxidative phosphorylation per $\frac{1}{2}O_2$ reduced (thus, per pair of electrons passed to O_2). Experimental values used in this text are 2.5 for passage of electrons from NADH to O_2, and 1.5 for passage of electrons from FADH to O_2.

porphyria: An inherited disease resulting from the lack of one or more enzymes required to synthesize porphyrins.

porphyrin: A complex nitrogenous compound containing four substituted pyrroles covalently joined in a ring; often complexed with a central metal atom.

positive cooperativity: A property of some multisubunit enzymes or proteins in which binding of a ligand or substrate to one subunit facilitates binding to another subunit.

positive-inside rule: The general observation that most plasma membrane proteins are oriented so that most of their positively charged residues (Lys and Arg) are on the cytosolic face.

posttranscriptional processing: The enzymatic processing of the primary RNA transcript to produce functional RNAs, including mRNAs, tRNAs, rRNAs, and many other classes of RNAs.

posttranslational modification: The enzymatic processing of a polypeptide chain after translation from its mRNA.

PPAR (peroxisome proliferator-activated receptor): A family of nuclear transcription factors, activated by lipidic ligands, that alter the expression of specific genes, including those encoding enzymes of lipid synthesis and breakdown.

prebiotic: A selectively fermented, nondigestible food ingredient that supports the growth of health-promoting bacteria.

precursor transcript: The immediate RNA product of transcription before any posttranscriptional processing reactions. Also called primary transcript.

preinitiation complex (PIC): The set of proteins necessary for positioning RNA polymerases at transcription start sites in eukaryotes. For RNA polymerase II, this can include nearly 100 factors including the polymerase, general transcription factors, and Mediator complex.

pre–steady state: In an enzyme-catalyzed reaction, the period preceding establishment of the steady state, often encompassing just the first enzymatic turnover.

primary structure: A description of the covalent backbone of a polymer (macromolecule), including the sequence of monomeric subunits and any interchain and intrachain covalent bonds.

primary transcript: *See* precursor transcript.

primases: Enzymes that catalyze formation of RNA oligonucleotides used as primers by DNA polymerases.

primer: A short oligomer (e.g., of sugars or nucleotides) to which an enzyme adds additional monomeric subunits.

primer terminus: The end of a primer to which monomeric subunits are added.

priming: (1) In protein phosphorylation, the phosphorylation of an amino acid residue that becomes the binding site and point of reference for phosphorylation of other residues in the same protein. (2) In DNA replication, the synthesis of a short oligonucleotide to which DNA polymerases can add additional nucleotides.

primosome: An enzyme complex that synthesizes the primers required for lagging strand DNA synthesis.

probiotic: A live microorganism that, ingested in adequate amounts, confers a health benefit on the host.

processivity: For any enzyme that catalyzes the synthesis of a biological polymer, the property of adding multiple subunits to the polymer without dissociating from the substrate.

prochiral molecule: A symmetric molecule that can react asymmetrically with an enzyme having an asymmetric active site, generating a chiral product.

projection formulas: *See* Fischer projection formulas.

prokaryote: A term used historically to refer to any species in the domains Bacteria and Archaea. The differences between bacteria (formerly "eubacteria") and archaea are sufficiently great that this term is of marginal usefulness. A tendency to use "prokaryote" when referring only to bacteria is common and misleading; "prokaryote" also implies an ancestral relationship to eukaryotes, which is incorrect. We do not use "prokaryote" and "prokaryotic" in this text.

promoter: A DNA sequence at which RNA polymerase may bind, leading to initiation of transcription.

proofreading: The correction of errors in the synthesis of an information-containing biopolymer by removing incorrect monomeric subunits after they have been covalently added to the growing polymer.

prostaglandin (PG): One of a class of polyunsaturated, cyclic eicosanoid lipids that act as paracrine hormones.

prosthetic group: A metal ion or organic compound (other than an amino acid) covalently bound to a protein and essential to its activity.

proteases: Enzymes that catalyze the hydrolytic cleavage of peptide bonds in proteins.

proteasome: A supramolecular assembly of enzyme complexes that function in the degradation of damaged or unneeded cellular proteins.

protein: A macromolecule composed of one or more polypeptide chains, each with a characteristic sequence of amino acids linked by peptide bonds.

Protein Data Bank: *See* PDB.

protein kinase A (PKA): *See* cAMP-dependent protein kinase.

protein kinases: Enzymes that transfer the terminal phosphoryl group of ATP or another nucleoside triphosphate to a Ser, Thr, Tyr, Asp, or His side chain in a target protein, thereby regulating the activity or other properties of that protein.

protein phosphatases: Enzymes that hydrolyze a phosphate ester or anhydride bond on a protein, releasing inorganic phosphate, P_i. Also called phosphoprotein phosphatases.

protein targeting: The process by which newly synthesized proteins are sorted and transported to their proper locations in the cell.

proteoglycan: A hybrid macromolecule consisting of a heteropolysaccharide joined to a polypeptide; the polysaccharide is the major component.

proteome: The full complement of proteins expressed in a given cell, or the complete complement of proteins that can be expressed by a given genome.

proteomics: Broadly, the study of the protein complement of a cell or organism.

proteostasis: The maintenance of a cellular steady-state collection of proteins that are required for cell functions under a given set of conditions.

protomer: A general term describing any repeated unit of one or more stably associated protein subunits in a larger protein structure. In a protomer with multiple subunits, the subunits may be identical or different.

proton acceptor: An anionic compound capable of accepting a proton from a proton donor; that is, a base.

proton donor: The donor of a proton in an acid-base reaction; that is, an acid.

proton-motive force: The electrochemical potential inherent in a transmembrane gradient of H^+ concentration; used in oxidative phosphorylation and photophosphorylation to drive ATP synthesis.

proto-oncogene: A cellular gene, usually encoding a regulatory protein, that can be converted into an oncogene by mutation.

PUFA: *See* polyunsaturated fatty acid.

purine: A nitrogenous heterocyclic base that is a component of nucleotides and nucleic acids; contains fused pyrimidine and imidazole rings.

puromycin: An antibiotic that inhibits polypeptide synthesis by incorporating into a growing polypeptide chain, causing its premature termination.

pyranose: A simple sugar containing the six-membered pyran ring.

pyridine nucleotide: A nucleotide coenzyme containing the pyridine derivative nicotinamide; NAD or NADP.

pyridoxal phosphate (PLP): A coenzyme containing the vitamin pyridoxine (vitamin B_6); functions in amino group transfer reactions.

pyrimidine: A nitrogenous heterocyclic base that is a component of nucleotides and nucleic acids.

pyrimidine dimer: A covalently joined dimer of two adjacent pyrimidine residues in DNA, induced by absorption of UV light; most commonly derived from two adjacent thymines (a thymine dimer).

pyrophosphatase: *See* inorganic pyrophosphatase.

pyrosequencing: A DNA sequencing technology in which each nucleotide addition by DNA polymerase triggers a series of reactions ending in a luciferase-generated flash of light.

q

Q: *See* mass-action ratio.

quantitative PCR (qPCR): A PCR procedure that allows determination of how much of an amplified template was in the original sample; also called real-time PCR.

quantum: The ultimate unit of energy.

quaternary structure: The three-dimensional structure of a multisubunit protein, particularly the manner in which the subunits fit together.

r

racemic mixture (racemate): An equimolar mixture of the D and L stereoisomers of an optically active compound.

radical: An atom or group of atoms possessing an unpaired electron; also called a free radical.

radioactive isotope: An isotopic form of an element with an unstable nucleus that stabilizes itself by emitting ionizing radiation.

radioimmunoassay (RIA): A sensitive, quantitative method for detecting trace amounts of a biomolecule, based on its capacity to displace a radioactive form of the molecule from combination with its specific antibody.

Ras superfamily of G proteins: Small (M_r ~20,000), monomeric guanosine nucleotide–binding proteins that regulate signaling and membrane trafficking pathways; inactive with GDP bound, activated by displacement of the GDP by GTP, then inactivated by their intrinsic GTPase. Also called small G proteins.

rate constant: The proportionality constant that relates the velocity of a chemical reaction to the concentration(s) of the reactant(s).

rate-limiting step: (1) Generally, the step in an enzymatic reaction with the greatest activation energy or with the transition state of highest free energy. (2) The slowest step in a metabolic pathway.

reaction intermediate: Any chemical species in a reaction pathway that has a finite chemical lifetime.

reactive oxygen species (ROS): Highly reactive products of the partial reduction of O_2, including hydrogen peroxide (H_2O_2), superoxide ($^{\bullet}O_2^-$), and hydroxyl free radical ($^{\bullet}OH$); minor byproducts of oxidative phosphorylation.

reading frame: A contiguous, nonoverlapping set of three-nucleotide codons in DNA or RNA.

real-time PCR: *See* quantitative PCR.

receptor Tyr kinase (RTK): A large family of plasma membrane proteins with a ligand-binding site on the extracellular domain, a single transmembrane helix, and a cytoplasmic domain with protein Tyr kinase activity controlled by the extracellular ligand.

recombinant DNA: DNA formed by the joining of genes into new combinations.

recombination: Any enzymatic process by which the linear arrangement of a nucleic acid sequence in a chromosome is altered by cleavage and rejoining.

recombinational DNA repair: Recombinational processes directed at the repair of DNA strand breaks or cross-links, especially at inactivated replication forks.

redox pair: An electron donor and its corresponding oxidized form; for example, NADH and NAD^+.

redox reaction: *See* oxidation-reduction reaction.

reducing agent (reductant): The electron donor in an oxidation-reduction reaction.

reducing end: The end of a polysaccharide that has a terminal sugar with a free anomeric carbon; the terminal residue can act as a reducing sugar.

reducing equivalent: A general term for an electron or an electron equivalent in the form of a hydrogen atom or a hydride ion.

reducing sugar: A sugar in which the carbonyl (anomeric) carbon is not involved in a glycosidic bond and can therefore undergo oxidation.

reduction: The gain of electrons by a compound or ion.

regulator of G protein signaling (RGS): A protein structural domain that stimulates the GTPase activity of heterotrimeric G proteins.

regulatory cascade: A multistep regulatory pathway in which a signal leads to activation of a series of proteins in succession, with each protein in the succession catalytically activating the next, such that the original signal is amplified exponentially. *See also* enzyme cascade.

regulatory enzyme: An enzyme with a regulatory function, through its capacity to undergo a change in catalytic activity by allosteric mechanisms or by covalent modification.

regulatory gene: A gene that gives rise to a product involved in regulation of the expression of another gene; for example, a gene encoding a repressor protein.

regulatory protein: Protein whose function is to regulate the activity of another protein or enzyme by binding to it.

regulatory sequence: A DNA sequence involved in regulating the expression of a gene; for example, a promoter or operator.

regulon: A group of genes or operons that are coordinately regulated, even though some, or all, may be spatially distant in the chromosome or genome.

relaxed DNA: Any DNA that exists in its most stable, unstrained structure, typically the B form under most cellular conditions.

release factors: Protein factors in the cytosol required for the release of a completed polypeptide chain from a ribosome; also known as termination factors.

renaturation: The refolding of an unfolded (denatured) globular protein so as to restore its native structure and function.

replication: Synthesis of daughter nucleic acid molecules identical to the parental nucleic acid.

replication fork: The Y-shaped structure generally found at the point where DNA is being synthesized.

replicative form: Any of the full-length structural forms of a viral chromosome that serve as distinct replication intermediates.

replisome: The multiprotein complex that promotes DNA synthesis at the replication fork.

repressible enzyme: In bacteria, an enzyme for which synthesis is inhibited when its reaction product is readily available to the cell.

repression: A decrease in the expression of a gene in response to a change in the activity of a regulatory protein.

repressor: The protein that binds to the regulatory sequence or operator for a gene, blocking its transcription.

residue: A single unit in a polymer; for example, an amino acid in a polypeptide chain. The term reflects the fact that sugars, nucleotides, and amino acids lose a few atoms (generally the elements of water) when incorporated in their respective polymers.

respirasome: A supercomplex of electron transfer Complexes I, III, and IV in the mitochondrial inner membrane.

respiration: Any metabolic process that leads to the uptake of oxygen and release of CO_2.

respiration-linked phosphorylation: ATP formation from ADP and P_i, driven by electron transfer through a series of membrane-bound carriers, with a proton gradient as the direct source of energy driving rotational catalysis by ATP synthase.

respiratory chain: The electron-transfer chain; a sequence of electron-carrying proteins that transfers electrons from substrates to molecular oxygen in aerobic cells.

response element: A region of DNA, near (upstream from) a gene, bound by specific proteins that influence the rate of transcription of the gene.

restriction endonucleases: Site-specific endonucleases that cleave both strands of DNA at points in or near the specific site recognized by the enzyme; important tools in genetic engineering.

restriction fragment: A segment of double-stranded DNA produced by the action of a restriction endonuclease on a larger DNA.

restriction-modification system: A paired enzyme system, generally in bacteria, that will either cleave (restrict) invading viral DNA at a particular sequence or methylate (modify) one or more nucleotides within the same sequence where it occurs in the host chromosome, so as to avoid chromosome cleavage.

retinal: A 20-carbon isoprene aldehyde derived from carotene, which serves as the light-sensitive component of the visual pigment rhodopsin. Illumination converts 11-*cis*-retinal to all-*trans*-retinal.

retrovirus: An RNA virus containing a reverse transcriptase.

reverse transcriptase: An RNA-directed DNA polymerase of retroviruses; capable of making DNA complementary to an RNA.

reverse transcriptase PCR (RT-PCR): A PCR procedure in which reverse transcriptase is used to convert RNA into cDNA in the first few steps. The cDNA is then amplified by standard PCR or qPCR. When coupled to qPCR, the method can provide a measure of RNA abundance.

reversible inhibition: Inhibition by a molecule that binds reversibly to the enzyme, such that the enzyme activity returns when the inhibitor is no longer present.

reversible terminator sequencing: A DNA sequencing technology in which nucleotide additions are detected and scored by the color of fluorescence displayed when a labeled nucleotide with a removable sequence terminator is added.

R group: (1) Formally, an abbreviation denoting any alkyl group. (2) Occasionally, used in a more general sense to denote virtually any organic substituent (e.g., the R groups of amino acids).

RGS: *See* regulator of G protein signaling.

rhodopsin: The visual pigment, composed of the protein opsin and the chromophore retinal.

RIA: *See* radioimmunoassay.

ribonuclease: A nuclease that catalyzes the hydrolysis of certain internucleotide linkages of RNA.

ribonucleic acid: *See* RNA.

ribonucleoprotein (RNP): Biomolecule with subunits of both RNA and protein. Examples include telomerase, spliceosomes, and ribosomes.

ribonucleotide: A nucleotide containing D-ribose as its pentose component.

ribosomal RNA (rRNA): A class of RNA molecules serving as components of ribosomes.

ribosome: A supramolecular complex of rRNAs and proteins, approximately 18 to 22 nm in diameter; the site of protein synthesis.

ribosome profiling: A technique employing next-generation DNA sequencing of cDNA fragments derived from RNA bound to cellular ribosomes, to determine what mRNAs are being translated at a given moment.

riboswitch: A structured segment of an mRNA that binds to a specific ligand and affects translation or processing of the mRNA.

ribozymes: Ribonucleic acid molecules with catalytic activities; RNA enzymes.

ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco): The enzyme that fixes CO_2 into organic form (3-phosphoglycerate) in organisms (plants and some microorganisms) capable of CO_2 fixation.

Rieske iron-sulfur protein: A type of iron-sulfur protein in which two of the ligands to the central iron ion are His side chains; act in many electron-transfer sequences, including oxidative phosphorylation and photophosphorylation.

RNA (ribonucleic acid): A polyribonucleotide of a specific sequence linked by successive 3′,5′-phosphodiester bonds.

RNA editing: Posttranscriptional modification of an mRNA that can alter the meaning of one or more codons during translation.

RNA polymerase: An enzyme that catalyzes formation of RNA from ribonucleoside 5′-triphosphates, using a strand of DNA or RNA as a template.

RNA recognition motif (RRM): A single-stranded nucleic acid–binding motif consisting of a four-strand antiparallel β sheet with two α helices on one face.

RNA-Seq: A method for determining the relative expression levels of all or selected groups of genes within a genome.

RNA splicing: Removal of introns and joining of exons in a primary transcript. Also called simply splicing.

RNA world hypothesis: A hypothesis that life on Earth originated with molecules like RNA capable of storing and replicating genetic information in addition to catalyzing biochemical reactions.

RNP: *See* ribonucleoprotein.

ROS: *See* reactive oxygen species.

rRNA: *See* ribosomal RNA.

RRM: *See* RNA recognition motif.

RTK: *See* receptor Tyr kinase.

RT-PCR: *See* reverse transcriptase PCR.

rubisco: *See* ribulose 1,5-bisphosphate carboxylase/oxygenase.

S

salvage pathway: A pathway for synthesis of a biomolecule, such as a nucleotide, from intermediates in the degradative pathway for the biomolecule; a recycling pathway, as distinct from a de novo pathway.

Sanger sequencing: A DNA sequencing method based on the use of dideoxynucleoside triphosphates, developed by Frederick Sanger; also called dideoxy sequencing.

sarcomere: A functional and structural unit of the muscle contractile system.

satellite DNA: *See* simple-sequence DNA.

saturated fatty acid: A fatty acid containing a fully saturated alkyl chain.

scaffold proteins: Noncatalytic proteins that nucleate formation of multienzyme complexes by providing two or more specific binding sites for those proteins.

scramblases: Membrane proteins that catalyze movement of phospholipids across the membrane bilayer, leading to uniform distribution of a lipid between the two membrane leaflets (monolayers).

scRNA-seq: *See* single-cell RNA-seq.

secondary metabolism: Pathways that lead to specialized products not found in every living cell.

secondary structure: The local spatial arrangement of the main-chain atoms in a segment of a polymer (polypeptide or polynucleotide) chain.

second law of thermodynamics: The law stating that, in any chemical or physical process, the entropy of the universe tends to increase.

second messenger: An effector molecule synthesized in a cell in response to an external signal (first messenger) such as a hormone.

sedimentation coefficient: A physical constant specifying the rate of sedimentation of a particle in a centrifugal field under specified conditions.

selectins: A large family of membrane proteins that bind oligosaccharides on other cells tightly and specifically and carry signals across the plasma membrane.

SELEX: A method for rapid experimental identification of nucleic acid sequences (usually RNA) that have particular catalytic or ligand-binding properties.

septins: A family of highly conserved GTP-binding proteins that act in processes that involve membrane bending, such as cytokinesis, exocytosis, phagocytosis, and apoptosis.

sequence polymorphisms: Any alterations in genomic sequence (base-pair changes, insertions, deletions, rearrangements) that help distinguish subsets of individuals in a population or distinguish one species from another.

sequencing depth: The number of times, on average, that a given genomic nucleotide is included in sequenced DNA segments.

serine proteases: One of four major classes of proteases, having a reaction mechanism in which an active-site Ser residue acts as a covalent catalyst.

sgRNA: *See* single guide RNA.

Shine-Dalgarno sequence: A sequence in an mRNA that is important for binding bacterial ribosomes.

short tandem repeat (STR): A short (typically 3 to 6 base pairs) DNA sequence, repeated many times in tandem at a particular location in a chromosome.

SH2 domain: A protein domain that binds tightly to a phosphotyrosine residue in certain proteins, such as the receptor Tyr kinases, initiating formation of a multiprotein complex that acts in a signaling pathway.

shuttle vector: A recombinant DNA vector that can be replicated in two or more different host species. *See also* vector.

sickle cell anemia: A human disease characterized by defective hemoglobin molecules in individuals homozygous for a mutant allele coding for the β chain of hemoglobin.

σ: (1) A subunit of the bacterial RNA polymerase that confers specificity for certain promoters; usually designated by a superscript indicating its size (e.g., σ^{70} has a molecular weight of 70,000). (2) *See* superhelical density.

signal sequence: An amino acid sequence, often at the amino terminus, that signals the cellular fate or destination of a newly synthesized protein.

signal transduction: The process by which an extracellular signal (chemical, mechanical, or electrical) is amplified and converted to a cellular response.

silent mutation: A gene mutation that causes no detectable change in the biological characteristics of the gene product.

simple diffusion: Movement of solute molecules across a membrane to a region of lower concentration, unassisted by a protein transporter.

simple protein: A protein yielding only amino acids on hydrolysis.

simple-sequence DNA: Highly repeated, nontranslated segments of DNA in eukaryotic chromosomes; most often associated with the centromeric region. Its function is unknown. Also called satellite DNA.

single cell RNA-Seq (scRNA-Seq): An adaptation of RNA-Seq that is focused on global levels of gene expression within a single cell.

single guide RNA (sgRNA): A combination of gRNA and tracrRNA that allows one RNA to both activate Cas nucleases (particularly Cas9) and target the system to a particular DNA sequence. Parts of the RNA can be engineered to target the system to any desired DNA sequence.

single-molecule real-time (SMRT) sequencing: A DNA sequencing technology in which nucleotide additions are detected as flashes of fluorescent colored light, with sensitivity enhanced so that long DNA molecules (up to 15,000 nucleotides) can be sequenced and the results displayed in real time.

single nucleotide polymorphism (SNP): A genomic base-pair change that helps distinguish one species from another or one subset of individuals in a population.

site-directed mutagenesis: A set of methods used to create specific alterations in the sequence of a gene.

site-specific recombination: A type of genetic recombination that occurs only at specific sequences.

size-exclusion chromatography: A procedure for separation of molecules by size, based on the capacity of porous polymers to exclude solutes above a certain size; also called gel filtration.

small G proteins: *See* Ras superfamily of G proteins.

small nuclear RNA (snRNA): A class of short RNAs, typically 100 to 200 nucleotides long, present in the nucleus; involved in the splicing of eukaryotic mRNAs.

small nucleolar RNA (snoRNA): A class of short RNAs, generally 60 to 300 nucleotides long, that guide modification of rRNAs in the nucleolus.

SMRT sequencing: *See* single-molecule real-time sequencing.

SNP: *See* single nucleotide polymorphism.

snRNA: *See* small nuclear RNA.

snoRNA: *See* small nucleolar RNA.

somatic cells: All body cells except the germ-line cells.

SOS response: In bacteria, a coordinated induction of a variety of genes in response to high levels of DNA damage.

Southern blot: A DNA hybridization procedure in which one or more specific DNA fragments are detected in a larger population by hybridization to a complementary, labeled nucleic acid probe.

specialized pro-resolving mediator (SPM): One of several eicosanoids, derived from essential fatty acids, that promote the resolution phase of the inflammatory response.

specific acid-base catalysis: Acid or base catalysis involving the constituents of water (hydroxide or hydronium ions).

specific activity: The number of micromoles (μmol) of a substrate transformed by an enzyme preparation per minute per milligram of protein at 25 °C; a measure of enzyme purity.

specificity: The ability of an enzyme or receptor to discriminate among competing substrates or ligands.

specific rotation: The rotation, in degrees, of the plane of plane-polarized light (D-line of sodium) by an optically active compound at 25 °C, with a specified concentration and light path.

sphingolipid: An amphipathic lipid with a sphingosine backbone to which are attached a long-chain fatty acid and a polar alcohol.

spliceosome: A complex of RNAs and proteins involved in the splicing of mRNAs in eukaryotic cells.

splicing: *See* RNA splicing.

SPM: *See* specialized pro-resolving mediator.

standard free-energy change ($\Delta G°$): The free-energy change for a reaction occurring under a set of standard conditions: temperature, 298 K; pressure, 1 atm (101.3 kPa); and all solutes at 1 M concentration. $\Delta G'°$ denotes the standard transformed free-energy change at pH 7.0 in 55.5 M water used by biochemists.

standard reduction potential ($E°$): The electromotive force exhibited at an electrode by 1 M concentrations of a reducing agent and its oxidized form at 25 °C; a measure of the relative tendency of the reducing agent to lose electrons. $E'°$ denotes the standard transformed reduction potential at pH 7.0 and 55.5 M water used by biochemists.

statin: Any of a class of drugs used to reduce blood cholesterol in humans; acts by inhibiting the enzyme HMG-CoA reductase, an early step in sterol synthesis.

steady state: A nonequilibrium state of a system through which matter is flowing and in which all components remain at a constant concentration.

stem cells: The common, self-regenerating cells in bone marrow that give rise to differentiated blood cells such as erythrocytes and lymphocytes.

stereoisomers: Compounds that have the same composition and the same order of atomic connections but different molecular arrangements.

sterol: A group of steroid lipids in which the 3 position of the A ring of the steroid nucleus has been modified with a hydroxyl group.

sticky ends: Two DNA ends in the same DNA molecule, or in different molecules, with short overhanging single-stranded segments that are complementary to one another, facilitating ligation of the ends; also known as cohesive ends.

stimulatory G protein (G_s): A trimeric regulatory GTP-binding protein that, when activated by an associated plasma membrane receptor, stimulates a neighboring membrane enzyme such as adenylyl cyclase; its effects oppose those of G_i. *Compare* inhibitory G protein (G_i).

stop codons: *See* termination codons.

STR: *See* short tandem repeat.

stroma: The space and aqueous solution enclosed within the inner membrane of a chloroplast, not including the contents of the thylakoid membranes.

structural gene: A gene coding for a protein or RNA molecule; as distinct from a regulatory gene.

substitution mutation: A mutation caused by replacement of one base by another.

substrate: The specific compound acted upon by an enzyme.

substrate channeling: Movement of the chemical intermediates in a series of enzyme-catalyzed reactions from the active site of one enzyme to that of the next enzyme in the pathway, without leaving the surface of the protein complex that includes the enzymes.

substrate cycle: A cycle of enzyme-catalyzed reactions that results in release of thermal energy by the hydrolysis of ATP; sometimes referred to as a futile cycle.

substrate-level phosphorylation: Phosphorylation of ADP or some other nucleoside 5′-diphosphate coupled to dehydrogenation of an organic substrate; independent of the respiratory chain.

suicide inactivator: A relatively inert molecule that is transformed by an enzyme, at its active site, into a reactive substance that irreversibly inactivates the enzyme.

sulfonylurea drugs: A group of oral medications used in the treatment of type 2 diabetes; act by closing K^+ channels in pancreatic β cells, stimulating insulin secretion.

supercoil: The twisting of a helical (coiled) molecule on itself; a coiled coil.

supercoiled DNA: DNA that twists upon itself because it is under- or overwound (and thereby strained) relative to B-form DNA.

superhelical density (σ): In a helical molecule such as DNA, the number of supercoils (superhelical turns) relative to the number of coils (turns) in the relaxed molecule.

supersecondary structure: *See* motif.

suppressor mutation: A mutation, at a site different from that of a primary mutation, that totally or partially restores a function lost by the primary mutation.

Svedberg (S): A unit of measure of the rate at which a particle sediments in a centrifugal field.

symbionts: Two or more organisms that are mutually interdependent and usually living in physical association.

symport: Cotransport of solutes across a membrane in the same direction.

syndecan: A heparan sulfate proteoglycan with a single transmembrane domain and an extracellular domain bearing three to five chains of heparan sulfate and, in some cases, chondroitin sulfate.

synteny: Conserved gene order along the chromosomes of different species.

synthases: Enzymes that catalyze condensation reactions that do not require nucleoside triphosphate as an energy source.

synthetases: Enzymes that catalyze condensation reactions using ATP or another nucleoside triphosphate as an energy source.

system: An isolated collection of matter; all other matter in the universe apart from the system is called the surroundings.

systems biology: The study of complex biochemical systems, integrating information from genomics, proteomics, and metabolomics.

t

TADs: *See* topologically associating domains.

tag: An extra segment of protein that is fused to a protein of interest by modification of its gene, usually for purposes of purification.

tautomers: Isomers that interconvert rapidly such that they exist in equilibrium.

TCA (tricarboxylic acid) cycle: *See* citric acid cycle.

T cell: *See* T lymphocyte.

telomerase: The ribonucleoprotein supramolecular complex responsible for addition of telomere repeat DNA to chromosome ends in eukaryotes.

telomere: A specialized nucleic acid structure at the ends of linear eukaryotic chromosomes.

template: A macromolecular mold or pattern for the synthesis of an informational macromolecule.

template strand: A strand of nucleic acid used by a polymerase as a template to synthesize a complementary strand, as distinct from the coding strand.

terminal transferase: An enzyme that catalyzes addition of nucleotide residues of a single kind to the 3′ end of DNA chains.

termination codons: UAA, UAG, and UGA; in protein synthesis, these codons signal termination of a polypeptide chain. Also known as stop codons.

termination factors: *See* release factors.

termination sequence: A DNA sequence, at the end of a transcriptional unit, that signals the end of transcription.

tertiary structure: The three-dimensional conformation of a polymer in its native, folded state.

tetrahydrobiopterin: The reduced coenzyme form of biopterin.

tetrahydrofolate: The reduced, active coenzyme form of the vitamin folate.

thermogenesis: The biological generation of heat by muscle activity (shivering), uncoupled oxidative phosphorylation, or the operation of substrate (futile) cycles.

thermogenin: *See* uncoupling protein 1.

thiamine pyrophosphate (TPP): The active coenzyme form of vitamin B_1; involved in aldehyde transfer reactions.

thiazolidinediones: A class of medications used in the treatment of type 2 diabetes; act to reduce circulating fatty acids and increase sensitivity to insulin. Also known as glitazones.

thioester: An ester of a carboxylic acid with a thiol or mercaptan.

thioredoxin: A small, ubiquitous protein with a pair of Cys residues that participate in redox reactions, alternating between their —SH and —S—S— forms. One role is to maintain the sulfhydryl residues of key proteins in their reduced state.

3′ end: The end of a nucleic acid that lacks a nucleotide bound at the 3′ position of the terminal residue.

thrombocytes: *See* platelets.

thromboxane (TX): Any of a class of eicosanoid lipids with a six-membered ether-containing ring; involved in platelet aggregation during blood clotting.

thylakoid: A closed, continuous system of flattened disks, formed by the pigment-bearing internal membranes of chloroplasts.

thymine dimer: *See* pyrimidine dimer.

tissue culture: A method by which cells derived from multicellular organisms are grown in liquid media.

titration curve: A plot of pH versus the equivalents of base added during titration of an acid.

T lymphocyte (T cell): One of a class of blood cells (lymphocytes) of thymic origin, involved in cell-mediated immune reactions.

tocopherol: Any of several forms of vitamin E.

topoisomerases: Enzymes that introduce positive or negative supercoils in closed, circular duplex DNA.

topoisomers: Different forms of a covalently closed, circular DNA molecule that differ only in linking number.

topologically associating domains (TADs): Large DNA loops within chromosomes, constrained at the base and encompassing 800,000 or more base pairs of DNA; found in both transcriptionally active and inactive chromosomal regions.

topology: The study of the properties of an object that do not change under continuous deformations such as twisting or bending.

topology diagram: A structural representation in which the connections between elements of secondary structure are depicted in two dimensions.

TPP: *See* thiamine pyrophosphate.

trace element: A chemical element required by an organism in only trace amounts.

trans-activating CRISPR RNA (tracrRNA): A bacterially encoded RNA required for the activation and function of the relatively simple CRISPR/Cas system in the human pathogen *Streptococcus pyogenes*.

transaminases: *See* aminotransferases.

transamination: Enzymatic transfer of an amino group from an α-amino acid to an α-keto acid.

transcription: The enzymatic process whereby the genetic information contained in one strand of DNA is used to specify a complementary sequence of bases in an mRNA.

transcriptional control: Regulation of the synthesis of a protein by regulation of the formation of its mRNA.

transcription factor: A protein that affects the regulation and transcription initiation of a gene by binding to a regulatory sequence near or within the gene and interacting with RNA polymerase and/or other transcription factors.

transcriptome: The entire complement of RNA transcripts present in a given cell or tissue under specific conditions.

transcriptomics: A discipline focused on the study of gene expression on a genomic scale, often following increases and decreases in transcription of various genes under different conditions.

transduction: (1) Generally, the conversion of energy or information from one form to another. (2) The transfer of genetic information from one cell to another by means of a viral vector.

transfer RNA (tRNA): A class of RNA molecules (M_r 25,000 to 30,000), each of which combines covalently with a specific amino acid as the first step in protein synthesis.

transformation: Introduction of an exogenous DNA into a cell, causing the cell to acquire a new phenotype.

transgenic: Describes an organism that has genes from another organism incorporated in its genome as a result of recombinant DNA procedures.

transition state: An activated form of a molecule in which the molecule has undergone a partial chemical reaction; the highest point on the reaction coordinate.

transition-state analog: A stable molecule that resembles the transition state of a particular reaction and therefore binds the enzyme that catalyzes the reaction more tightly than does the substrate in the enzyme-substrate complex.

translation: The process in which the genetic information in an mRNA molecule specifies the sequence of amino acids during protein synthesis.

translational control: Regulation of the synthesis of a protein by regulation of the rate of its translation on the ribosome.

translational frameshifting: A programmed change in the reading frame during translation of an mRNA on a ribosome, occurring by any of several mechanisms.

translational repressor: A repressor that binds to an mRNA, blocking translation.

translocase: An enzyme that causes movement, such as movement of a ribosome along an mRNA.

transpiration: Passage of water from the roots of a plant to the atmosphere via the vascular system and the stomata of leaves.

transport constant (K_t; $K_{\text{transport}}$): A kinetic parameter for a membrane transporter, analogous to the Michaelis constant, K_m, for an enzymatic reaction. The rate of substrate uptake is half-maximal when the substrate concentration equals K_t.

transporters: Proteins that span a membrane and transport specific nutrients, metabolites, ions, or proteins across the membrane; sometimes called permeases.

transposition: Movement of a gene or set of genes from one site in the genome to another. Also called DNA transposition.

transposon (transposable element): A segment of DNA that can move from one position in the genome to another.

triacylglycerol: An ester of glycerol with three molecules of fatty acid; also called a triglyceride or neutral fat.

tricarboxylic acid (TCA) cycle: *See* citric acid cycle.

trimeric G proteins: Members of the G protein family with three subunits; function in a variety of signaling pathways. They are inactive with GDP bound, activated by associated receptors as the GDP is displaced by GTP, then inactivated by their intrinsic GTPase activity.

triose: A simple sugar with a backbone containing three carbon atoms.

tRNA: *See* transfer RNA.

tropic hormones (tropins): Peptide hormones that stimulate a specific target gland to secrete its hormone; for example, thyrotropin produced by the pituitary stimulates secretion of thyroxine by the thyroid.

t-SNAREs: Protein receptors in a targeted membrane (typically the plasma membrane) that bind to v-SNAREs in the membrane of a secretory vesicle, mediating fusion of the vesicle and target membranes.

tumor suppressor gene: One of a class of genes that encode proteins that normally regulate the cell cycle by suppressing cell division. Mutation of one copy of the gene is usually without effect, but when both copies are defective, the cell continues dividing without limitation, producing a tumor.

turnover number: The number of times an enzyme molecule transforms a substrate molecule per unit time, under conditions giving maximal activity at saturating substrate concentrations.

TX: *See* thromboxane.

type 2 diabetes mellitus: A metabolic disorder characterized by insulin resistance and poorly regulated blood glucose level; also known as adult-onset diabetes or noninsulin-dependent diabetes (NIDD).

u

ubiquitin: A small, highly conserved eukaryotic protein that targets an intracellular protein for degradation by proteasomes. Several ubiquitin molecules are covalently attached in tandem to a Lys residue of the target protein by a ubiquitinating enzyme.

ultraviolet (UV) radiation: Electromagnetic radiation in the region of 200 to 400 nm.

uncompetitive inhibition: The reversible inhibition pattern resulting when an inhibitor molecule can bind to the enzyme-substrate complex but not to the free enzyme.

uncoupling protein 1 (UCP1): A protein of the inner mitochondrial membrane in brown and beige adipose tissue that allows transmembrane movement of protons, short-circuiting the normal use of protons to drive ATP synthesis and dissipating the energy of substrate oxidation as heat. Also called thermogenin.

uniport: A transport system that carries only one solute, as distinct from cotransport.

unsaturated fatty acid: A fatty acid containing one or more double bonds.

urea cycle: A cyclic metabolic pathway in vertebrate liver that synthesizes urea from amino groups and carbon dioxide.

ureotelic: Excreting excess nitrogen in the form of urea.

uricotelic: Excreting excess nitrogen in the form of urate (uric acid).

v

V_0: Initial velocity, the initial rate of a reaction.

V_m: *See* membrane potential.

V_{max}: The maximum velocity of an enzymatic reaction when the binding site is saturated with substrate.

van der Waals interactions: Weak intermolecular forces between molecules as a result of each inducing polarization in the other.

vector: A DNA molecule known to replicate autonomously in a host cell, to which a segment of DNA may be spliced to allow its replication in a cell; for example, a plasmid or an artificial chromosome.

vectorial: Describes an enzymatic reaction or transport process in which the protein has a specific orientation in a biological membrane such that the substrate is moved from one side of the membrane to the other as it is converted into product.

vectorial metabolism: Metabolic transformations in which the location (not the chemical composition) of a substrate changes relative to the plasma membrane or organellar membrane; for example, the action of transporters and the proton pumps of oxidative phosphorylation and photophosphorylation.

vesicle: A small, spherical, membrane-bounded particle with an internal aqueous compartment that contains components such as hormones or neurotransmitters to be moved within or out of a cell.

viral vector: A viral DNA altered so that it can act as a vector for recombinant DNA.

virion: A virus particle.

virus: A self-replicating, infectious, nucleic acid–protein complex that requires an intact host cell for its replication; its genome is DNA or RNA.

vitamin: An organic substance required in small quantities in the diet of some species; generally functions as a component of a coenzyme.

v-SNAREs: Protein receptors in the membrane of a secretory vesicle that bind to t-SNAREs in a targeted membrane (typically the plasma membrane) and mediate fusion of the vesicle and target membranes.

w

water-splitting center: *See* oxygen-evolving center.

Western blotting: *See* immunoblotting.

white adipose tissue (WAT): Nonthermogenic adipose tissue rich in triacylglycerols, stored and mobilized in response to hormonal signals. Transfer of electrons in the respiratory chain of WAT mitochondria is tightly coupled to ATP synthesis. *Compare* beige adipose tissue; brown adipose tissue.

wild type: The normal (unmutated) genotype or phenotype.

wobble: The relatively loose base pairing between the base at the 3′ end of a codon and the complementary base at the 5′ end of the anticodon.

x

x-ray crystallography: The analysis of x-ray diffraction patterns of a crystalline compound, used to determine the molecule's three-dimensional structure.

z

zinc finger: A specialized protein motif of some DNA-binding proteins, involved in DNA recognition; characterized by a single atom of zinc coordinated to four Cys residues or to two His and two Cys residues.

Z scheme: In oxygenic photosynthesis, the path of electrons from water through photosystem II and the cytochrome b_6f complex to photosystem I and finally to NADPH. When the sequence of electron carriers is plotted against their reduction potentials, the path of electrons looks like a sideways Z.

zwitterion: A dipolar ion with spatially separated positive and negative charges.

zymogen: An inactive precursor of an enzyme; for example, pepsinogen, the precursor of pepsin.

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Resources

Abbreviations for Amino Acids

A	Ala	Alanine	N	Asn	Asparagine
B	Asx	Asparagine or aspartate	P	Pro	Proline
			Q	Gln	Glutamine
C	Cys	Cysteine	R	Arg	Arginine
D	Asp	Aspartate	S	Ser	Serine
E	Glu	Glutamate	T	Thr	Threonine
F	Phe	Phenylalanine	V	Val	Valine
G	Gly	Glycine	W	Trp	Tryptophan
H	His	Histidine	X	—	Unknown or nonstandard amino acid
I	Ile	Isoleucine			
K	Lys	Lysine	Y	Tyr	Tyrosine
L	Leu	Leucine	Z	Glx	Glutamine or glutamate
M	Met	Methionine			

Asx and Glx are used in describing the results of amino acid analytical procedures in which Asp and Glu are not readily distinguished from their amide counterparts, Asn and Gln.

The Standard Genetic Code

UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys
UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys
UUA	Leu	UCA	Ser	UAA	Stop	UGA	Stop
UUG	Leu	UCG	Ser	UAG	Stop	UGG	Trp
CUU	Leu	CCU	Pro	CAU	His	CGU	Arg
CUC	Leu	CCC	Pro	CAC	His	CGC	Arg
CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg
CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg
AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser
AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser
AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg
AUG	Met*	ACG	Thr	AAG	Lys	AGG	Arg
GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly
GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly
GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly
GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly

*AUG also serves as the initiation codon in protein synthesis.

1 **H** 1.008																		2 **He** 4.0
3 **Li** 6.94	4 **Be** 9.01												5 **B** 10.81	6 **C** 12.011	7 **N** 14.01	8 **O** 16.00	9 **F** 19.00	10 **Ne** 20.18
11 **Na** 22.99	12 **Mg** 24.31												13 **Al** 26.98	14 **Si** 28.09	15 **P** 30.97	16 **S** 32.06	17 **Cl** 35.45	18 **Ar** 39.95
19 **K** 39.10	20 **Ca** 40.08		21 **Sc** 44.96	22 **Ti** 47.90	23 **V** 50.94	24 **Cr** 52.00	25 **Mn** 54.94	26 **Fe** 55.85	27 **Co** 58.93	28 **Ni** 58.71	29 **Cu** 63.55	30 **Zn** 65.37	31 **Ga** 69.72	32 **Ge** 72.59	33 **As** 74.92	34 **Se** 78.96	35 **Br** 79.90	36 **Kr** 83.30
37 **Rb** 85.47	38 **Sr** 87.62		39 **Y** 88.91	40 **Zr** 91.22	41 **Nb** 92.91	42 **Mo** 95.94	43 **Te** 98.91	44 **Ru** 101.07	45 **Rh** 102.91	46 **Pd** 106.4	47 **Ag** 107.87	48 **Cd** 112.40	49 **In** 114.82	50 **Sn** 118.69	51 **Sb** 121.75	52 **Te** 126.70	53 **I** 126.90	54 **Xe** 131.30
55 **Cs** 132.91	56 **Ba** 137.34	57–70 *	71 **Lu** 174.97	72 **Hf** 178.49	73 **Ta** 180.95	74 **W** 183.85	75 **Re** 186.2	76 **Os** 190.2	77 **Ir** 192.2	78 **Pt** 195.09	79 **Au** 196.97	80 **Hg** 200.59	81 **Tl** 204.37	82 **Pb** 207.19	83 **Bi** 208.98	84 **Po** (209)	85 **At** (210)	86 **Rn** (222)
87 **Fr** (223)	88 **Ra** 226.03	89–102 **	103 **Lr** 262.11	104 **Rf** 261.11	105 **Db** 262.11	106 **Sg** 263.12	107 **Bh** 264.12	108 **Hs** 265.13	109 **Mt** 268	110 **Ds** 281	111 **Rg** 281	112 **Cn** 285	113 **Nh** 286	114 **Fl** 289	115 **Mc** 289	116 **Lv** 293	117 **Ts** 293	118 **Og** 294

*Lanthanides	57 **La** 138.91	58 **Ce** 140.12	59 **Pr** 140.91	60 **Nd** 144.24	61 **Pm** 144.91	62 **Sm** 150.36	63 **Eu** 151.96	64 **Gd** 157.25	65 **Tb** 158.93	66 **Dy** 162.50	67 **Ho** 164.93	68 **Er** 167.26	69 **Tm** 168.93	70 **Yb** 173.04
**Actinides	89 **Ac** 227.03	90 **Th** 232.04	91 **Pa** 231.04	92 **U** 238.03	93 **Np** 237.05	94 **Pu** 244.06	95 **Am** 243.06	96 **Cm** 247.07	97 **Bk** 247.07	98 **Cf** 251.08	99 **Es** 252.08	100 **Fm** 257.10	101 **Md** 258.10	102 **No** 259.10

Bioinformatics Databases

National Center for Biotechnology Information (NCBI)	www.ncbi.nlm.nih.gov
UniProt	www.uniprot.org
ExPASy Bioinformatics Resource Portal	www.expasy.org
GenomeNet	www.genome.jp

Structure Data Banks and Databases

Protein Data Bank (PDB)	www.rcsb.org
EMDataBank Unified Resource for 3DEM	www.emdataresource.org
National Center for Biomedical Glycomics	www.glycomics.uga.edu
LIPIDMAPS Lipidomics Gateway	www.lipidmaps.org
Nucleic Acid Database (NDB)	www.ndbserver.rutgers.edu
Modomics database of RNA modification pathways	www.genesilico.pl/modomics

Other Resources and Tools

Structural Classification of Proteins database (SCOP2)	http://scop2.mrc-lmb.cam.ac.uk
PROSITE Sequence logo	http://prosite.expasy.org/sequence_logo.html
ProtScale hydrophobicity and other profiles of amino acids	http://web.expasy.org/protscale
Predictor of Natural Disordered Regions (PONDR)	www.pondr.com
Enzyme nomenclature	www.qmul.ac.uk/sbcs/iubmb/enzyme
Ensembl genome databases	www.ensembl.org
PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System	www.pantherdb.org
Basic Local Alignment Search Tool (BLAST)	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Kyoto Encyclopedia of Genes and Genomes (KEGG)	www.genome.jp/kegg
KEGG pathway maps	www.genome.ad.jp/kegg/pathway/map/map01100.html
Biochemical nomenclature	www.qmul.ac.uk/sbcs/iubmb
Online Mendelian Inheritance in Man	www.omim.org

Unit Abbreviations

A	ampere	L	liter
Å	angstrom	M	molar (concentration)
atm	atmosphere	m	meter
°C	degree Celsius	min	minute
cal	calorie	mm Hg	millimeters of mercury (pressure)
cpm	counts per minute	mol	mole
Da	dalton	*N*	normal (concentration)
dpm	disintegrations per minute	Pa	pascal
F	faraday	r	revolution
g	gram	S	Svedberg unit
h	hour	s	second
J	joule	V	volt
K	kelvin	yr	year

Prefixes

10^{9}	giga	G
10^{6}	mega	M
10^{3}	kilo	k
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f

Some Conversion Factors

Length	$1\text{ cm} = 10\text{ mm} = 10^{4}\ \mu\text{m} = 10^{7}\text{ nm}$ $= 10^{8}\ \text{Å} = 0.394\text{ in}$ $1\text{ in} = 2.54\text{ cm}$ $1\text{ yard} = 0.9144\text{ meters}$ $1\text{ mile} = 1.609\text{ kilometers}$
Mass	$1\text{ g} = 10^{-3}\text{ kg} = 10^{3}\text{ mg} = 10^{6}\ \mu\text{g}$ $= 3.53 \times 10^{-2}\text{ oz}$ $1\text{ oz} = 28.3\text{ g}$
Temperature	$°\text{C} = 5/9(°\text{F} - 32)$ $\text{K} = °\text{C} + 273$
Energy	$1\text{ J} = 10^{7}\text{ erg} = 0.239\text{ cal}$ $1\text{ cal} = 4.184\text{ J}$
Pressure	$1\text{ torr} = 1\text{ mm Hg} = 1.32 \times 10^{-3}\text{ atm}$ $= 1.333 \times 10^{2}\text{ Pa}$ $1\text{ atm} = 758\text{ torr} = 1.01 \times 10^{5}\text{ Pa}$

Some Physical Constants, with Symbols and Values

Atomic mass unit (dalton)	amu	1.661×10^{-24} g
Avogadro's number	*N*	6.022×10^{23}/mol
Boltzmann constant	**k**	1.381×10^{-23} J/K; 3.298×10^{-24} cal/K
Faraday constant	*F*	96,480 J/V • mol
Gas constant	*R*	1.987 cal/mol • K; 8.315 J/mol • K
Planck's constant	*h*	1.584×10^{-34} cal • s; 6.626×10^{-34} J • s
Speed of light (in vacuum)	*c*	2.998×10^{10} cm/s

ON THE COVER:

In this artistic rendering, a protocell composed of a bilayer of fatty acids surrounds an aqueous compartment containing an RNA ribozyme and nucleic acids. Protocells are used by biochemists to explore reactions that could mimic those that led to the origin of life on Earth. Billions of years ago, hydrophobic molecules may have formed cell-like assemblies like protocells in which molecules capable of replicating themselves could be concentrated and protected from the external environment. These likely provided the origins of the membranes, nucleic acids, and catalysts found in today's cells.

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